BIOLOGY OF REPRODUCTION 69, 338–346 (2003)
Published online before print 2 April 2003.
DOI 10.1095/biolreprod.102.013953

Overexpression of Human Chorionic Gonadotropin Causes Multiple Reproductive Defects in Transgenic Mice[1]

Martin M. Matzuk,[3,4,5] Francesco J. DeMayo,[4] Lou Ann Hadsell,[4] and T. Rajendra Kumar[2,3,4]

Departments of Pathology,[3] Molecular and Cellular Biology,[4] and Molecular and Human Genetics,[5] Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

Human CG is a pregnancy marker secreted by the placenta, and it utilizes the same receptors as does LH. Human CG is a heterodimer, and its subunits are expressed in tissues other than placenta. Similarly, LH/hCG receptors are also expressed in multiple tissues; however, the physiological significance of this expression is unknown. Free hCGβ is efficiently secreted in vitro in transfected cells and is highly expressed in many human cancers; however, the biological effects of free hCGβ in vivo are unknown. To study in vivo consequences of elevated levels of free hCGβ and hCG dimer in both male and female reproductive physiology, we used mouse metallothionein 1 promoter to generate multiple lines of transgenic mice that overexpressed either one or both subunits of hCG. Although mice expressing the glycoprotein hormone α subunit are normal and fertile, both male and female transgenic mice overexpressing only the hormone-specific hCGβ subunit are infertile. The hCGβ subunit-expressing transgenic female mice progressively develop cystic ovaries, whereas the male transgenic mice are infertile but otherwise are not phenotypically discernible. In contrast, both the male and female transgenic mice coexpressing high levels of the hCG subunits (i.e., the hCG dimer) demonstrate multiple reproductive defects. The male transgenic mice have Leydig cell hyperplasia, very high levels of serum testosterone, reduced testis size, and dramatically enlarged seminal vesicles and are infertile and display overly aggressive behavior when caged with females. The female transgenic mice are also infertile, have elevated levels of serum estradiol, and progressively develop hemorrhagic and cystic ovaries with thecal layer enlargement and stromal cell proliferation and degenerating kidneys. These results suggest that the in vivo biological effects of ectopically expressed free hCGβ subunit are distinct from those of the hCG dimer and are gender specific. These transgenic mice are useful models for studying the biology of free hCGβ subunit, for further analyzing the gain of function effects of hCG during early Leydig cell development, and for studying the roles of hCG in ovarian and kidney pathophysiology and function.

[1]These studies were supported in part by funds (to T.R.K.) from the Moran Foundation (Department of Pathology, Baylor College of Medicine) and by NIH grant CA 60651 and Specialized Cooperative Centers Program in Reproduction Research grant HD-07495 (to M.M.M.). The Hormone Assay Core at the University of Virginia is supported by an NIH grant (U54-HD28-934) to the Center for Cellular and Molecular Studies in Reproduction.

[2]Correspondence: T. Rajendra Kumar, Department of Pathology, Baylor College of Medicine, Houston, TX 77030. FAX: 713 798 7505; e-mail: tkumar@bcm.tmc.edu

Received: 3 December 2002.
First decision: 17 December 2002.
Accepted: 7 March 2003.

ISSN: 0006-3363. http://www.biolreprod.org

female reproductive tract, male reproductive tract, ovary, pituitary, testis

INTRODUCTION

The placental gonadotropin hCG is a member of the heterodimeric glycoprotein hormone superfamily that includes LH, FSH, and thyroid-stimulating hormone [1]. These members share a common α subunit that is noncovalently associated with a hormone/receptor-specific β subunit to form biologically active heterodimers. The hCGβ and the human LHβ (hLHβ) genes are organized into a multigene cluster on chromosome 19; these genes and their encoded polypeptide sequences share significant identity [2]. Furthermore, LH and hCG bind to identical receptors that are coupled to G-proteins in the gonads of both sexes. Structurally, hCGβ is characterized by an *O*-glycosylated carboxy terminal peptide (CTP) that is not present in hLHβ [3]. This CTP sequence confers an extended serum half-life to hCG or to other family members such as LH and FSH when genetically fused and expressed in vitro or in vivo [3, 4]. Accordingly, more potent analogs have been generated and clinically tested for their efficiency in various in vitro fertilization protocols [5]. The CTP is a critical determinant for polarized apical secretion of hCG in Madin-Darby canine kidney cells, in contrast to the basolateral secretion of LH, which lacks this sequence [6].

Human CG is a pregnancy-specific hormone; it is not normally synthesized in men. Although hCG expression from the placenta is detected during the early stages of pregnancy, with peak levels attained during the first trimester, free hCGβ subunit is known to be expressed in many other tissues in women [7, 8]. Similarly, the LH/hCG receptor has a wide tissue expression pattern, including the uterus, brain, and duodenum [7, 8]. However, the biological significance of this expression is unknown. Moreover, free hCGβ subunit is expressed at high levels in multiple cancers and has been a useful diagnostic marker at least in some cases [9, 10]. It is not known what function the free β subunit exerts in this pathological condition. Similarly, elevated levels of pituitary gonadotropins are associated with ovarian cancer [11].

Our research group and others have used transgenic and knockout mouse technology to generate models for human diseases involving the gonadotropin-signaling cascade. Female FSHβ and FSH receptor (FSH-R) knockout mice phenocopy human ovarian dysgenesis, whereas the male mutant mice have apparently normal fertility (FSHβ knockouts) [12] or reduced fertility (FSH-R knockouts) [13, 14]. Human FSH-overexpressing male transgenic mice are infertile and have elevated testosterone levels. The female transgenic mice are also infertile and demonstrate symptoms similar to those of human ovarian hyperstimulation syndrome, including disrupted ovarian folliculogenesis

leading to hemorrhage and cyst formation and accompanied by urinary tract abnormalities [15]. Male transgenic mice that overexpress an LHβ-CTP analog are reportedly normal, whereas the female transgenic mice develop strain-dependent granulosa/stromal cell tumors, mammary tumors, pituitary adenomas, and defects in adrenal cortex development [16, 17]. Hypersecretion of LH in women causes polycystic ovarian syndrome, and this phenotype is attributed to a perturbed secretion of nonsteroidal ovarian hormones [18]. Two research groups have recently reported phenotypes of LH receptor (LH-R) knockout mice that phenocopy human LH-R-inactivating mutations [19, 20]. Typical features of these mutant mice include Leydig cell hypoplasia, reduced testosterone levels, and infertility in males. The female mice are infertile because of a blockade of ovarian folliculogenesis at the antral follicle stage. Activating mutations in the human LH-R gene cause male-limited precocious puberty, but women harboring the same mutation have normal fertility [21–24]. Clearly, these studies with mouse models and clinical cases suggest that the effects of gain and loss of function for gonadotropins are gender specific.

To distinguish the biological effects of free hCGβ and hCG dimer in vivo and to analyze the consequences of overexpression of hCGβ and hCG dimer in both male and female reproductive tract development and function, we used mouse metallothionein 1 (mMT-1) promoter to generate transgenic mice expressing either the hCGβ subunit or both hCG subunits together in multiple tissues. Here, we report distinct gender-specific phenotypes of these transgenic mice.

MATERIALS AND METHODS

Transgene Construction

The metallothionein (MT)-hCGα transgene was constructed as described previously [15]. A 1.8-kilobase (kb) mMT-1 promoter was inserted upstream of the 3.6-kb hCGβ gene [25]. The transgene fragments were released from the vector backbone with appropriate restriction enzyme digestions, purified using the GENECLEAN kit (Bio 101, Carlsbad, CA), and microinjectioned into fertilized eggs to produce transgenic mice [26].

Transgenic Mice

Independent lines of mice carrying either the MT-hCGα transgene or the MT-hCGβ transgene were separately generated by standard pronuclear injections into fertilized eggs from C57BL/6/C3H × ICR hybrid mice. Stable pedigrees of MT-hCGα transgenic mice were obtained by crossing Southern blot-positive founder mice with control wild-type littermates. Additionally, mice expressing hCG heterodimer were generated by coinjection of both MT-hCGα and MT-hCGβ transgenes. All animal studies were conducted in accordance with the NIH guidelines for Care and Use of Experimental Animals, as approved by Baylor College of Medicine.

Southern Blot Analysis

For genotype analysis of founders and offspring, Southern blot analysis was performed on tail DNA using ^{32}P-labeled probes as previously described [12, 15]. MT-hCGα transgenic mice were screened as described previously [15]. The MT-hCGβ transgene was detected with a probe made from 700 base pairs of exon 3 amplified by polymerse chain reaction (PCR) that does not cross-react with the endogenous mouse LHβ gene sequence.

Northern Blot Analysis

Total RNA was extracted from different tissues of wild-type, MT-hCG, or MT-hCGβ transgenic mice by the TRI-reagent (Leedo Medical Laboratories, Houston, TX) method [15]. RNA was denatured, separated on 1.4% agarose-formaldehyde gels, and transferred to nylon membranes. The membranes were hybridized with hCGα or hCGβ probes, washed, and exposed to autoradiographic film. Blots were then stripped and rehybridized with an 18S rRNA probe to check for equal loading of RNA [15].

Reverse Transcription PCR

Total RNA was isolated from multiple tissues of wild-type and transgenic mice and used in a reverse transcription (RT) reaction as previously described [15]. The first strand cDNA templates were used in a PCR with hCGβ-specific exon 3 primers (forward: 5′ ACC CGC GTG CTG CAG GGG 3′; reverse: 5′ TTA TTG TGG GAG GAT CGG 3′). The amplified products were separated on 1.5% agarose gels and transferred to GeneScreen plus (Perkin Elmer, Boston, MA) membranes, and Southern blot analysis was performed using a hCGβ-specific probe.

Hormone Assays

Mice under anesthesia were exsanguinated by closed cardiac puncture. Serum samples were collected and stored frozen at −20°C until further use. RIAs for LH and FSH were performed using National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) hormone assay kits according to the methods previously described [12, 15, 27] or by the standard protocols of the Ligand Assay and Analysis Core Laboratory (University of Virginia, Charlottesville, VA). Serum samples were analyzed for dimeric hCG by an ELISA using paired monoclonal antibodies or for hCGβ free subunit using an IRMA kit (Diagnostic Systems Laboratories, Webster, TX), according to the manufacturer's instructions. The testosterone and estradiol assays were performed as described previously using solid phase RIA kits [12, 15, 27].

Histological Analysis

Testes were fixed in Bouin reagent overnight and rinsed several times in $LiCO_3$-saturated 70% ethanol. Kidneys and ovaries were fixed in formalin. The tissues were processed and embedded in paraffin, and 4-μm sections were cut and stained with periodic acid-Schiff-hematoxylin reagents as previously described [12, 15, 27].

Statistical Anaylsis

Statistical analysis was performed with a Student *t*-test using a Microsoft Corp. (Redford, WA) Excel (version 6.0) software program. Differences were considered significant at $P < 0.05$.

RESULTS

Generation of Mice Carrying the MT-hCGα and MT-hCGβ Transgenes

We previously used a 1.2-kb fragment of the mMT-1 promoter to successfully overexpress various transgenes, including the hCG α subunit, in multiple tissues of mice [15, 28]. Using the same promoter sequences, we produced transgenic mice that carry the hCGβ subunit (Fig. 1, A–C). In addition, we generated mice that carry both the hCGα and hCGβ subunits by coinjecting the two transgenes into one-cell embryos. Southern blot analysis with specific probes identified transgenic founders that carried multiple copies of the hCGβ subunit or both the α and β subunits together in low (~5–10 copies) and high (>50 copies) numbers. Stable pedigrees could be established for MT-hCGα transgenic mice but not for transgenic mice that carried either the MT-hCGβ subunit or both subunits.

MT-hCGβ Subunit Transgenic Mice Are Infertile and Demonstrate Gonadal Defects

All of the transgenic male (three of three) and female mice (three of three) that carried the MT-hCGβ subunit were infertile when mated to either wild-type or hCGα subunit transgenic mice over a period of 6 mo, suggesting that the free hCGβ subunit, secreted from cells where it was produced, interfered with fertility in these mice. Expression of the hCGβ subunit was found in multiple tissues of trans-

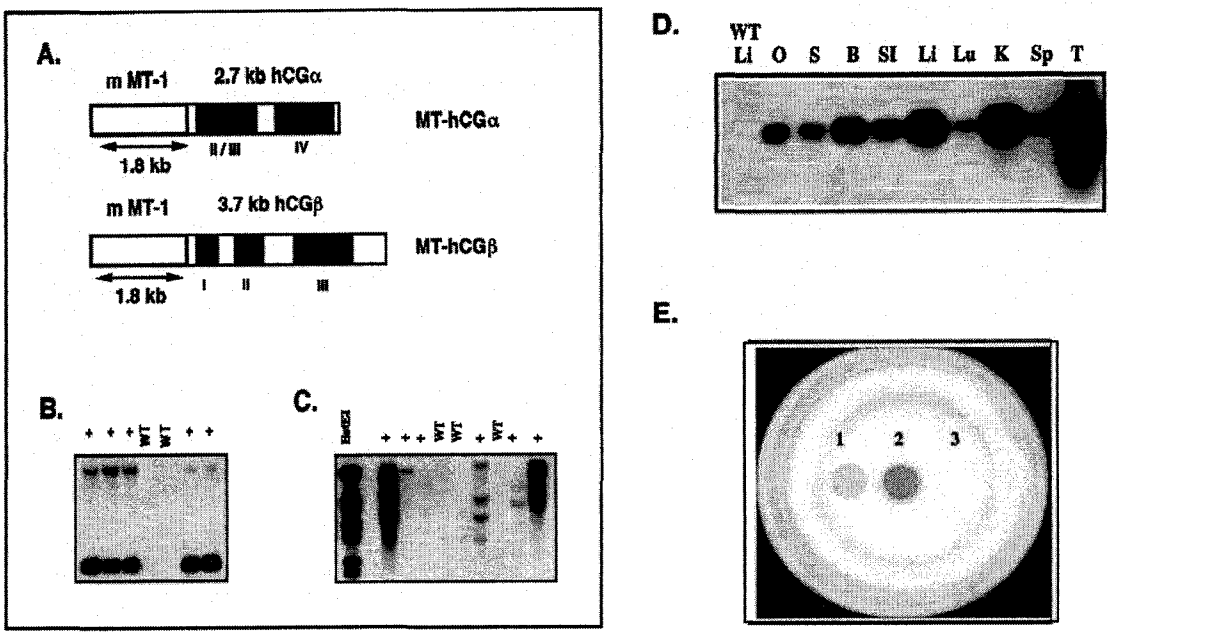

FIG. 1. Transgene identification and hCG expression analysis. The hCG subunit genes were cloned downstream of a 1.8-kb mMT-1 promoter (**A**). A 2.7-kb hCGα subunit minigene is expressed both in vitro and in vivo [4, 15]. Tail DNA (~5 μg) was digested with either *Hin*dIII or *Eco*RI/*Bam*HI enzymes, separated on agarose gels, transferred onto nitrocellulose membranes, and hybridized with a 700-bp hCGα subunit (**B**) or a 700-bp hCGβ subunit specific probe (**C**). Multiple transgenic founder mice were identified that carry the hCG subunit transgenes. WT, Wild-type; +, transgene positive. For RT-PCR analysis of transgene expression in adult tissues (**D**), total RNA was isolated from various tissues of adult transgenic mice and reverse transcribed, and hCGβ subunit-specific primers were used to amplify the cDNA templates in a PCR. The amplified products were subjected to Southern blot hybridization. Note the expression of hCGβ subunit in multiple tissues of a transgenic mouse (**D**). Previous Northern blot studies have similarly identified the expression of hCGα subunit in multiple tissues [15]. Total RNA from the wild-type mouse liver (WT-Li) and from the following tissues from transgenic mice were used for RT-PCR analysis: ovary (O), stomach (S), brain (B), small intestine (SI), liver (Li), lung (Lu), kidney (K), spleen (Sp), and testis (T). To confirm the hCG dimer expression, a urine sample was collected from an adult transgenic male founder carrying both MT-hCGα and MT-hCGβ subunits and evaluated with the ICON pregnancy spot test (Hybritech, San Diego, CA) (**E**) 1, positive control (equivalent to 25 IU of hCG); 2, urine sample from a male founder; 3, negative control. Note the bright spot from the transgenic mouse urine sample (lane 2) indicating the expression of intact hCG heterodimer.

genic mice, similar to the expression of the hCGα subunit [15], by RT-PCR assay (Fig. 1D) and Northern blot analyses [15] (data not shown). A dimer-specific immunoradiometric assay did not detect any hCG dimer in the serum of the hCGβ subunit transgenic mice (data not shown). Although testes of the infertile MT-hCGβ male transgenic mice appeared normal morphologically and histologically, ovaries from the female transgenic mice demonstrated a block in folliculogenesis. These ovaries did not contain antral follicles or corpora lutea, indicating that the mice did not cycle. In some cases, the female transgenic mice developed cysts and hemorrhages and enlargement of the uterine horns. These results demonstrate that the hCGβ subunit-expressing mice are infertile, and the female transgenic mice have ovarian defects.

Low-Level hCG Dimer-Expressing Transgenic Mice Develop Progressive Infertility

Because the MT-hCGβ-expressing transgenic mice were infertile, to study the gain of function effects of the hCG dimer, we coinjected both the MT-hCGα and MT-hCGβ transgenes into mouse embryos to generate hCG dimer-expressing transgenic mice. Male and female mice that carry both the transgenes in low copy numbers (~5–10 copies) were fertile initially and produced offspring that carried both the transgenes. The litter sizes were normal, with an average of 8.3 ± 0.3 pups/litter (35 litters, five mating pairs), when compared with those from wild-type matings (8.3 ± 0.2 pups/litter, 104 litters, 12 mating pairs). However, these transgenic male and female mice progressively became infertile by 6–7 mo of age but displayed no other apparent differences. The gonads were normal, with no discernible evidence of histological abnormalities (data not shown). Thus, constitutive low-level expression of hCG causes progressive infertility of unknown etiology in both male and female transgenic mice.

High-Level hCG Dimer-Expressing Male Transgenic Mice Are Infertile and Develop Leydig Cell Hyperplasia

In contrast to the low-level hCG dimer-expressing mice, male founder mice that expressed high levels of hCG dimer were all (five of five) infertile. These male mice never mated with females (no vaginal plugs) of any genotype (wild-type or transgenic) over a period of 6 mo. They had very high levels of serum hCG dimer and consequently elevated testosterone levels (Table 1). As a result of the elevated levels of testosterone, these male transgenic mice demonstrated massive enlargement of fluid-filled seminal vesicles (Table 1 and Fig. 2, A and B). The testis size in these mice was reduced (Fig. 2, A and B), and histological analysis revealed significant Leydig cell hyperplasia (Fig. 2, F and H), with some tubules demonstrating a "Sertoli cell only" phenotype (Fig. 2G) characteristic of germ cell loss in mice and humans. Occasionally, many vacuolated tubules were also observed near the periphery of the tubules (Fig. 2, F and H). Serum LH and FSH levels were suppressed in these

male mice compared with the wild-type controls (Table 1), presumably because of heightened steroid feedback (Table 1). These results suggest that high levels of hCG cause multiple male reproductive defects, including Leydig cell hyperplasia, seminal vesicle enlargement, and infertility.

High-Level hCG Dimer-Expressing Female Transgenic Mice Are Infertile and Develop Polycystic Ovaries and Ovarian Thecomas

MT-hCG female transgenic mice that express high levels of the hCG dimer were all (four of four) infertile when mated to wild-type male mice. Serum analysis of these mice demonstrated elevated levels of estradiol (Table 1) consistent with the presence of enlarged uterine horns, compared with levels in wild-type mice (Table 1 and Fig. 3, A and G). These transgenic mice also displayed a dramatic ovarian phenotype by 6–7 wk of age, with massive hemorrhage and the presence of multiple cysts (Fig. 3, G–I). Histological analysis revealed multiple aberrant ovarian follicles with enlarged thecal cell layers (Fig. 3, J and K) and proliferating stromal tissue. Many of these stromal cells appeared multinucleated (Fig. 3K).

Additional Phenotypes in High-Level hCG Dimer-Expressing Transgenic Mice

In addition to the gonadal phonotypes described above, the high-level hCG dimer-expressing mice demonstrated phenotypes in nongonadal tissues. Male mice overexpressing hCG dimer at high levels were behaviorally very aggressive; they were ferocious and displayed mutilating behavior when caged with either transgenic or nontransgenic control males or females. The aggressive behavior was noted only in males and not in females expressing hCG dimer, suggesting that the high testosterone was causal for this behavioral finding. Serum estradiol levels were not elevated in these male mice

TABLE 1. Comparison of wild-type and hCG dimer-overexpressing transgenic mice.[a]

Characteristic	Wild type	Transgenic
Adult males (≥12–14 wk old)		
Testis weight (mg)	121 ± 5.4	56.8 ± 8.6[b]
Seminal vesicle weight (g)	0.3 ± 0.02	2.6 ± 0.8[b]
Urinary hCG (ng/ml)	Undetectable	1600 ± 500
Serum hCG (ng/ml)	Undetectable	237 ± 5.5
Serum testosterone (ng/ml)	0.6 ± 0.1	14.5 ± 1.3[b]
Serum LH (ng/ml)	7.3 ± 0.6	0.5 ± 0.3[b]
Serum FSH (ng/ml)	64.8 ± 4.8	20.4 ± 1.4
Serum estradiol (pg/ml)	<5	<5
Adult females (≥12–14 wk old)		
Urinary hCG (ng/ml)	Undetectable	7100 ± 300
Serum hCG (ng/ml)	Undetectable	610 ± 45
Serum estradiol (pg/ml)[c]	<5.0	69 ± 4[b]

[a] Obtained from two separate microinjection experiments by coinjecting equimolar concentrations of MT-hCGα and MT-hCGβ subunit transgenes. Five of five male and four of four female founders were infertile. All reported values are mean ± SEM from three to five mice.
[b] Significantly different from the value in wild-type mice ($P < 0.05$).
[c] Estimated by a second generation ultrasensitive assay (sensitivity is 5 pg/ml).

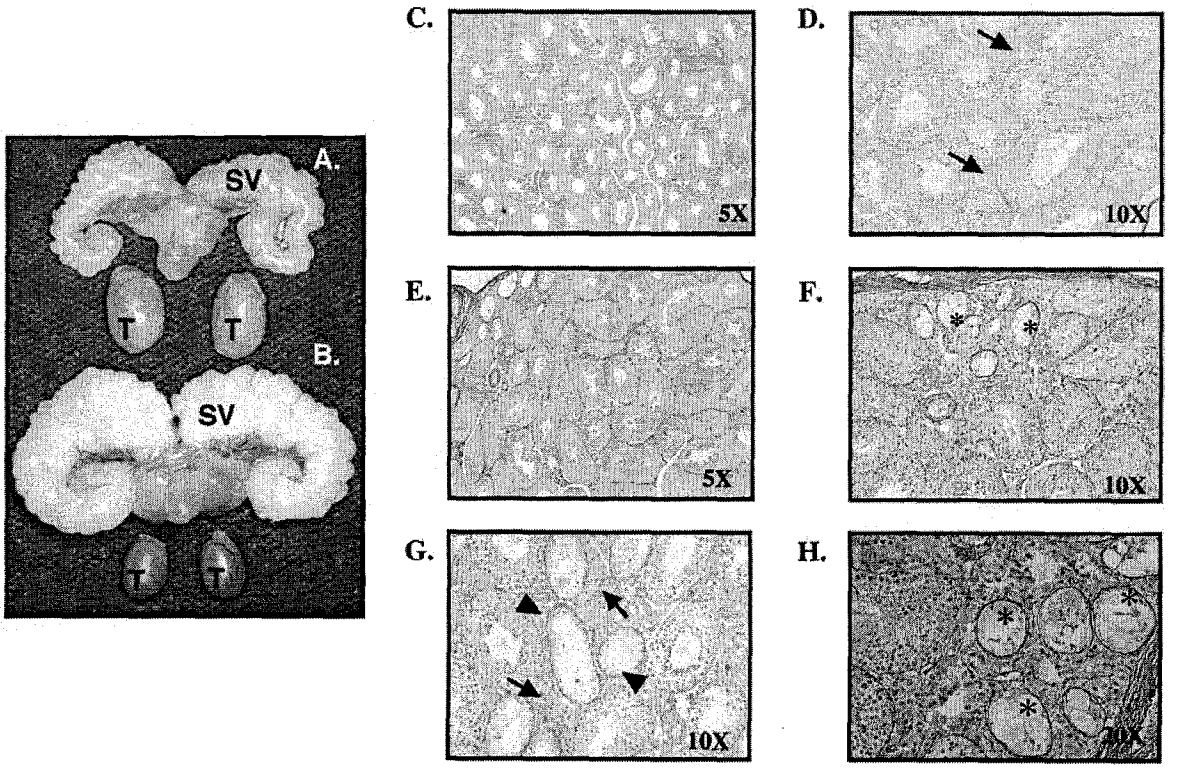

FIG. 2. Male reproductive phenotypes of MT-hCG dimer-expressing transgenic mice. Gross morphology of testes and seminal vesicles of adult wild-type (WT) (**A**) and MT-hCG transgenic (**B**) mice. Note the enlarged seminal vesicles and reduced size of the testes obtained from the hCG transgenic mouse compared with those from the WT mouse. Histological analysis revealed normal Leydig cells and tubule architecture in the testis of a WT mouse (**C** and **D**) and the presence of Leydig cell hyperplasia (**E–H**), vacuolated tubules (**F–H**), and Sertoli cell-only tubules (**G**) in the testis of an hCG transgenic mouse. Arrows indicate Leydig cell islands in the WT and transgenic mouse testes. Arrowheads (**G**) indicate Sertoli cell-only tubules. Asterisks (**F**, low power; **H**, high power) indicate vacuoles in tubules. T, Testis; SV, seminal vesicle.

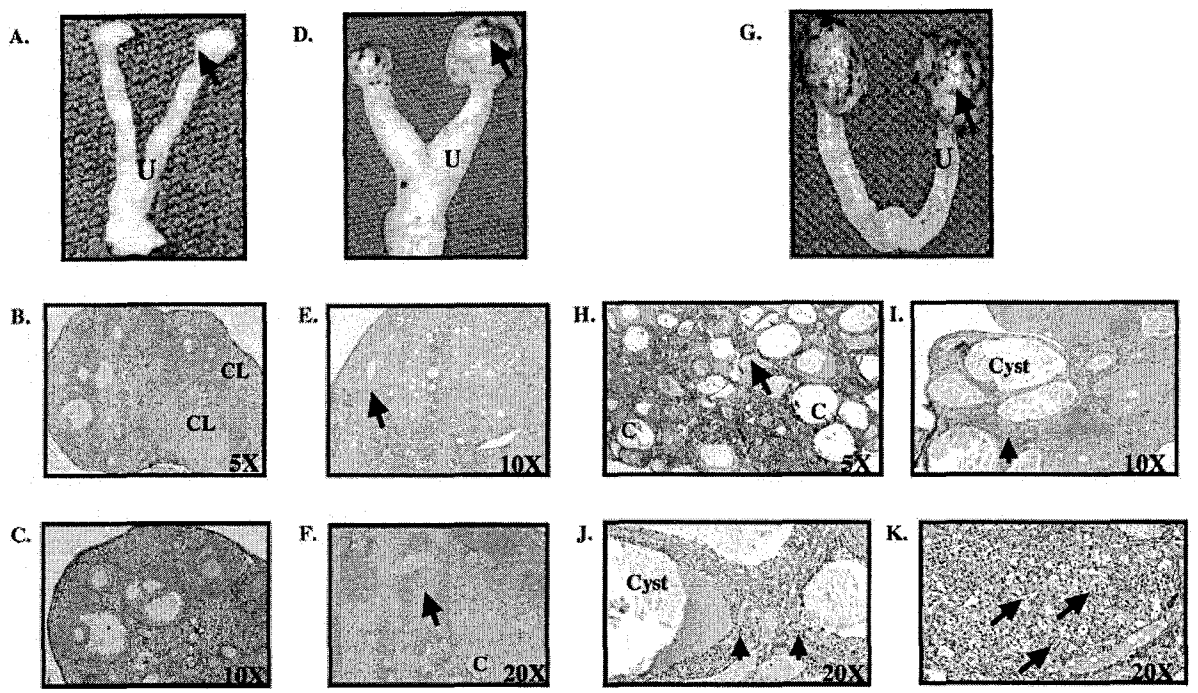

FIG. 3. Gross and histological analysis of the ovaries in wild-type (**A–C**), MT-hCGβ transgenic (**D–F**), and hCG dimer-expressing transgenic (**G–K**) mice. Female transgenic mice carrying the MT-hCGβ subunit were infertile because of ovarian defects. Gross morphology of the ovary and the uterine horns of a 12 wk-old female MT-hCGβ transgenic mouse (**D**) reveals enlarged uteri and enlarged cystic and hemorrhagic ovaries compared with those in a wild-type female mouse (**A**). Histological analysis of the ovary (**E** and **F**) reveals disrupted folliculogenesis, many small cysts (one designated C in **F**), and multiple hemorrhagic spots. There was a rare tubule-like structure in this ovarian section (arrow). Similar to mice expressing only MT-hCGβ subunit, MT-hCG heterodimer-expressing female mice are also infertile. Note the enlarged uterine horns and ovaries with multiple hemorrhagic spots in a MT-hCG dimer-expressing mouse (**G**). Histological analysis of the ovary (**H–K**) indicates disrupted folluculogenesis with hemorrhage and multiple cysts. Many of the disrupted follicles have multiple thecal layers instead of the normal single layer in the wild-type ovary (**B**, **C**, **I** and **J**). No corpora lutea are visible in MT-hCGβ and MT-hCG dimer transgenic mouse ovaries in contrast to the wild-type ovarian section, in which corpora lutea are present (**B** and **C**). Multiple layers of thecal cells in two adjacent follicles are visible (**I** and **J**). Multinucleated stromal cells are evident in the interstitium between two follicles (**K**). U, Uterus; CL, corpus luteum; C, cyst.

(Table 1), suggesting that the observed aggressive phenotype in male transgenic mice is not due to an estrogen effect.

In contrast to these behavioral defects in males, the female transgenic mice overexpressing hCG developed urinary tract defects, including marked enlargement of the kidneys and bladder coincident with the ovarian hemorrhage and cysts (Fig. 4, A–D) (data not shown). Histological analysis of the enlarged kidneys indicated total disruption of the glomerular architecture, and many cystic spaces suggested degeneration of the glomeruli (Fig. 4, D and E) compared with normal glomerular architecture in the wild-type mouse kidneys (Fig. 4C).

Despite the above phenotypes resulting from prolonged hCG stimulation, the mammary and the adrenal glands in these mice were morphologically and histologically normal at all ages examined (data not shown).

DISCUSSION

We previously generated and analyzed the phenotypes of MT-hCGα, MT-FSHβ, and MT-FSH transgenic mice. High levels of expression of these transgenes was achieved using an mMT-1 promoter. The MT-1 promoter is activated very early during mouse embryogenesis and is ubiquitously expressed. It is also highly expressed in the liver. We used this same promoter (~1.8 kb) to generate multiple MT-hCGβ transgenic mice. Similar to our earlier results, we obtained mice strains for both low and high transgene copy numbers. The MT-hCGβ transgene was expressed at high levels in multiple tissues, consistent with the high basal activity of the MT-1 promoter in multiple tissues of the mouse.

In contrast to the normal fertility of male and female MT-hCGα and male MT-hFSHβ transgenic mice, both male and female transgenic mice expressing MT-hCGβ were infertile. This result is probably related to the intracellular assembly and secretion behavior of glycoprotein hormone free subunits. Free hFSHβ is not efficiently secreted from cells, whereas free hCGβ is efficiently secreted in vivo and in vitro [25, 29, 30]. Free hCGβ subunit is secreted into serum in the MT-hCGβ transgenic mice, interfering with endogenous LH binding to LH/hCG receptors on gonads and thus causing male infertility. There is some in vitro evidence to suggest that free hCGβ subunit can bind LH/hCG receptors and block steroidogenesis [31, 32]. However it is not known what effects large amounts of hCGβ alone can elicit when secreted from multiple tissues, including the testis. Male mice expressing only hCGβ only are infertile even though hCGβ acts as a weak agonist in LH/hCG receptor binding assays and in vitro its activity is only 1–2% that of the intact heterodimer. Contrary to the expected regressed testis phenotype, these male mice demonstrated apparently normal testis histology. The infertility in these male mice could be the result of quantitative and functional differences in spermatogenesis, although qualitatively all stages of spermatogenesis appear normal. In female transgenic mice expressing only hCGβ, which were also infer-

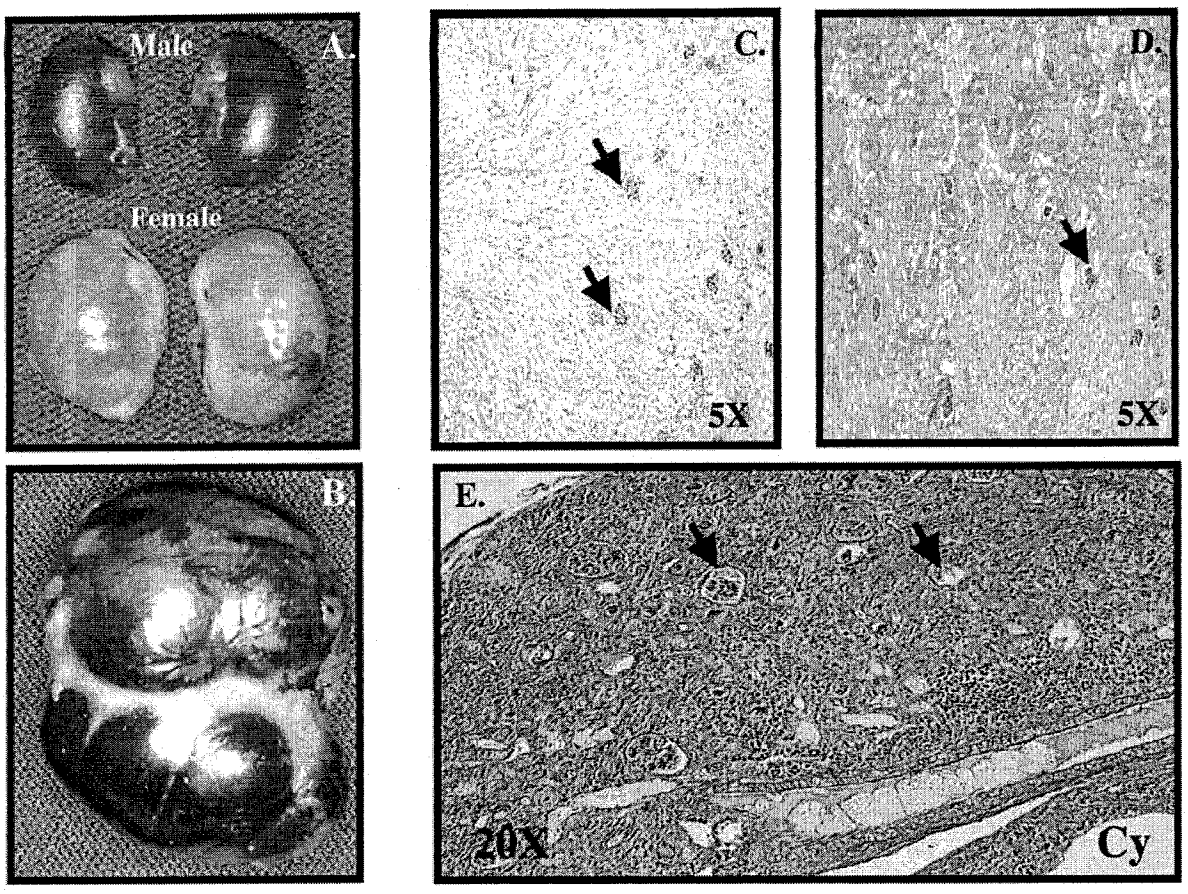

FIG. 4. Gross views of the kidneys from hCG dimer-expressing male (top) and female (bottom) transgenic mice at 4 (**A**) and 7 (**B**) mo of age. Note the significant enlargement of the cystic kidneys from female hCG transgenic mouse compared with those in the transgenic male mouse. Histological analysis of the enlarged kidney of the female transgenic mouse revealed dilated tubules with disrupted glomeruli (**D** and **E**, arrows) compared with normal tubules and glomeruli in the kidney of a wild-type female (**C**) or a transgenic male mouse (data not shown). Majority of the kidney contained a large cyst (cy) with many damaged glomeruli at the periphery (**E**).

tile, additional ovarian cystic and hemorrhagic phenotypes may be caused by alteration of local factors or by a direct effect of locally produced hCGβ subunit within the ovary (mMT-1 promoter is active in the ovary) [15]. Furthermore, the phenotypes of mice expressing only hCGβ and those expressing the hCG dimer are distinct, suggesting that ubiquitous expression of hCGβ does not cause bioactive production of hCG heterodimer by assembling with the mouse α-glycoprotein subunit (GSU) in the pituitary, as has been recently reported in ubiquitin C-hCGβ transgenic mice. Bioactive hCG dimer was not produced in these MT-hCGβ transgenic mice, consistent with previous observations indicating MT-1 promoter is not active in pituitary gonadotrope cells [15].

Because the transgenic male and female mice expressing MT-hCGβ subunit were infertile, we coinjected the individual subunit transgenes to generate mice expressing the hCG dimer. This strategy resulted in production of female mice that expressed low and high levels of hCG dimer. Although, initially normal and fertile, the low-level hCG dimer-expressing mice progressively became infertile by 5–6 mo of age. This finding suggests that sustained production of hCG from multiple tissues can cause infertility, although the exact mechanism remains to be elucidated. Similar production of low levels of FSH from the same promoter does not affect male or female fertility [15, 33]. However, in each case, it is difficult to compare levels of FSH and hCG, their receptor binding capacities, and their bioactivity in the target cells. It is also difficult to assess how the steroid and nonsteroidal gonadal hormone feedback mechanisms operate at the hypothalamus-pituitary level in each case.

High-level hCG dimer-expressing male mice had multiple reproductive defects. Most of these appeared to be secondary to elevated serum testosterone levels and included suppression of endogenous LH and FSH levels and greatly enlarged seminal vesicles. Similar to hFSH transgenic male mice, hCG-overexpressing male mice were infertile. The important difference between these two strains of mice is that testes were normal size in hFSH-overexpressing mice but were small in hCG-overexpressing male mice. Furthermore, unlike hFSH-overexpressing mice, these mice were highly aggressive and displayed abnormal sexual behavior. The phenotypes in hCG dimer-expressing male mice are in sharp contrast to normal fertility in transgenic male mice in which the expression of an LH analog (LHβ-CTP) was targeted to the gonadotropes using a bovine glycoprotein hormone α subunit promoter [34]. Similarly, these phenotypes are not present in male human pa-

TABLE 2. Gain of function mouse models for LH/hCG.

Promoter	Gene/cDNA	Sites of production	Major findings	References
MT-1	α-GSU	Multiple tissues	No defects, normal fertility	15, 33
MT-1	hCGβ	Multiple tissues	Males and females infertile, cystic and hemorrhagic ovaries	Present study
MT-1	α-GSU + hCGβ: low levels	Multiple tissues	Progressive infertility in males and females	Present study
	α-GSU + hCGβ: high levels	Multiple tissues	Males infertile, testis size reduced, Leydig cell hyperplasia, enlarged seminal vesicles, serum testosterone levels elevated, and aggressive behavior; females infertile, serum estrogen levels elevated, hemorrhagic and cystic ovaries, thecal and stromal cell expansion, and cystic kidneys	Present study
hCGβ	hCGβ cluster	Multiple tissues	No defects	35
bLHα	bLHβ-CTP	Pituitary	Males normal; females exhibit precocious puberty, serum estrogen levels elevated, ovarian granulosa/stromal cell tumors, pituitary adenomas, mammary tumors, and pyelonephritis	34, 43
Ubiquitin C	hCGβ	Multiple tissues	Male phenotypes unknown; females exhibit increased bioactive hCG in serum, precocious puberty, serum estrogen levels elevated, prolactinomas, mammary adenocarcinomas	42

tients who demonstrate precocious puberty due to constitutively active LH-R signaling [23]. The aggressive phenotype in hCG-overexpressing mice could have two causes: 1) high levels of testosterone are known to affect male behavior, and 2) local production of hCG in regions of the brain known to be important for aggressive and sexual behavior might cause alterations in LH/hCG receptor signaling. In transgenic mice that harbor a 36-kb cosmid transgene consisting of six hCGβ genes driven by the homologous promoter, a different subset of CGβ genes, CGβ1 and CGβ 2, normally not transcribed in human placenta, are abundantly expressed in the cerebral cortex [35]. Similarly, some evidence suggests that LH/hCG receptors and orphan receptors belonging to the glycoprotein hormone receptor family, the leucine-rich repeat-containing G protein-coupled receptors, are present within the hypothalamus and brain, respectively [36, 37]. The high levels of hCG in the brain of our hCG transgenic mice might be interfering with these signaling pathways, resulting in behavioral defects.

Another important testis phenotype of hCG-overexpressing mice is Leydig cell hyperplasia. Leydig cells develop as two distinct populations during testis development and are dependent upon LH stimulation. Because the MT-1 promoter is active very early during mouse embryogenesis, continuous production/stimulation by hCG (which binds the LH receptor) can result in Leydig cell hyperplasia, leading to the high testosterone production that we observed. The Leydig cell phenotype of hCG-overexpressing transgenic mice is consistent with previous observations from in vitro and in vivo studies, in which continuous injections of pharmacological doses of hCG for a prolonged time in rats and mice resulted in accelerated Leydig cell hyperplasia with a corresponding elevation in serum testosterone levels. Future studies could focus on determining the aberrant cell cycle events triggered by overexpression of hCG (continuously from embryo to adult stage) that lead to the Leydig cell phenotype in these mice.

Female transgenic mice that overexpress hCG demonstrated reproductive and urinary tract defects. These mice had very high serum estradiol levels and enlarged uterine horns. Similar to the LHβ-CTP transgenic mice, these female mice were infertile and had hemorrhagic and cystic ovaries [38, 39]. In contrast to granulosa cell tumors that develop in a genetic strain-dependent manner in LHβ-CTP female transgenic mice, hCG-overexpressing female mice developed thecomas with excessive interstitial cell proliferation, and the ovaries demonstrated disrupted folliculogenesis. Other similarities between these two strains of transgenic mice are the kidney defects that are specific to females. The renotropic activity of LH has been reported; however, the biological basis for this has not been explored [40, 41]. Transgenic female mice overexpressing hCG develop enlarged cystic kidneys with progressive degeneration of glomerular architecture (Fig. 4C). Prolonged hCG stimulation of the kidneys in these female transgenic mice might be causing the glomerular degeneration.

More recently, female transgenic mice overexpressing the hCGβ subunit driven by a ubiquitin C promoter have been generated [42]. Unlike the 100% infertility that was observed with our MT-hCGβ mice, two of the five founder ubiquitin C-hCGβ transgenic mice were fertile. These mice had high levels of bioactive serum hCG (because of the heterodimeric assembly of the hCGβ with the mouse α-GSU in the pituitary), leading to increased secretion of ovarian steroids. Additional phenotypes in these mice resemble those seen in bovine glycoprotein hormone α-LHβ-CTP mice and include precocious puberty, pituitary (lactotrope) adenomas accompanied by hyperprolactinemia, and mammary adenocarcinoma. No kidney changes were reported. It is not known whether male transgenic mice expressing hCGβ from the ubiquitin C promoter demonstrate any reproductive anomalies.

The distinct phenotypes observed in our hCG dimer-expressing male mice suggest that the extent of hCG overexpression may be critical for functional activity. Because MT-1 promoter is active very early during mouse devel-

opment, and LH-Rs are expressed at this stage, we hypothesize that the defects we observed in our transgenic mice are a result of both abnormal morphogenesis and hyperaction of the hormone. Further, because the hCG-overexpressing founder male and female mice were all infertile, it was not possible to evaluate the progression of phenotypes with age. In future studies, we plan to use a tetracyclin on/off system to produce mice that express hCG in a temporal fashion and to evaluate the resultant phenotypes during multiple developmental stages.

We used the mMT-1 promoter to develop transgenic mice that constitutively expressed either hCGβ subunit or hCG dimer in multiple tissues. The phenotypes of mice expressing only hCGβ are distinct from those of mice expressing the hCG dimer. Furthermore, hCG dimer-expressing mice are similar to other existing gonadotropin mouse models and display additional unique reproductive, urinary tract, and behavioral phenotypes (Table 2), suggesting the importance of pituitary targeted versus ectopic expression of gonadotropic hormones in reproductive physiology. The hCG-overexpressing transgenic mice may be useful for further study of the mechanisms of Leydig cell hyperplasia and ovarian hyperstimulation syndrome accompanied by kidney defects.

ACKNOWLEDGMENTS

We thank Ms. Jennifer Newton for preparing the manuscript, Dr. Irving Boime and Ms. Kathleen Burns for critically reading the manuscript, Ms. Susan Huang for helping with the genotype analysis of mice, Mr. Carlos Talavera for advice on digital imaging, and Ms. Grace Hamilton and Mr. Bliss Walker for assistance with histology of specimens. We thank Dr. William Moyle for performing hCG dimer ELISAs and Dr. A.F. Parlow (NHPP, NIDDK) for providing the gonadotropin RIA kits.

REFERENCES

1. Bousfield GR, Perry WM, Ward DN. Gonadotropins: chemistry and biosynthesis. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1994:1749–1792.
2. Albanese C, Colin IM, Crowley WF, Ito M, Pestell RG, Weiss J, Jameson JL. The gonadotropin genes: evolution of distinct mechanisms for hormonal control. Recent Prog Horm Res 1996; 51:23–58.
3. Boime I, Ben-Menahem D. Glycoprotein hormone structure-function and analog design. Recent Prog Horm Res 1999; 54:271–288.
4. Matzuk MM, Hsueh AJ, Lapolt P, Tsafriri A, Keene JL, Boime I. The biological role of the carboxyl-terminal extension of human chorionic gonadotropin [corrected] beta-subunit. Endocrinology 1990; 126:376–383.
5. Garcia-Campayo V, Boime I. Novel recombinant gonadotropins. Trends Endocrinol Metab 2001; 12:72–77.
6. Jablonka-Shariff A, Garcia-Campayo V, Boime I. Evolution of lutropin to chorionic gonadotropin generates a specific routing signal for apical release in vivo. J Biol Chem 2002; 277:879–882.
7. Rao CV. Multiple novel roles of luteinizing hormone. Fertil Steril 2001; 76:1097–1100.
8. Rao CV. An overview of the past, present, and future of nongonadal LH/hCG actions in reproductive biology and medicine. Semin Reprod Med 2001; 19:7–17.
9. Gallo RC, Bryant J, Lundardi-Iskandar Y. Antitumor effects of hCG in KS. Nat Biotechnol 1998; 16:218.
10. Gill PS, Lunardi-Ishkandar Y, Louie S, Tulpule A, Zheng T, Espina BM, Besnier JM, Hermans P, Levine AM, Bryant JL, Gallo RC. The effects of preparations of human chorionic gonadotropin on AIDS-related Kaposi's sarcoma. N Engl J Med 1996; 335:1261–1269.
11. Konishi I, Kuroda H, Mandai M. Review: gonadotropins and development of ovarian cancer. Oncology 1999; 57(suppl 2):45–48.
12. Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 1997; 15:201–204.
13. Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. Endocrinology 2000; 141:1795–1803.
14. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci U S A 1998; 95:13612–13617.
15. Kumar TR, Palapattu G, Wang P, Woodruff TK, Boime I, Byrne MC, Matzuk MM. Transgenic models to study gonadotropin function: the role of follicle-stimulating hormone in gonadal growth and tumorigenesis. Mol Endocrinol 1999; 13:851–865.
16. Keri RA, Lozada KL, Abdul-Karim FW, Nadeau JH, Nilson JH. Luteinizing hormone induction of ovarian tumors: oligogenic differences between mouse strains dictates tumor disposition. Proc Natl Acad Sci U S A 2000; 97:383–387.
17. Risma KA, Clay CM, Nett TM, Wagner T, Yun J, Nilson JH. Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. Proc Natl Acad Sci U S A 1995; 92:1322–1326.
18. Balen AH. Hypersecretion of luteinizing hormone and the polycystic ovary syndrome. Hum Reprod 1993; 8(suppl 2):123–128.
19. Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV. Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. Mol Endocrinol 2001; 15:184–200.
20. Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. Mol Endocrinol 2001; 15:172–183.
21. Huhtaniemi I. Activating and inactivating hormone receptor mutations. Horm Res 2000; 53:9–16.
22. Kosugi S, Van Dop C, Geffner ME, Rabl W, Carel JC, Chaussain JL, Mori T, Merendino JJ Jr, Shenker A. Characterization of heterogeneous mutations causing constitutive activation of the luteinizing hormone receptor in familial male precocious puberty. Hum Mol Genet 1995; 4:183–188.
23. Shenker A, Laue L, Kosugi S, Merendino JJ Jr, Minegishi T, Cutler GB Jr. A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. Nature 1993; 365:652–654.
24. Themmen AP, Martens JW, Brunner HG. Activating and inactivating mutations in LH receptors. Mol Cell Endocrinol 1998; 145:137–142.
25. Keene JL, Matzuk MM, Boime I. Expression of recombinant human choriogonadotropin in Chinese hamster ovary glycosylation mutants. Mol Endocrinol 1989; 3:2011–2017.
26. Kumar TR, Fairchild-Huntress V, Low MJ. Gonadotrope-specific expression of the human follicle-stimulating hormone beta-subunit gene in pituitaries of transgenic mice. Mol Endocrinol 1992; 6:81–90.
27. Kumar TR, Wiseman AL, Kala G, Kala SV, Matzuk MM, Lieberman MW. Reproductive defects in gamma-glutamyl transpeptidase-deficient mice. Endocrinology 2000; 141:4270–4277.
28. Guo Q, Kumar TR, Woodruff T, Hadsell LA, DeMayo FJ, Matzuk MM. Overexpression of mouse follistatin causes reproductive defects in transgenic mice. Mol Endocrinol 1998; 12:96–106.
29. Corless CL, Matzuk MM, Ramabhadran TV, Krichevsky A, Boime I. Gonadotropin beta subunits determine the rate of assembly and the oligosaccharide processing of hormone dimer in transfected cells. J Cell Biol 1987; 104:1173–1181.
30. Keene JL, Matzuk MM, Otani T, Fauser BC, Galway AB, Hsueh AJ, Boime I. Expression of biologically active human follitropin in Chinese hamster ovary cells. J Biol Chem 1989; 264:4769–4775.
31. Dighe RR, Muralidhar K, Moudgal NR. Ability of human chorionic gonadotropin beta-subunit to inhibit the steroidogenic response to lutropin. Biochem J 1979; 180:573–578.
32. Moudgal NR, Li CH. Beta subunits of human choriogonadotropin and ovine lutropin are biologically active. Proc Natl Acad Sci U S A 1982; 79:2500–2503.
33. Kumar TR, Low MJ, Matzuk MM. Genetic rescue of follicle-stimulating hormone beta-deficient mice. Endocrinology 1998; 139:3289–3295.
34. Nilson JH, Abbud RA, Keri RA, Quirk CC. Chronic hypersecretion of luteinizing hormone in transgenic mice disrupts both ovarian and pituitary function, with some effects modified by the genetic background. Recent Prog Horm Res 2000; 55:69–89.
35. Strauss BL, Pittman R, Pixley MR, Nilson JH, Boime I. Expression of the beta subunit of chorionic gonadotropin in transgenic mice. J Biol Chem 1994; 269:4968–4973.
36. Lei ZM, Rao CV. Neural actions of luteinizing hormone and human chorionic gonadotropin. Semin Reprod Med 2001; 19:103–109.

37. Hsu SY, Kudo M, Chen T, Nakabayashi K, Bhalla A, van der Spek PJ, van Duin M, Hsueh AJ. The three subfamilies of leucine-rich repeat-containing G protein-coupled receptors (LGR): identification of LGR6 and LGR7 and the signaling mechanism for LGR7. Mol Endocrinol 2000; 14:1257–1271.
38. Kero J, Poutanen M, Zhang FP, Rahman N, McNicol AM, Nilson JH, Keri RA, Huhtaniemi IT. Elevated luteinizing hormone induces expression of its receptor and promotes steroidogenesis in the adrenal cortex. J Clin Invest 2000; 105:633–641.
39. Risma KA, Hirshfield AN, Nilson JH. Elevated luteinizing hormone in prepubertal transgenic mice causes hyperandrogenemia, precocious puberty, and substantial ovarian pathology. Endocrinology 1997; 138: 3540–3547.
40. Nomura K, Demura H, Shizume K. Stimulation of renal deoxyribonucleic acid synthesis by a pituitary-derived renotropin and its inhibition by testosterone and thyroxine. Endocrinology 1985; 116:616–621.
41. Nomura K, Nakamura Y, Ujihara M, Ohmura K, Toraya S, Horiba N, Demura H. Renotropic and gonadotropic activity in homologous and heterologous hybrids of ovine luteinizing hormone and human chorionic gonadotropin subunits. Acta Endocrinol (Copenh) 1991; 125: 590–594.
42. Rulli SB, Kuorelahti A, Karaer O, Pelliniemi LJ, Poutanen M, Huhtaniemi I. Reproductive disturbances, pituitary lactotrope adenomas, and mammary gland tumors in transgenic female mice producing high levels of human chorionic gonadotropin. Endocrinology 2002; 143: 4084–4095.
43. Milliken EL, Ameduri RK, Landis MD, Behrooz A, Abdul-Karim FW, Keri RA. Ovarian hyperstimulation by LH leads to mammary gland hyperplasia and cancer predisposition in transgenic mice. Endocrinology 2002; 143:3671–3680.