

# Mutations of Gonadotropins and Gonadotropin Receptors: Elucidating the Physiology and Pathophysiology of Pituitary-Gonadal Function

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## ABSTRACT

The recent unraveling of structures of genes for the gonadotropin subunits and gonadotropin receptors has provided reproductive endocrinologists with new tools to study normal and pathological functions of the hypothalamic-pituitary-gonadal axis. Rare inactivating mutations that produce distinctive phenotypes of isolated LH or FSH deficiency have been discovered in gonadotropin subunit genes. In addition, there is a common polymorphism in the LH $\beta$  subunit gene with possible clinical significance as a contributing factor to pathologies of LH-dependent gonadal functions. Both activating and inactivating mutations have been detected in the gonadotropin receptor genes, a larger number in the LH re-

ceptor gene, but so far only a few in the gene for the FSH receptor. These mutations corroborate and extend our knowledge of clinical consequences of gonadotropin resistance and inappropriate gonadotropin action. The information obtained from human mutations has been complemented by animal models with disrupted or inappropriately activated gonadotropin ligand or receptor genes. These clinical and experimental genetic disease models form a powerful tool for exploring the physiology and pathophysiology of gonadotropin function and provide an excellent example of the power of molecular biological approaches in the study of pathogenesis of diseases. (*Endocrine Reviews* 21: 551–583, 2000)

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## I. Introduction

THE INFORMATION of genetic causes of human diseases is accumulating with increasing speed and volume. This development has become possible through unraveling of struc-

tures and functions of genes present in the human genome, a task that is almost complete. Although infertility is not conventionally considered an inherited condition, a growing number of mutations specifically affecting reproductive function have been detected in humans and characterized in genetically modified animals. Among single-gene mutations affecting hypothalamic-pituitary-gonadal function (1, 2), we know today those of the orphan nuclear receptor DAX1, extracellular protein KAL (anosmin), the receptor of GnRH, the homeodomain genes HES1 and PROP1, the receptors for estrogen and androgen, the steroidogenic acute regulatory protein (StAR), a number of steroidogenic enzymes, and finally those of gonadotropins and their receptors, the topic of this review.

Mutations of genes concerned with hypothalamic-pituitary-gonadal function, due to their critical role in the development and regulation of reproductive functions, are understandably very rare and therefore not of major concern within the clinical practice of infertility treatment. However, they form today a class of diagnoses that must be taken into account upon differential diagnostics of aberrant and delayed sexual differentiation and development, as well as infertility. In addition, by displaying distinct phenotypes, these conditions have turned out to be very elucidating with regard to the main facets and certain poorly characterized details of the hormonal control of reproduction. These naturally occurring mutations are often corroborated by genetically manipulated animal models with astonishingly similar phenotypes to those of the human diseases. The same applies to the currently known human mutations of gonadotropin and gonadotropin receptor genes, as well as to their animal models.

Whereas the hormone ligand mutations that have been

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found to date usually represent loss-of-function mutations (homozygotes or compound heterozygotes have a phenotype), the receptor mutations can be both of the gain-of-function (also heterozygotes have a phenotype) and loss-of-function type. In addition to clear-cut disease-causing mutations, the genome of all individuals is full of small structural variations, polymorphisms, which usually are associated with repeats in noncoding regions of the genome or point mutations within the genes. They may or may not cause alterations in gene function or structure of the encoded protein, and, consequently, often have no clear-cut phenotypic expression. However, subtle changes in function of the encoded protein, with mild phenotypic expression, are also possible in these cases.

## II. Structure-Function Relationships of Gonadotropins and Gonadotropin Receptors

Together with TSH, the gonadotropins LH, human CG (hCG), and FSH form the family of glycoprotein hormones. LH, FSH, and TSH are produced in the pituitary gland, while the LH homolog hCG originates from the placenta. The members of this family of relatively large proteins (molecular mass, 30–40 kDa) consist of a common  $\alpha$ -subunit and a hormone-specific  $\beta$ -subunit that are associated through non-covalent interactions. The mature  $\alpha$ -subunit consists of 92 amino acid residues and is encoded by a single gene, comprising four exons, which is localized on chromosome 6q12.21 (Fig. 1). The  $\alpha$ -subunit protein contains 10 cysteines, which are involved in intrasubunit disulfide linkages and two N-linked glycosylation sites. Although the  $\beta$ -subunits confer functional specificity of the hormones, they show considerable amino acid identity, ranging from 32% for the LH-TSH pair to 83% for the LH-hCG pair (excluding the non-homologous C-terminal extension of hCG). The  $\beta$ -subunit genes are located on different chromosomes: the LH/hCG $\beta$  gene cluster on chromosome 19q13.32, FSH $\beta$  on chromosome 11p13, and TSH $\beta$  on chromosome 1p13. The LH/hCG $\beta$  gene cluster consists of one LH $\beta$  gene and six hCG $\beta$  genes and pseudogenes (3). At least five of the hCG $\beta$  genes are expressed in choriocarcinoma cells and placenta, but most of steady-state hCG $\beta$  mRNAs appear to be transcribed from genes 3, 5, and 8 (4).

The mature  $\beta$ -subunit proteins contain 12 cysteine pairs that form six intrasubunit disulfide bridges, two N-linked glycosylation sites (one in LH $\beta$ ), and range in length from 111–145 amino acid residues. The hCG $\beta$  gene is thought to have been recently evolved from the LH $\beta$  gene through a frameshift mutation in the last exon of the gene causing extension of the reading frame (5). Thus, the hCG $\beta$  protein is larger than the LH $\beta$  protein, containing a C-terminal extension of 29 amino acids, with four additional O-linked glycosylation sites not present in LH $\beta$ . This structural difference explains the longer circulating half-life and higher biopotency of hCG over LH.

The crystal structure of deglycosylated hCG (6) has revealed that the  $\alpha$ -subunit and the  $\beta$ -subunits both contain a so-called cystine knot structure, similar to some remotely related signaling molecules such as transforming growth

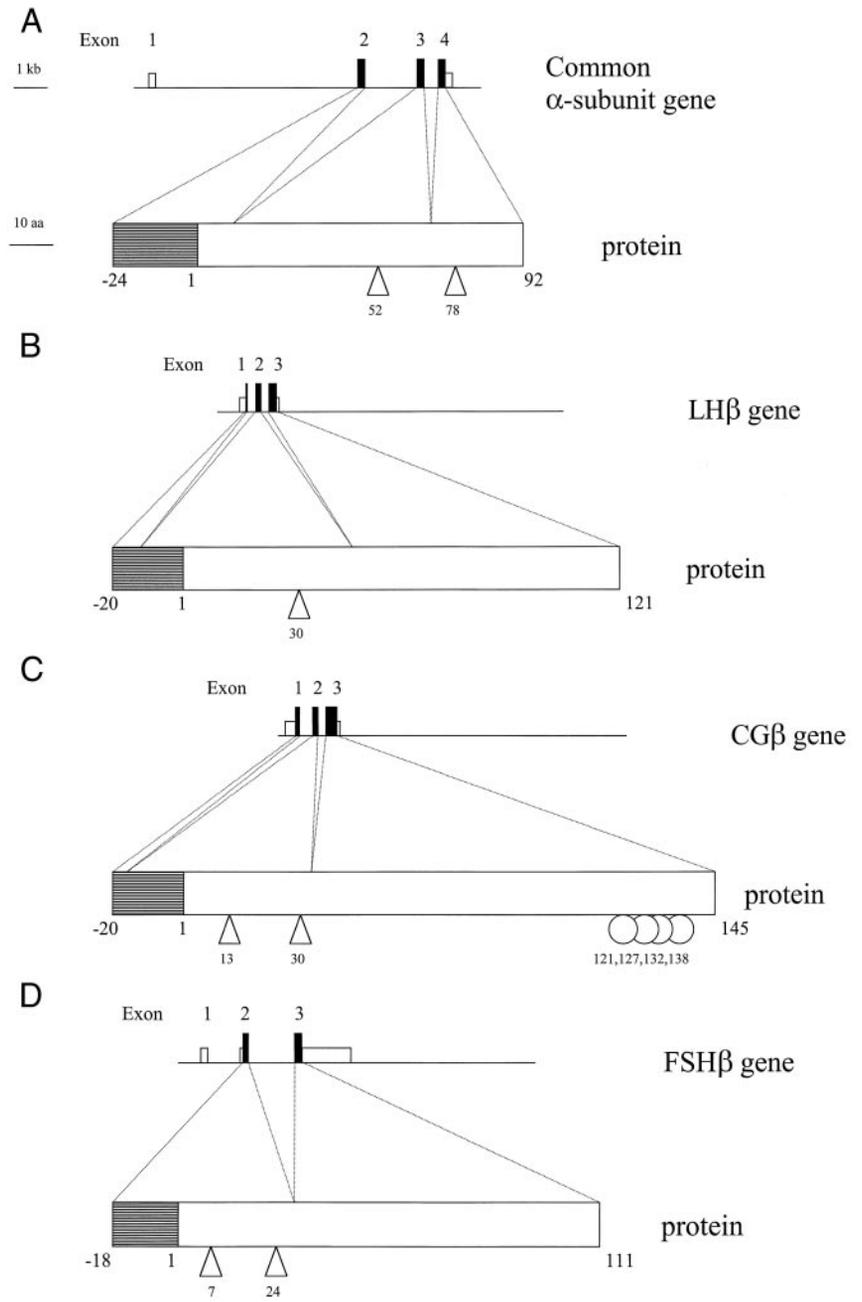
factor- $\beta$  (TGF $\beta$ ), nerve growth factor (NGF), and platelet-derived growth factor (PDGF). Each subunit has elongated shape with two  $\beta$ -hairpin loops on one side of the central cystine knot and a long loop on the other side. The non-covalent interaction between the two subunits is stabilized by a segment of the  $\beta$ -subunit that extends like a "seat-belt" around the  $\alpha$ -subunit and is "locked" by a disulfide bridge.

Just as their ligands, the receptors for the glycoprotein hormones have related structures (Fig. 2). The receptors belong to the large family of G protein-coupled receptors, whose members all have a transmembrane domain that consists of seven-membrane traversing  $\alpha$ -helices connected by three extracellular and three intracellular loops. The glycoprotein hormone receptors form a separate subgroup within this large family on the merit of their large extracellular hormone-binding domain at the N terminus. FSH and TSH bind to the FSH and TSH receptors, respectively, while LH and hCG both bind to the same LH receptor. The LH and FSH receptor genes are located on chromosome 2p21 (7) and 2p21–16, respectively (8, 9), while the TSH receptor is found on a different chromosome, 14q31 (10, 11). The relationship of the glycoprotein hormone receptors to the other G protein-coupled receptors is indicated by their sequence homology in the C-terminal half of the receptor. This domain, encoded by a single, last exon, contains the seven-transmembrane segments and the G protein-coupling domain. The extracellular domain of the glycoprotein hormone receptors is encoded by the preceding 9 or 10 exons.

The 5'-terminal part of the open reading frame of exon 1 encodes the signal peptide that directs the protein to the luminal side of the endoplasmic reticulum and eventually to the extracellular side of the plasma membrane. The amino acid sequence encoded by the following exons 2–9 (2–10 in LH receptor) has been shown to confer hormone specificity and binding to the gonadotropin receptors (12), and it contains a number of so-called leucine-rich repeats, which are found in a diverse group of proteins (13, 14). The crystal structure of a ribonuclease inhibitor, which contains a number of leucine-rich repeat units, revealed a nonglobular, flexible crescent-shaped molecule in which the leucine-rich repeats correspond to  $\beta\alpha$ -structural units that may be responsible for the protein-binding function of ribonuclease inhibitor. The extracellular domain of the glycoprotein hormone receptors with nine such leucine-rich repeats may have a similar structure, and this feature was used as an aid in studies of the interaction of hCG with the LH receptor (15–17). The leucine-rich repeat units are flanked by motifs that appear to be structurally stabilized by cysteine disulfide bridges (17).

In the extracellular domain of the LH and FSH receptors, a number of potential N-linked glycosylation sites have been identified. There are six sites in the LH receptor: Asn 99, 174, 195, 291, 299, and 313; and four sites in the FSH receptor: Asn 191, 199, 293, and 318, although the last site is not conserved among species (Fig. 2; the amino acids in the receptor proteins are numbered by taking the first methionine of the signal peptide as 1). The role of N-linked glycosylation in receptor function is not completely elucidated, and some seemingly contradictory results have been presented. Chem-

FIG. 1. Schematic representation of the human gonadotropin subunit genes. In the top part of each scheme the gene structure is depicted. The open bars indicate sequences that do not encode protein (noncoding). The closed bars indicate the sequences that comprise the open reading frame. The genes are drawn to scale. In the bottom part of each scheme the protein structure is shown. The signal peptide is indicated by the shaded bar, while the mature protein is depicted by the open bar. The numbers below the protein signify the start and end of the signal peptide and the length of the mature protein product, taking the first amino acid of the mature protein as 1. Below the protein the positions (and number of amino acid) of the N-linked glycosylation sites are indicated by inverted triangles and, in the case of CG $\beta$ , the O-linked glycosylation sites (circles). The connecting lines between the coding exons in the top part of the scheme and the protein structure in the bottom part serve to indicate the sections of the protein encoded by the respective exon. Note that the  $\beta$ -genes consist of three exons, and that the common  $\alpha$ -subunit gene is much larger mainly because of addition of the first, noncoding exon and long intron 1. In contrast to the other  $\beta$ -genes, the first exon of FSH $\beta$  is non-coding and exon 3 encodes a long 3'-untranslated region (open bar). The information shown in this figure is taken mainly taken from Ref. 3 and GenBank.



ical deglycosylation of the rat LH receptor, or inhibition of glycosylation by tunicamycin treatment, did not prevent correct LH receptor folding, hormone binding, and signal transduction (18, 19). Mutational analysis of the rat LH receptor revealed a decrease or even complete loss of hormone binding activity upon elimination of the potential glycosylation sites at Asn<sup>103</sup>, Asn<sup>178</sup>, and Asn<sup>199</sup> (equivalent to Asn<sup>99</sup>, Asn<sup>174</sup>, and Asn<sup>195</sup> in the human), indicating the presence of functional carbohydrate chains at these positions in the rat ovarian LH/hCG receptor (20). Additional studies of the rat LH receptor using more extensive mutational dissection showed that all potential consensus glycosylation sites are N-glycosylated, but also revealed that the deleterious effects of the mutated N-linked glycosylation sites on rat LH receptor function result from the amino acid substitutions *per*

*se*, and not from absent glycosylation (19). In the case of the rat FSH receptor, two of the three glycosylation sites (Asn<sup>191</sup> and Asn<sup>293</sup>) are actually glycosylated, and a carbohydrate at either residue is required for efficient and correct folding of the receptor (21).

The extracellular ligand-binding domain of the gonadotropin receptors is connected to the transmembrane signaling domain by a hinge region. It is not clear whether this structure has functions other than serving as a connecting peptide, although the part of the hinge region closest to the first transmembrane segment is well conserved between the glycoprotein hormone receptors, suggesting a special role. Interestingly, in the marmoset monkey, exon 10 of the LH receptor gene, although present in the genome, is always completely spliced out from the mature mRNA (22). Exon 10

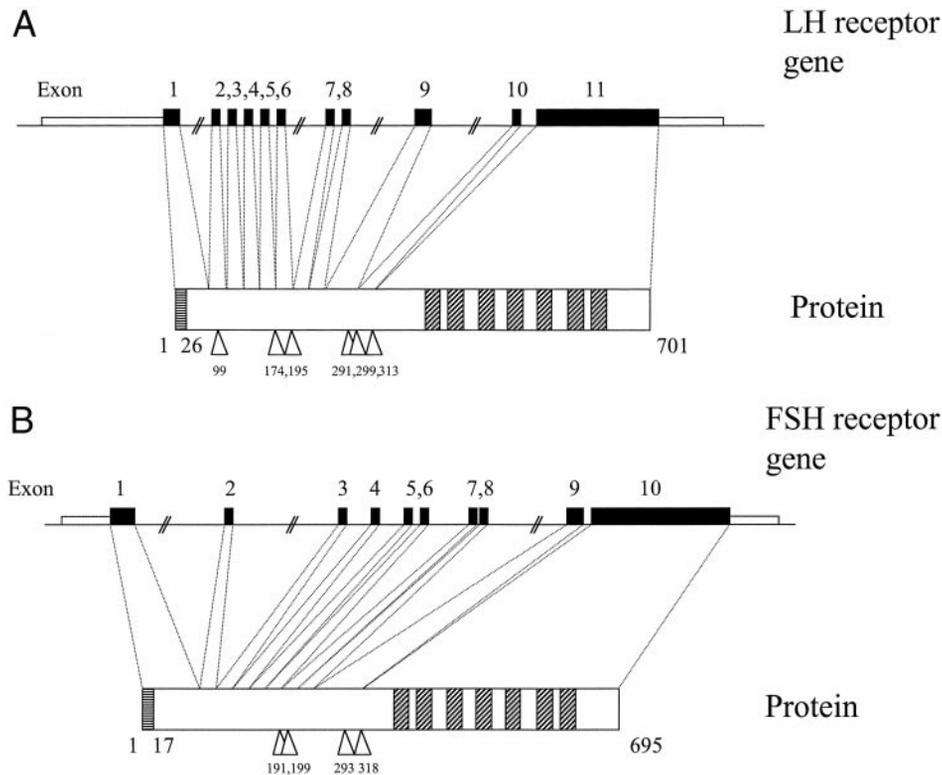


FIG. 2. Schematic representation of the human gonadotropin receptor genes. The structure of the genes is depicted in the *top of the drawings*. The *open bars* indicate sections of the exons that encode untranslated regions of the mRNA, while the *closed bars* indicate those sequences that encode the protein. Although the genes are not drawn to scale, exons that are grouped in the gene are also drawn grouped. Both genes are at least 80 kb in size. The relation between the intron/exon structure of the gene and the domains of the protein are indicated by the *broken lines*. The *horizontally hatched* part of the protein indicates the signal peptide, and the *cross-hatched bars* signify the seven segments of the transmembrane domain. The *numbers below the protein* indicate the start and end of the signal peptide and the length of the total protein product including the signal peptide. The *numbered inverted triangles below the protein* indicate the positions of N-linked glycosylation sites. Note that the receptor genes are very similar in structure with the exception of an additional exon 11 in the LH receptor gene (see text). Exon 1 encodes the signal peptide and a small part of the extracellular domain; the following eight or nine exons encode the rest of the extracellular domain, including the leucine-rich repeat motifs. In both receptor genes, the final exon is the largest and contains the information for the transmembrane signal transduction domain. The information shown in this figure is taken mainly from Ref. 8 and GenBank.

encodes the N-terminal part of the cystine cluster that is proposed to flank the leucine-rich repeat structure at its C terminus (17). This cluster has a chemokine-like structure, indicating an important function in the LH receptor. Nevertheless, the marmoset LH receptor (22) appears to function normally, suggesting that this cystine motif is not important in this species for receptor action and may act merely as a spacer allowing correct location of the extracellular domain in relation to the transmembrane domain. However, if exon 10 is deleted from the human LH receptor, the transit of the mutated receptor to the cell membrane is hampered (23), and the same is found to occur in a naturally occurring human LH receptor mutation in which the sequence encoding exon 10 is spliced out (Ref. 24; see below, *Section V.B*).

The transmembrane domain with its seven membrane-spanning  $\alpha$ -helices, connected by three extracellular and three intracellular loops, is similar to the other members of the large family of G protein-coupled receptors. Evidence has been presented that the transmembrane domain is sufficient for hormone binding (25), but this report has not been substantiated by other investigators. A molecular model of the LH receptor has proposed that some parts of the extracellular loops may function as contact points for the hormone bound

to the extracellular binding domain and/or to this domain itself, relaying the hormonal signal to the intracellular face of the receptor (15). Together with the cytoplasmic parts of the transmembrane  $\alpha$ -helices, the intracellular loops and the C-terminal tail of the receptor form the interaction domain with G proteins. As with other members of the G protein-coupled receptor family, the third intracellular loop and the cytoplasmic tail are most closely involved in G protein coupling and in the selectivity of coupling to specific types of G proteins (26, 27).

The LH and FSH receptors are mainly coupled to Gs, the G protein that activates the various adenylyl cyclase isoenzymes, resulting in elevation of intracellular cAMP levels. Both of these receptors, however, are also able to activate other signal transduction pathways, and in *in vitro* experiments with cells isolated from experimental animals (rat, mouse, porcine) or transfected with LH or FSH receptor cDNA (human, rat, mouse), increased phosphatidylinositol turnover, elevated intracellular  $\text{Ca}^{2+}$ , and activation of mitogen-activated protein kinases have been found (26, 28–40). Coupling to Gi proteins has been demonstrated for bovine and murine LH receptor (28), while the porcine LH receptor is able to activate Gq/11 and G13 in addition to Gi (32, 41,

42). Further studies will be necessary to elucidate the identity of G proteins coupling to the FSH receptor and to unravel possible species specificity of G protein coupling of either receptor type. The alternate intracellular pathways are in most cases activated at higher hormone concentrations than the cAMP pathway and may depend on high receptor densities (33). Therefore, their physiological relevance often remains unclear, although high serum levels of hCG and LH during pregnancy and around the time of ovulation, respectively, may make use of them.

Upon hormonal stimulation, the LH and FSH receptors desensitize, *i.e.*, the hormonal signal is relayed in a less efficient manner. This process is caused by uncoupling of the receptor from the intracellular transducing G proteins, and by internalization of the receptor, resulting in decreased density of extracellularly exposed hormone binding sites. Most experiments addressing desensitization mechanisms have been carried out with rodents, either *in vitro* with isolated gonadal cells or *in vivo* following hormonal stimulation. Incubating purified testicular Sertoli (43, 44) or Leydig cells (45, 46) with FSH or LH, respectively, leads to rapid loss of hormone binding and cAMP response caused by loss of membrane receptors through internalization of the hormone-bound receptors. Similar findings have been made with various ovarian cell models *in vitro* (47–50). In most experimental conditions, treatment of animals with LH, hCG, or FSH results in decreased responses of their testicular (51–53) and ovarian target cells (54–56). However, these mechanisms are not operative in all tissues. In the human corpus luteum, LH receptors or their mRNA are not lost under conditions of increasing hCG levels (57) or in fetal Leydig cells in the presence of high levels of placental hCG or increased LH secretion by the fetal pituitary (58–60).

In addition to the loss of receptors through internalization, a reduction in density of gonadotropin membrane receptors can also be caused by decreased receptor synthesis. In cultured immature rat Sertoli cells, a 4-h incubation with FSH or a cAMP analog causes complete disappearance of FSH receptor mRNA, probably through a posttranscriptional process involving a change in FSH receptor mRNA stability (44). Less strong effects of FSH that did not appear to involve mRNA stability changes were observed in another study with rat Sertoli cells (61). In cultured granulosa cells from rat and porcine origin, both stimulating and inhibitory effects of FSH on FSH receptor mRNA have been observed (62–65). However, these observations may be a reflection of the differentiating actions of FSH on the granulosa cells, rather than rapid sensitization or desensitization effects. In similar culture studies with porcine Leydig cells (66), mouse tumor Leydig cells (67, 68), and rat granulosa cells (69), LH/hCG caused rapid LH receptor mRNA loss. Also in the case of the down-regulation of LH receptor mRNA levels, it remains unclear whether mRNA stability is involved in all cases.

Agonist-induced phosphorylation of G protein-coupled receptors by second messenger kinases such as protein kinase A or by G protein-coupled receptor kinases (GRKs) leads to receptor uncoupling and internalization through a process that involves binding of inhibitory proteins (arrestins) to the receptor and targeting to clathrin-coated pits

(70, 71). This process has been best studied for the adrenergic receptors, but it is also involved in uncoupling and internalization of the gonadotropin receptors. Both rat LH and FSH receptors are uncoupled and internalized after stimulation, and subsequent phosphorylation of the C-terminal tail [LH receptor (72, 73)] or the first and third intracellular loops [FSH receptor (74)] has been implicated to play a role in this process. *In vitro* coexpression of rat LH or FSH receptors with several different GRKs or  $\beta$ -arrestins results in increased uncoupling and internalization, demonstrating the involvement of these proteins in the regulation of gonadotropin receptor function (75–78). Phosphorylation of the LH and FSH receptors is not always sufficient or necessary for the desensitization processes (42, 79, 80), as is also found for other G protein-coupled receptors (81, 82). Changes in conformation of the receptor may also be important (83).

Recently, two more distantly related members of the glycoprotein hormone receptor family, named LGR4 and HG38/LGR5, were identified from expressed sequence tag (EST) databases based on their similarities to the other family members (84, 85). The most conspicuous difference between LGR4 and HG38/LGR5 and the other family members was found to be the addition of an extra eight leucine-rich repeat in the extracellular domain. LGR4 and HG38/LGR5, whose ligands and function are as yet unknown, show a less strict tissue distribution of expression (LGR4: ovary, testis, adrenal, placenta, thymus, spinal cord, thyroid; LGR5: muscle, placenta, brain, spinal cord) than the LH, FSH (ovary, testis), and TSH (thyroid) receptors (84, 85). Several groups, however, have reported that the LH receptor gene also is expressed in several nonclassical gonadotropin target tissues, such as placenta, brain, adrenal gland, and prostate (86–89) and in normal and malignant breast tissue (90). However, the physiological significance of this “ectopic” LH receptor expression still remains unclear, since the effects of the absence of LH receptor function found in patients do not indicate effects on other tissues than the gonads, although these observations are limited by the effects of the pseudohermaphroditism or amenorrhea found in such cases (see below).

### III. Normal and Pathological Gonadotropin Function

#### A. During sexual differentiation

The advances in molecular biology techniques have allowed major progression in our knowledge of the regulation and control of sex differentiation and gonadal function. Several of the genes that function at the very basis of the gonadal development are now known (*e.g.*, WT1, SF1), as well as the proteins that govern whether a gonad develops in the male or female direction (*e.g.*, Sry, Sox9, Dax1) (91).

During the first phase of sex differentiation, *i.e.*, commitment of its direction, two testicular hormones come into play, signaling the direction of gonadal development to other genital structures of the developing fetus. The Sertoli cell product anti-Müllerian hormone (AMH; also known as Müllerian inhibiting substance, MIS), causes the regression of the *an-*

lagen of the female internal genitalia, *i.e.*, the Müllerian ducts, preventing the development of these female organs in the male. The growth and differentiation of the male internal genitalia, which develop from the Wolffian ducts and the urogenital sinus, is stimulated by the androgen testosterone, produced by Leydig cells of the fetal testis, and in some target tissues by  $5\alpha$ -dihydrotestosterone, a metabolite of testosterone through conversion by the  $5\alpha$ -reductase enzyme. In the human fetus, after an initial gonadotropin-independent phase, this activation of steroidogenesis, as well as Leydig cell growth and differentiation, is completely dependent on placental hCG. Although the majority of hCG is secreted to the maternal circulation, its concentration in fetal blood is high enough to stimulate fetal testicular steroidogenesis (59). The role of fetal pituitary gonadotropins in the regulation of gonadal function *in utero* is apparently negligible. As will be elaborated below, male development is largely dependent on correct hormone production by the fetal and postnatal testis. In contrast, the fetal ovary is hormonally silent and fetal differentiation into the female direction appears to be totally independent of gonadotropins and gonadal function. The fetal ovary apparently does not express gonadotropin receptors (92).

A short period of high gonadotropin secretion after birth in both sexes is probably responsible for the postnatal peak of testosterone measured in baby boys during the first 3–4 months of postnatal life (93), whereas nothing is known about its possible effects on the ovary. After this postnatal peak, gonadotropin secretion is suppressed to very low levels until the advent of puberty. However, pulsatile release of low levels of gonadotropins, predominately at night, is also found in prepubertal children (94). The prepubertal testes apparently have both LH and FSH receptors, since they show clear testosterone and growth responses to LH/hCG and FSH, respectively (95, 96). In contrast, no data are available on the presence of gonadotropin receptors in the prepubertal ovary. However, also prepubertal ovaries may express gonadotropin receptors, since ovarian follicles of girls with a central activation of the hypothalamus/pituitary start producing estrogens.

Puberty can be envisaged as the second phase of sexual differentiation. Reactivation of the hypothalamic-pituitary-gonadal axis results in increased secretion of LH and FSH from the pituitary, with stimulation of the cognate gonadal target cells. Again, steroid hormone production plays a central role in signaling the maturation of gonadal function to extragonadal tissues. The androgen production of Leydig cells is now activated by pituitary LH and induces the secondary sex characteristics of the adult male. Feminization and attainment of fertility of the female occur under control of estrogens and progesterone, produced by the stimulated ovarian follicles and corpus luteum through combined actions of FSH and LH.

### B. Mature function

In the ovary, granulosa cells are the only target cells of FSH action, thus expressing the FSH receptor, whereas both theca, stromal, late-stage (luteinizing) granulosa, and luteal cells contain LH receptors. The known role of FSH in the ovary is

to stimulate follicular maturation, including follicular estrogen production through aromatization of androgens. LH stimulates androgen production in theca cells, thus providing substrate for granulosa cell estrogen production. LH also triggers ovulation, and thereafter maintains the progesterone production of corpus luteum. In the testis, Sertoli cells are the target of FSH action and Leydig cells are the target of LH action. The specific role of FSH in testicular function is still somewhat unclear, but functions such as stimulation of Sertoli cell proliferation in the immature testis and maintenance of qualitatively and quantitatively normal spermatogenesis, through indirect effects mediated by Sertoli cells, have been proposed. The role of LH is to stimulate Leydig cell androgen production and thereby to maintain the endocrine (extra-testicular) and paracrine (spermatogenic) effects of androgens.

The synthesis and secretion of gonadotropins are under positive control of the hypothalamic GnRH (GnRH), and gonadal steroid and peptide (mainly inhibin) hormones exert negative and positive feedback effects on gonadotropin synthesis and secretion, either directly at the pituitary level or indirectly via the hypothalamus, mainly by modulating GnRH secretion. For GnRH to stimulate gonadotropin secretion, it is important that it is released in pulsatile fashion from the hypothalamus to the hypophysial portal circulation. This causes pulsatile secretion of gonadotropins, which is clearer with LH, due to its shorter half-time in circulation. However, the pulsatile mode of gonadotropin action at the gonadal level is apparently not important. Recent studies on male rats, either by follow-up of endogenous LH and testosterone pulses, or by pulsatile treatment with recombinant rat LH, demonstrate that gonadal stimulation is achieved by trains of multiple LH peaks of sufficient size (97, 98). However, the pulsatility of these effects may not be critical in view of effective gonadal stimulation by tonic gonadotropin injections in experimental animals and in humans. The importance of pulsatility in FSH secretion is even less clear, due to its longer half-life in circulation.

This pituitary-gonadal function remains basically similar in the female until menopause, after which estrogen production ceases in the absence of follicles, and gonadotropin secretion increases in the absence of ovarian negative feedback effects. In the male, there is gradual suppression of testicular androgen production and reciprocal increase of gonadotropins upon aging, beyond 50–60 yr of age.

A number of diseases at the hypothalamic and pituitary levels can impair the synthesis and secretion of gonadotropins. Aberrations in the hypothalamic regulation of gonadotropin synthesis, of which the best example is the absence of GnRH neurons, can result in Kallmann's syndrome. This syndrome is due to disturbed migration of the GnRH neurons from the olfactory placode to their final location in the hypothalamus. This migration of GnRH neurons is disturbed in the most common X-linked form of Kallmann's syndrome (hypogonadotropic hypogonadism and anosmia) through mutation in the gene of an extracellular matrix protein, anosmin, resulting in disturbance in development of olfactory bulbs and tracts (99). The other causes of abnormally low gonadotropin secretion include craniopharyngioma and a variety of other tumors, infiltrative diseases (*e.g.*, sarcoid-

osis), trauma, vascular disease, radiation therapy, pituitary infarction, metabolic diseases (e.g., hemochromatosis), and functional causes such as stress and anorexia nervosa (100, 101). Pathologically increased levels of gonadotropins are observed in connection with paraneoplastic gonadotropin secretion, central precocious puberty, and primary hypogonadism (100, 101). We can also include in this category hyperthyroidism associated with pregnancy and trophoblastic tumors, where the highly elevated levels of hCG, due to structural similarity of LH/hCG, TSH, and their cognate receptors, are able to stimulate thyroid function by binding to the TSH receptor (102). More detailed discussion of these conditions is beyond the scope of this review.

A rare condition of suppressed gonadotropin action is caused by disturbance of gonadotropin glycosylation. This step of gonadotropin synthesis normally occurs through action of a group of specific enzymes, which in rare cases are inactivated by mutations. Although the actions of the glycosylation enzymes are not specific for gonadotropins, their mutations nevertheless appear to influence the formation of functionally competent glycoprotein hormones. The condition is called carbohydrate-deficient glycoprotein syndrome, where several different autosomal recessive enzyme deficiencies can result in incomplete glycosylation of plasma proteins (103). The syndrome appears to cause hypergonadotropic hypogonadism in women, where the high circulating levels of immunoreactive FSH have been found to have very low bioactivity (104–106). Male patients with this syndrome virilize at puberty but display suppressed testicular volume. LH levels and LH action seem to be only marginally, or not at all, affected in the subjects. This syndrome emphasizes the importance of proper glycosylation of gonadotropins for their bioactivity. Although it remains unclear why FSH is more affected than LH, the phenotypic expression of this disease is reminiscent of genetic inactivation of FSH or its receptor (see below).

In addition to the genetic alterations of gonadotropin receptor genes, the topic of the present review, there are also other causes for the end-organ gonadotropin resistance, i.e., hypergonadotropic hypogonadism. The apparent causes include anatomical aberrations of gonadal development and structure in various forms of gonadal dysgenesis and agenesis (107). The most common form of ovarian dysgenesis is Turner's syndrome (45, XO). The other forms include diagnoses such as pure gonadal dysgenesis, ovarian steroidogenic enzyme defects, and premature ovarian failure, but their exact pathogenesis often remains open. Testicular resistance to gonadotropins can be caused by various developmental abnormalities. The most common chromosomal aberrations are Klinefelter's (XXY) and XX male syndromes, while the other diagnoses include various forms of idiopathic and acquired arrest of spermatogenesis, acquired immunodeficiency syndrome (AIDS), various neurological diseases, trisomy 21, effects of drugs, radiation, and environmental toxins, autoimmunity, and a number of systemic diseases (108). Gonadotropin resistance of both sexes is also possible, if there are defects in gonadal actions of other circulating hormones or of para- or autocrine effectors (109). However, distinct clinical conditions with disturbances in these functions are not yet known.

#### IV. Mutations in Human Gonadotropin Subunit Genes

##### A. Common $\alpha$ -subunit

Although there are several reports on restriction fragment length polymorphisms (RFLP) of the human glycoprotein hormone common  $\alpha$ -subunit gene (110–113), none of them appear to influence the encoded amino acid sequence. Some studies (112, 114, 115), though not all (116), report that particular common  $\alpha$  genotypes are disproportionately represented in DNA derived from trophoblastic malignancies. Paired normal and tumorous tissues from the same subject showed similar RFLP patterns, suggesting that particular common  $\alpha$  alleles predispose toward a variety of neoplasias, rather than represent somatic mutations in tumors (115). How exactly common  $\alpha$ -polymorphism is related to tumorigenesis remains obscure. It could be linked to mutation of a neighboring gene with clear causal relationship to the malignancy, although it may also represent a spurious association.

The only genetic alteration so far reported in the  $\alpha$ -subunit protein is a single Glu<sup>56</sup>Ala amino acid substitution in  $\alpha$ -subunit ectopically secreted by a human carcinoma (Table 1) (117). This mutated protein failed to associate with the  $\beta$ -subunit and appeared to have significantly higher mol wt than the native  $\alpha$ -subunit. It was proposed that the detected mutation causes altered tertiary structure, self-dimerization, or altered glycosylation, which could then be responsible for the ectopic subunit's increased size and failure to dimerize with LH $\beta$ .

The lack of proven germ line mutations in the  $\alpha$ -subunit gene could mean that such changes are lethal. In addition to gonadotropins, they would also affect the formation of CG and TSH. The fact that mice with targeted disruption of the common  $\alpha$ -gene are viable (128) (see below) speaks against this possibility. However, the mouse, not producing CG, may not be an adequate model for the human in this respect. The question about possible presence of common  $\alpha$ -mutations and their phenotypic expression in the human thus remains open.

##### B. Mutations of the LH $\beta$ subunit

The only true human mutation of the LH $\beta$  gene causing total functional inactivation is that described by Weiss *et al.* (118) (Table 1). The proband was a male, who presented with delayed puberty at the age of 17 yr. He was a member of a previously identified kindred with several infertile men (129) and had low testosterone and high immunoreactive LH serum concentrations. His testosterone secretion responded normally to exogenous LH and hCG, but in an *in vitro* bioassay serum LH was found to be devoid of bioactivity (130). These findings, together with the occurrence of infertility in three maternal uncles (with slightly lowered testosterone and increased LH) and a family history of consanguinity, suggested that the man had an inherited defect in the structure of LH, although his mother and sister had no symptoms of reproductive inadequacy. After two years of testosterone treatment, no sign of spontaneous puberty was seen after withdrawal of the treatment. Testicular biopsy revealed ar-

TABLE 1. The currently known mutations and polymorphisms, altering protein structure, that have been detected in gonadotropin subunit genes

Gene	Location	Type	Base change	Amino acid change	Effect at protein level	Ref.
Common $\alpha$	Exon 3	Missense	CA <sup>239</sup> G→CCG <sup>a</sup>	Glu <sup>56</sup> Ala	No association with $\beta$ -subunit	(117)
	LH $\beta$	Exon 3	Missense	GG <sup>221</sup> C→GAC	Glu <sup>54</sup> Arg	Absent bioactivity
LH $\beta$	Exon 2	Two missense mutations in the same allele	T <sup>82</sup> GG→CGG	Trp <sup>8</sup> Arg	Normal immunoreactivity	(119,120)
			AT <sup>104</sup> C→ACC	Ile <sup>15</sup> Thr	Poorly detected by $\alpha/\beta$ specific antibodies	
	Exon 3	Missense	A <sup>364</sup> GT→GGT	Ser <sup>102</sup> Gly	Increased <i>in vitro</i> bioactivity	(121)
CG $\beta$	Exon 3	Missense	G <sup>295</sup> TG→ATG	Val <sup>79</sup> Met	Decreased circulatory T <sub>1/2}</sub>	
			G <sup>295</sup> TG→ATG	Val <sup>79</sup> Met	Inefficient assembly with common $\alpha$	
FSH $\beta$	Exon 3	2-bp Deletion/premature STOP codon	GTG→GX <sup>236,237</sup>	STOP <sup>87</sup>	Slightly elevated <i>in vitro</i> bioactivity	(123–125)
	Exon 3	Missense	TG <sup>206</sup> T→GGT	Cys <sup>51</sup> Gly	No bioactivity or immunoreactivity	
	Exon 3	Missense	T <sup>298</sup> GT→CGT	Cys <sup>82</sup> Arg	No bioactivity or immunoreactivity	

<sup>a</sup> Apparently a somatic mutation in tumor.

rest of spermatogenesis and absence of Leydig cells. Long-term treatment with hCG resulted in testicular enlargement, normal virilization, and onset of spermatogenesis.

Upon sequencing of the LH $\beta$  subunit gene of the subject, a homozygous A-to-G missense mutation was found in codon 54, causing a Glu-to-Arg substitution (118). The subject's mother, sister, and three uncles were found to be heterozygous for the same mutation. A gene conversion whereby sequences from the CG $\beta$  gene are exchanged with or incorporated in the LH $\beta$  gene was excluded, which indicated that the alteration in LH $\beta$  structure represented a spontaneous germ line mutation. Coexpression of the mutated LH $\beta$  gene with normal  $\alpha$ -subunit gene in CHO cells resulted in formation of immunoreactive LH  $\alpha/\beta$  heterodimers, with no activity in RRA, *i.e.*, the mutated hormone was devoid of biological activity because of inability to bind to the LH receptor. In the heterozygous family members, as expected, the bioactivity of LH was reduced in relation to immunoreactivity, since half of their LH $\beta$  was encoded by the mutated gene.

This rare case clarifies some points about the developmental role of pituitary LH. Since the proband was apparently normally masculinized at birth with descended testes, pituitary LH is not needed for the stimulation of testicular testosterone production *in utero*. Indeed, testosterone production is initiated autonomously, but becomes subsequently dependent on placental hCG (58, 59), and fetal pituitary LH apparently plays no role in regulation of fetal testicular function. However, the endocrine function of the postnatal testes is critically dependent on pituitary LH secretion, as was demonstrated by the total absence of spontaneous puberty in this subject lacking bioactive LH. It is intriguing that the heterozygous male family members had impaired steroidogenesis and high incidence of infertility despite normal pubertal masculinization. However, since the proband's father was an obligate heterozygote, the importance of the heterozygosity for testicular function remains open. The heterozygous women, including the proband's mother, were apparently free of symptoms. It is curious that no other human subjects homozygous for this type of mutation have yet been detected. The female phenotype would probably resemble those with inactivating LH receptor mu-

tation (see below). Comparison of these two conditions would elucidate the role of intrauterine LH/hCG action, if any, in ovarian development and function.

### C. Genetic variants of LH $\beta$ subunit

Sequence variability of the LH $\beta$  chain was observed in early reports on chemical sequencing of this protein (131), but the existence of polymorphic alleles of the LH $\beta$  gene has only been recently recognized.

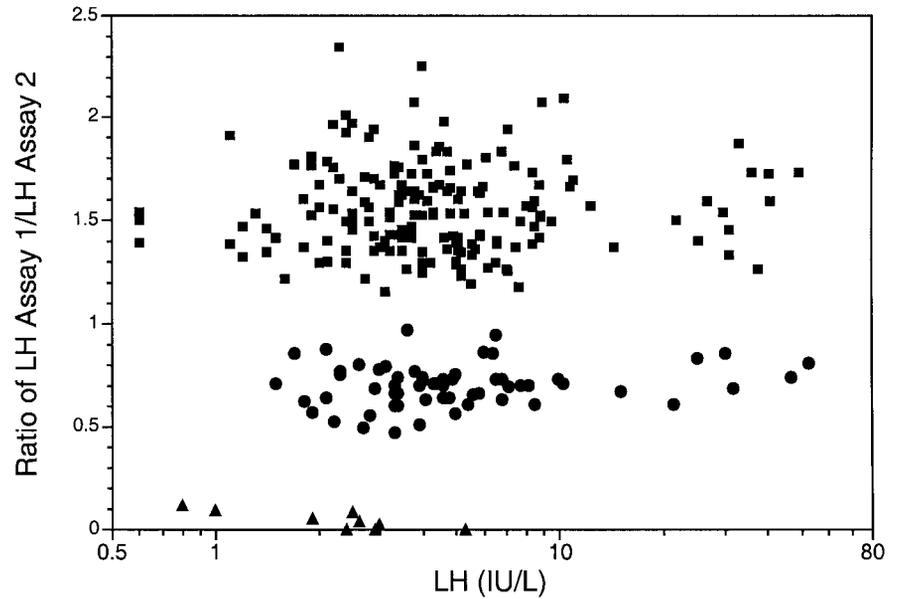
Upon testing the applicability of various monoclonal antibodies (Mabs) for the detection of LH, using the immunofluorometric assay (IFMA) principle, Pettersson and colleagues (132–134) described a healthy woman with two children, whose LH was undetectable using a Mab directed against an antigenic epitope present only in the intact LH  $\alpha/\beta$  dimer (assay 1). The woman's LH bioactivity and the ratio of bioactivity to immunoreactivity (using a subunit-specific IFMA for immunoreactive LH measurement, assay 2) were normal and in accordance with her normal fertility. Since her TSH and FSH levels were also normal, indicating no abnormalities in the common  $\alpha$ -subunit gene, the LH $\beta$  gene was sequenced (119, 135). The LH $\beta$  gene of the subject was found to represent a genetic variant (V) allele of the LH $\beta$  gene, with two missense mutations: Trp<sup>8</sup>Arg (TGG → CGG) and Ile<sup>15</sup>Thr (ATC → ACC) [Table 1 and Fig. 3 (119, 135)]. Recently, the same LH $\beta$  allele was reported from Japan (120, 136) in female patients with infertility, and their LH likewise was unmeasurable with an immunometric assay kit using two Mabs. There is a complete linkage of the two mutations in all samples so far analyzed from various populations (135).

In addition to RFLP and allele-specific oligonucleotide hybridization (135), IFMA assays offer a quick and robust way to detect the V-LH $\beta$  allele. By calculating the ratio of LH measured by IFMA assay 1/assay 2 (see above), the population can be subdivided into three groups: normal ratio [1–2, homozygotes for wild-type (WT) LH $\beta$ ], low ratio (0.5–0.75, heterozygotes) and zero ratio (close to 0, homozygotes for V-LH $\beta$ ) (Fig. 4). It was found that in the normal Finnish population, the carrier frequency of the V-LH $\beta$  allele was about 28% (137).

The Ile<sup>15</sup>Thr mutation in V-LH $\beta$  introduces an extra gly-



FIG. 4. Occurrence of WT and variant LH $\beta$  alleles in the Finnish population. The distribution of 249 normal Finnish subjects into the normal (WT, ■), low (heterozygote, ●), and zero (homozygote, ▲) ratio groups according to the results of the ratios of LH measured by assay 1 (measuring only WT-LH) and assay 2 (measuring equally WT and V-LH). The LH level measured by assay 2 is shown on the *abscissa*. As no sex differences were detected, the male and female data are compiled. [Reproduced with permission from: A. M. Haavisto *et al.*: *J Clin Endocrinol Metab* 80:1257–1263, 2000 (137). © The Endocrine Society.]



duces the pathological ovarian responses. However, this finding was not corroborated in the UK population, despite similar diagnostic criteria (148, 149). This is in keeping with the multifactorial pathogenesis of PCOS (152) and emphasizes that its pathogenesis in different populations may vary according to genetic background. Even though V-LH may not be directly related to PCOS, determination of V-LH may improve the prediction of risk of PCOS, especially in obese women. Its high frequency in various populations must be kept in mind because many widely used immunoassay reagents do not detect this LH form. One diagnostic criterion for PCOS, the elevated LH/FSH ratio, may remain undetected if such an LH assay is used, as has been emphasized recently (153). Other phenotypic associations with V-LH include the delayed tempo of pubertal progression in boys heterozygous for V-LH $\beta$  (154), and in elderly men, it was more common in those with low testosterone and high LH concentrations (150). However, larger numbers of observations from various ethnic groups are needed to resolve the role of V-LH in pathologies of gonadal function and infertility.

Hence, there is some evidence for association of V-LH, as a protective or predisposing factor, with various pathologies of LH action. Since findings in various ethnic groups do not always agree, the overall genetic background of the population may be important for the phenotypic expression and penetrance of this relatively mild polymorphic alteration in structure and function of the LH molecule. V-LH may thus be an example of influence of genetic heterogeneity on reproductive functions. Additional polymorphisms affecting reproductive endocrine functions are likely to be found. Interestingly, another polymorphism of LH $\beta$ , detected recently (Ser<sup>102</sup>Gly) in Singapore, has also been implicated in female infertility (155).

#### D. hCG $\beta$ subunit

Several polymorphisms have been detected in the hCG $\beta$ /LH $\beta$  gene complex by RFLP analysis (156, 157), but whether

they result in sequence differences in LH or hCG has not been studied in detail. Layman *et al.* (157) were unable to detect large deletions or duplications of the hCG $\beta$ /LH $\beta$  gene complex by genomic Southern blotting in patients with suspected disorders of hCG production, such as recurrent abortion, primary unexplained infertility, and gestational trophoblastic neoplasia. A very recent study (122) showed that of the six hCG $\beta$  genes present in the human genome, the one most highly expressed, number 5 (158), is highly conserved. Altogether six polymorphisms were detected in this gene in a random population, and they were, with the exception of one, either silent or located in introns. An A-to-G transition in exon 3 of hCG gene 5 was found to alter the amino acid: Val<sup>79</sup> Met (Table 1). When the mutated  $\beta$ -subunit was coexpressed with the common  $\alpha$ -subunit gene in CHO cells, the assembly of the two subunits was found to be inefficient, although those dimers that did form had normal bioactivity. This mutation was found in 4.2% of randomly chosen healthy subjects, but only in heterozygous form. A limited search in subjects with infertility ( $n = 41$ ) yielded one additional silent mutation, but the above mentioned amino acid change was not found in this population. This may be due to the limited sample size or to the possibility that the mutation is embryonic-lethal because of insufficient production of biologically active hCG. Whether the intronic mutations detected were truly silent, or affected the rate of transcription or mRNA splicing, remains to be studied.

#### E. FSH $\beta$ subunit

A total of five subjects (three women and two men) with different inactivating mutations of the FSH $\beta$  gene have so far been described in the literature (Table 1). The first mutation reported was a homozygous 2-bp deletion in codon 61 (Val<sup>61</sup>) of the FSH $\beta$  gene in a woman suffering from primary amenorrhea and infertility (123). The mutation gave rise to a completely altered amino acid sequence between codons 61 and 86 of the FSH $\beta$  chain, which was followed by a premature stop codon, and lack of translation of amino acids 87–

111. Consequently, the translated FSH $\beta$  protein was truncated and unable to associate with common  $\alpha$ -subunit to form bioactive or immunoreactive  $\alpha/\beta$ -dimers. The affected woman had apparently had normal adrenarche, but no menarche or telarche. Treatment of the patient with exogenous FSH resulted in follicular maturation, ovulation, and successful pregnancy. Her mother, heterozygous for the mutation, had suffered from menstrual irregularity and infertility, but these symptoms were unlikely related to the mutation, since the heterozygous relatives of other similar patients have been reported to be free of symptoms (see below).

The second case of inactivating FSH $\beta$  mutation was also a female with similar phenotype, *i.e.*, primary amenorrhea and poorly developed secondary sex characteristics (126). She had undetectable serum FSH and estradiol, high LH, and absent FSH response to GnRH stimulation test. Upon DNA sequencing, she appeared to be a compound heterozygote for two mutations in the FSH $\beta$  subunit gene. One was the same as the mutation described by Matthews *et al.* (123), and the other was a missense T-to-G mutation, causing a Cys<sup>51</sup>-to-Gly transition in the mature FSH $\beta$  protein. Cells transfected with the FSH $\beta$  gene carrying this mutation failed to produce immunoreactive FSH, apparently because of the loss of a cysteine critical for formation of proper disulfide bonds, as well as for synthesis and secretion of the hormone. No symptoms were found in the relatives heterozygous for either of the two FSH $\beta$  mutations of the proband, suggesting that one intact FSH $\beta$  gene is sufficient to maintain functionally adequate FSH secretion.

In a third female patient with isolated FSH deficiency, reported earlier (159), the cause was originally suggested to be due to circulating FSH antibodies (160). However, the molecular pathogenesis of this case was recently "re-revisited," and it was found to be due to the same homozygous 2-bp deletion as that of the first detected FSH $\beta$  mutation (124). The FSH antibodies apparently developed in response to treatment with urinary gonadotropins (161), which were recognized as foreign protein by the patient's immune system.

The female cases with FSH $\beta$  inactivation are in good agreement and demonstrate that FSH is necessary for normal follicular development, ovulation, and fertility. Likewise, pubertal development is hampered in the absence of sufficient numbers of later stage follicles to harbor the granulosa cells needed for adequate estrogen production. As will be described below, this phenotype is practically identical to that caused by inactivating FSH receptor mutation.

Very recently, two men with FSH $\beta$  mutations have been described (125, 127, 162). The report from Sweden by Lindstedt *et al.* (127, 162) described a 32-yr-old man of Serbian origin with azoospermia and normal puberty, but with selective absence of FSH. The LH-testosterone axis of the patient was apparently normal. Genetic analysis demonstrated a homozygous T-to-C mutation, predicting a Cys<sup>82</sup>-to-Arg substitution in the FSH $\beta$  protein. The second male, described from Israel (125), was an 18-yr-old man with slightly delayed puberty, small testes, azoospermia, and plasma FSH concentration below 0.5 IU/liter. Conspicuously, his testosterone level was low (4.5 nmol/liter) and LH high (24.5 IU/liter). Upon DNA sequencing, the same homozygous 2-bp deletion in codon 61 was found as reported before with the

female patients (123, 124, 126). It was postulated, on the basis of studies on hCG biosynthesis (6, 163), that in the Cys<sup>82</sup>Arg mutation, elimination of cysteine would result in inability to form the first intramolecular disulfide bond of FSH $\beta$ . This would then result in abnormal tertiary structure during FSH $\beta$  synthesis, with extensive intracellular degradation of the products, inability to associate with common  $\alpha$ -subunit, defective glycosylation, and finally inability to form biologically active hormone.

As will be elaborated below, the phenotypes of women with inactivating FSH $\beta$  and FSH receptor mutations, as well as the female knockout mice for FSH ligand and receptor, are in perfect agreement. However, there is an apparent discrepancy between phenotypes of men with inactivating FSH $\beta$  and FSH receptor mutations, which still leaves open the final word about the role of FSH in testicular function. Since the number of males with FSH $\beta$  mutation so far reported is only two, and that of men with FSH receptor mutation five (164), the information about the role of FSH in the male that can be obtained from earlier descriptions of men with idiopathic isolated FSH deficiency can be valuable. These studies were done at the time when genetic diagnostics was not yet available. Some of the subjects with "isolated FSH deficiency" had associated disorders, such as cryptorchidism, hypospadias, omphalocele, deafness, the olfactory-genital dysplasia syndrome, chromosomal alterations, autoimmunity, or short stature (160, 165–168), and therefore such patients may not be representative of truly isolated FSH deficiency. However, many of them seem to fulfill the diagnostic criteria. Those reported in full-length articles are summarized in Table 2, together with the two males with genetic proof of FSH $\beta$  mutation. In addition, one abstract exists on isolated FSH deficiency in a male (169).

A problem of the early studies, in addition to the lack of genetic information on structure of the FSH $\beta$  gene, is the suboptimal specificity and sensitivity of the FSH assays available, for which reason the real level of the low FSH concentrations remains unclear. This is partly compensated for by data on absent or subnormal FSH response to GnRH or clomiphene stimulation, indicating genuine FSH deficiency. Of the nine men presented in Table 2, two had evidence of prior fertility and had normal sperm counts with poor motility and morphology. Four men had severe oligozoospermia, and three were azoospermic. Testis biopsies displayed variable types of spermatogenic arrest, and the testis sizes varied from small to normal. Taken together, the phenotypic array of these men is very similar to the recently reported five men with inactivating FSH receptor mutation (164), displaying slightly to severely impaired spermatogenesis, but no azoospermia or obligatory infertility. In the absence of genetic data, one can naturally question the extent of FSH suppression in the men with the mildest phenotypes. However, they strengthen the sparse genetically verified data on at least some degree of spermatogenesis in the absence of FSH.

On the other hand, the two men with documented FSH $\beta$  mutations (see above) were possibly only detected because of their azoospermia and/or delayed puberty. The Swedish patient, unlike some other FSH-deficient men (173), was resistant to FSH treatments for periods of 120 and 210 days

TABLE 2. Men with idiopathic isolated FSH deficiency, previously reported in the literature

Age	Pubertal development	Fertility	FSH (IU/liter)	Other pituitary hormones	Testosterone	Sperm ( $10^6/ml$ )	Testis biopsy	Testis size	Reference
26	Normal	1 Child	<3	Normal	Normal	32-130	Spermatogenic arrest at spermatids	Normal	(170)
30	Normal	2 Children	2	Normal	Normal	Poor motility + morphology 25-170		Normal	(170)
39	Normal	Infertile	0	Normal	Normal	Poor motility + morphology 6-10	Normal	Normal	(171)
26	Normal	Infertile	low	Normal	Normal	2.7-7	Arrested spermatogenesis	Normal	(172)
30	Normal	Infertile	<0.5	Normal	Normal	Poor motility + morphology 0.9-1.7	Severe hypopermatogenesis	Normal	(173)
37	Normal	Infertile	3.0	Normal	Normal	0.6-<1.0	Disorders in spermatogenesis	Normal	(173)
34	Normal	Infertile	0	Normal	Normal	Azoospermia		Normal	(174)
28	Normal	Infertile	0	Normal	Normal	Azoospermia	Spermatogenic arrest	Small	(127)
18	Delayed	Not studied	<0.5	High LH (24.5 IU/liter) Low inhibin B (15.6 ng/liter)	Low (4.5 nmol/liter)	Azoospermia		Small	(125)

With the exception of the two last subjects, the reports are from the time before molecular mutation diagnostics were available.

(162), which may indicate additional contributing factor(s) to his azoospermia. The Israeli patient, in addition, had Leydig cell hypofunction, not demonstrated in any of the other FSH-deficient subjects, or those with FSH receptor defect (164), indicating the likelihood of an additional FSH-independent pathology of his testicular function.

In summary, the majority of information available indicates that FSH action *per se* is not mandatory for the pubertal initiation of spermatogenesis and fertility. However, qualitatively and quantitatively fully normal spermatogenesis apparently needs FSH action. The phenotype of men with defective FSH action varies from severe to mild impairment of spermatogenesis, in the face of apparently normal Leydig cell function. The azoospermia found in some of the men may be due to additional contributing factors, and not to truly isolated FSH deficiency. However, it is apparent that additional cases of genetically proven FSH deficiency are needed, before the existing discrepancy between phenotypes of the ligand and receptor deficiency, as well as the animal models with disrupted FSH $\beta$  and FSH receptor genes (see below), can be resolved. At the moment, it may be warranted to state that treatment of men with idiopathic oligozoospermia and normal to elevated FSH concentration with FSH has no scientific basis, and that prospects of a male contraceptive method based on inhibition of FSH secretion or action are not promising.

## V. Mutations in Human Gonadotropin Receptor Genes

In both LH and FSH receptor genes, activating and inactivating mutations have been identified with very different phenotypic effects. In the case of loss of function, it can be expected that the inactivating gene mutations range from missense changes of single amino acid residues, small deletions or insertions, frameshift mutations, and nonsense mutations that cause receptor truncation, to deletions of large parts of the receptor gene. Inactivating receptor gene mutations are found in homozygous or compound heterozygous states and the syndromes that are caused by these gene alterations follow a recessive pattern of inheritance. In contrast, activating mutations are much more limited in their character or position in the receptor gene. Actually, all activating receptor mutations in the gonadotropin receptors have been identified in the exon that encodes a small extracellular extension, the complete transmembrane domain of the receptor, and its intracellular C terminal tail (exon 11 in the LH receptor and exon 10 in the FSH receptor; see Fig. 2). Maps of the currently known LH and FSH receptor mutations are presented in Figs. 5 and 6.

### A. Activating mutations in the LH receptor

Activating mutations in the LH receptor gene were the first to be identified (175, 176). In the early 1980s, a unique form of pituitary-independent precocious puberty was described (177-179), characterized by symmetric testicular enlargement before 3 or 4 yr of age, increased testosterone levels, and low gonadotropins with prepubertal response to GnRH challenge. This familial form of male-limited precocious puberty

(FMPP) showed an autosomal dominant pattern of inheritance, and a majority of cases of familial male sexual precocity appeared to belong to this syndrome (180). The autonomous hypersecretion of testosterone could not be explained by increased gonadotropins since no immunoreactivity of these hormones could be identified in the patients' serum (178, 181, 182), although some evidence was presented of a factor in the serum of these boys that could stimulate monkey Leydig cells (183). Two groups demonstrated that these familial cases of LH-independent precocious puberty in boys were caused by mutations resulting in single amino acid changes in the LH receptor protein (175, 176). *In vitro* studies with the  $\alpha$ -1B adrenergic receptor had shown that changing a single alanine residue in the third intracellular loop to any other amino acid caused partial activation of the phosphoinositide pathway in the absence of ligand (184). In addition, mutations in the mouse MSH receptor gene had been described that caused dominant fur-color traits as a result of constitutive adenylyl cyclase activation (185). Indeed, in keeping with the hypothesis, the first mutations of the LH receptor were identified in the sixth transmembrane segment (TM6) and the flanking third intracellular loop (IL3), indicating, as had been found for other G protein-coupled receptors, that this region of the transmembrane domain was important for G protein coupling (Fig. 5 and Table 3).

Using *in vitro* transfection experiments, the mutant LH

receptor proteins were found to increase adenylyl cyclase activity in the absence of added ligand (hCG or LH) (176, 204), as measured by increased cAMP levels or by elevated luciferase activity when a reporter plasmid was cotransfected, containing six cAMP-response elements in front of the luciferase cDNA (Fig. 7) (208). Expression of mutant LH receptor molecules in the mouse Leydig tumor cell line MA-10 (227) resulted in increased cholesterol side-chain cleaving enzyme activity, as determined by the elevated levels of basal and hCG-stimulated pregnenolone production (228). Although these experiments do not provide formal proof that the LH receptor mutations cause precocious puberty, the transfected MA-10 cells in part mimic the situation in the Leydig cells of an FMPP patient, since in both situations WT and mutant LH receptor alleles are expressed.

Now that more activating LH receptor mutations have been identified, it can be seen that TM6 and the third intracellular loop are indeed the mutational hot spot of these alterations, although amino acid changes have been found in the other transmembrane segments as well, with the exception of TM4 and TM7 (Fig. 5). No activating mutations have been found in the other exons of the LH receptor gene that encode the signal peptide, extracellular hormone binding domain, and hinge region. Although exons 1–10 have not been investigated in all FMPP patients, it appears that in those patients in which no exon 11 mutations could be iden-

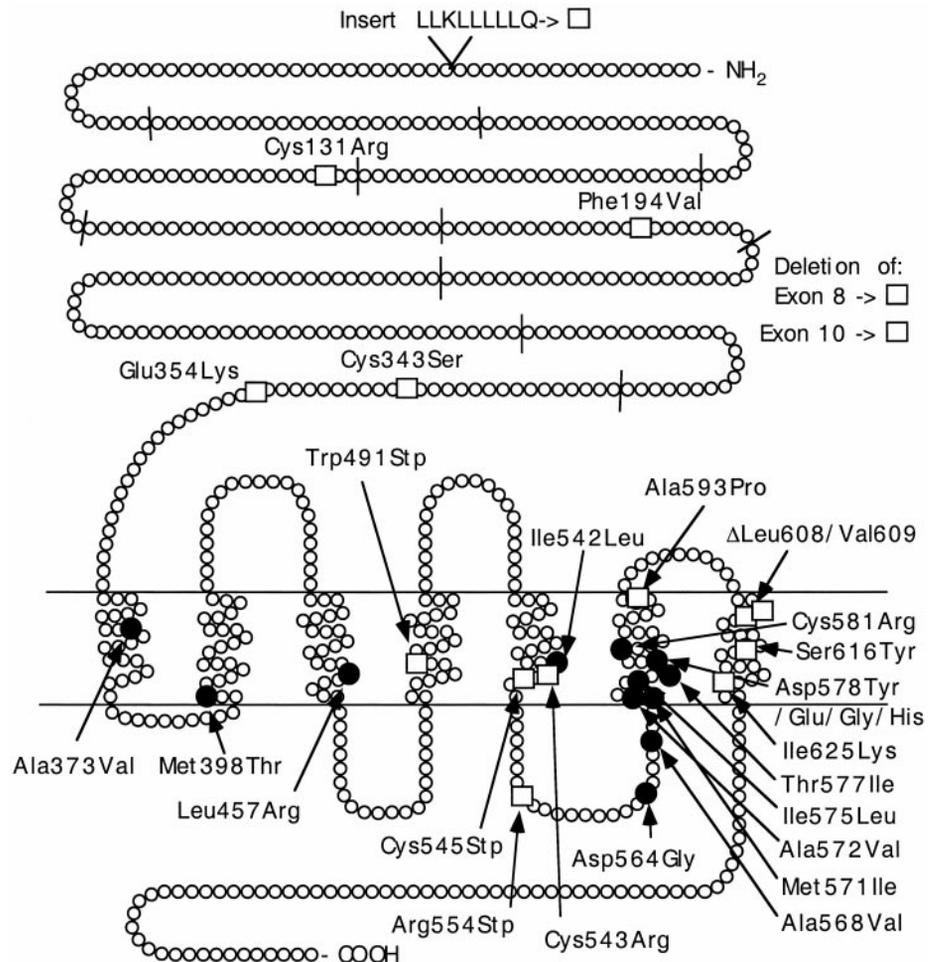


FIG. 5. Mutations in the LH receptor protein. Schematic structure of the LH receptor protein and localization of the inactivating (open squares) and activating (filled circles) mutations currently known in the human LH receptor. The short lines across the amino acid chain separate the 11 exons. For references, see Table 3.

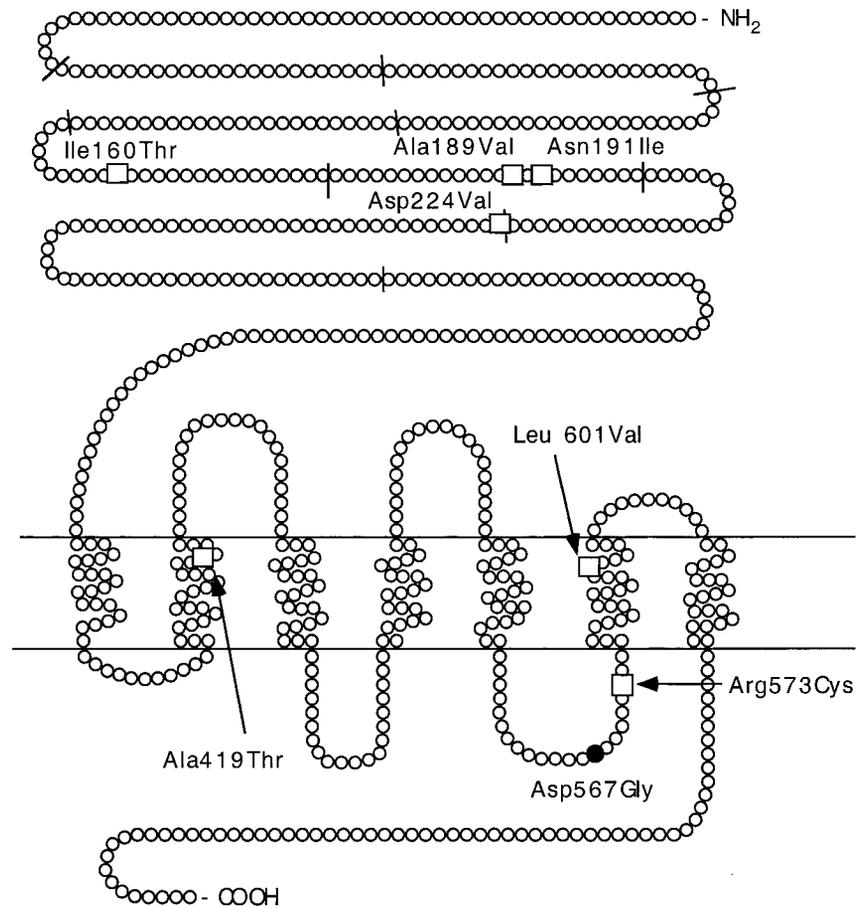


FIG. 6. Mutations in the FSH receptor protein. Schematic structure of the FSH receptor protein and localization of the inactivating (*open squares*), and one activating (*filled circle*), mutations currently known in the human FSH receptor. The *short lines* across the amino acid chain separate the 10 exons. For references, see Table 3.

tified, most likely other causes of LH-independent precocious puberty are operative (208). However, the finding of an activating mutation in the extracellular domain of the TSH receptor protein (229, 230) indicates that also for the gonadotropin receptors such mutations cannot be excluded, although they may be rare (231). Their probable scarcity supports the notion that in the glycoprotein hormone receptors, the function of hormone binding and signal transduction are separated. Mutations in the extracellular domain may increase the affinity of the receptor for the hormone but are probably without effect, since the increased sensitivity of the receptor would result in a negative feedback response of the pituitary, lowering ligand (LH or FSH) secretion and, as a result, the receptor would still be inactive in the absence of ligand. Only in those mutant receptors that show increased activity in the absence of hormone does the negative feedback action not play a role.

Comparison of the hCG dose-response activity of the receptor mutants and the WT-LH receptor reveals that, in many cases, the mutant LH receptor molecules display a lower response to a maximal stimulatory dose of hCG than the WT receptor (see *e.g.*, Refs. 204, 206, and 216). A decreased number of plasma membrane binding sites in cells expressing the mutant receptor molecules may be the reason for such a decrease (27, 190, 205, 210, 211, 213). A low number of cell surface expression may be caused by mutational effects on posttranslational modification and transport of the LH re-

ceptor protein (232) or increased internalization of the activated receptors (83, 233–235). However, a clear correlation between number of binding sites and maximal response is not always present. Comparing WT and two mutant LH receptors, Yano *et al.* (211) found that although both mutant receptors showed similar maximal responses to hCG as the WT LH receptor, one mutant (Ala<sup>572</sup>Val) displayed 3-fold less cell surface binding than the other mutant receptor (Asp<sup>578</sup>Gly). Similar discrepancy between binding and maximal activity was found in a study of the effect of the Ile<sup>575</sup>Leu mutation on LH receptor function (190). In keeping with the separated function of binding and signal transduction in the gonadotropin receptors, in almost all cases the affinity of the mutant receptors for the hormone remains unchanged (190, 209, 210, 213).

The Asp<sup>578</sup>Gly transition in the LH receptor protein is the most frequently observed amino acid change in FMPP patients (176, 206), and it appears that there is a strong founder effect for this mutation in the United States, since it has not been found in any of the European cohorts studied (208). Similarly, the Ile<sup>542</sup>Leu mutation was present in four Dutch kindreds, suggesting a common ancestor as the cause for this clustering (208), whereas the Met<sup>398</sup>Thr mutation seems to have a broader range of occurrence. This mutation has been found in kindreds from Germany and a patient from Sicily (208), in an FMPP kindred and a patient from the United Kingdom (191) and in an FMPP patient from Japan (205).

TABLE 3. Gonadotropin receptor gene variants and mutations

Gene	Location <sup>a</sup>	Type	Base change	Amino acid change	Effect	Reference
<b>LH receptor</b>						
Polymorphisms						
	Exon 1	Insertion (codon 18)	CTGCAG	LQ	No change	(186–188)
	Intron 1 (nucleotide 28)	Base Change	G↔C	No change	<sub>b</sub>	<sup>g</sup>
	Exon 8	Silent	C <sup>610</sup> TA↔TTA	Leu <sup>204</sup>	<sub>b</sub>	<sup>g</sup>
	Exon 10	Missense	AA <sup>872</sup> T↔AGT	Asn <sup>291</sup> Ser	No change	(188, 190)
	Exon 10	Missense	AA <sup>935</sup> T↔AGT	Asn <sup>312</sup> Ser	No change	(188, 190)
	Exon 11	Silent	GAC <sup>1065</sup> ↔GAT	Asp <sup>355</sup>	<sub>b</sub>	(190, 191)
Inactivating mutations						
	EC domain Exon 1	Insertion (codon 18)	CTGCTGAAGCTG CTGCTGCTGCAG	LLKLLLLLQ	Inactivating	( <sup>g</sup> , 192)
	EC domain Exon 5	Missense	T <sup>391</sup> GT→CGT	Cys <sup>131</sup> Arg	Inactivating	(193)
	EC domain Exon 7	Missense	T <sup>580</sup> TC→GTC	Phe <sup>194</sup> Val	Inactivating	(194)
	EC domain Exon 8	Deletion		Δexon 8	Inactivating	(195)
	EC domain Exon 10	Deletion		Δexon 10	Inactivating	(24)
	EC domain Exon 11	Missense	T <sup>1027</sup> GT→AGT	Cys <sup>343</sup> Ser	Inactivating	<sup>c</sup>
	EC domain Exon 11	Missense	G <sup>1060</sup> AA→AAA	Glu <sup>354</sup> Lys	Inactivating	(196)
	TM4	Nonsense	TGG <sup>1473</sup> →TGA	Trp <sup>491</sup> *	Inactivating	<sup>g</sup>
	TM5	Missense	T <sup>1627</sup> GT→CGT	Cys <sup>543</sup> Arg	Inactivating	<sup>c</sup>
	TM5	Nonsense	TGC <sup>1635</sup> →TGA	Cys <sup>545</sup> *	Inactivating	(197)
	IL3	Nonsense	C <sup>1660</sup> GA→TGA	Arg <sup>554</sup> *	Inactivating	(198)
	TM6	Missense	G <sup>1777</sup> CC→CCC	Ala <sup>593</sup> Pro	Inactivating	(199, 200)
	TM7	Deletion	ΔC <sup>1822</sup> TGGTT	ΔLeu <sup>608</sup> Val <sup>609</sup>	Inactivating	(201)
	TM7	Missense	TC <sup>1847</sup> T→TAT	Ser <sup>616</sup> Tyr	Inactivating	(195, 198)
	TM7	Missense	AT <sup>1874</sup> A→AAA	Ile <sup>625</sup> Lys	Inactivating	(202)
Activating mutations						
	TM1	Missense	GC <sup>1118</sup> C→GTC	Ala <sup>373</sup> Val	Activating	(203)
	TM2	Missense	AT <sup>1193</sup> G→ACG	Met <sup>398</sup> Thr	Activating	(191, 204–206)
	TM3	Missense	CT <sup>1370</sup> C→CGC	Leu <sup>457</sup> Arg	Activating	(207)
	TM5	Missense	A <sup>1624</sup> TT→CTT	Ile <sup>542</sup> Leu	Activating	(206)
	IL3	Missense	GA <sup>1691</sup> T→GGT	Asp <sup>564</sup> Gly	Activating	(206, 208)
	IL3	Missense	GC <sup>1703</sup> T→GTT	Ala <sup>568</sup> Val	Activating	(209)
	TM6	Missense	ATG <sup>1713</sup> →ATA	Met <sup>571</sup> Ile	Activating	(175, 210)
	TM6	Missense	GC <sup>1715</sup> A→GTA	Ala <sup>572</sup> Val	Activating	(211)
	TM6	Missense	A <sup>1723</sup> TC→CTC	Ile <sup>575</sup> Leu	Activating	(190, 208)
	TM6	Missense	AC <sup>1730</sup> C→ATC	Thr <sup>577</sup> Ile	Activating	(210, 212)
	TM6	Missense	GA <sup>1733</sup> T→GGT	Asp <sup>578</sup> Gly	Activating	(175, 176, 206, 213, 214)
	TM6	Missense	G <sup>1732</sup> AT→TAT	Asp <sup>578</sup> Tyr	Activating	(205, 206, 215)
	TM6	Missense	GAT <sup>1734</sup> →GAA	Asp <sup>578</sup> Glu	Activating	(216)
	TM6	Missense	G <sup>1732</sup> AT→CAT	Asp <sup>578</sup> His	Activating	(217)
	TM6	Missense	T <sup>1741</sup> GC→CGC	Cys <sup>581</sup> Arg	Activating	(206)
<b>FSH receptor</b>						
Polymorphisms						
	EC Domain Exon 10	Missense	A <sup>919</sup> CT→GCT	Thr <sup>307</sup> Ala	No change	(218–221)
	EC domain Exon 10	Missense	GT <sup>1022</sup> G→GCG	Val <sup>341</sup> Ala	No change	(218) <sup>d</sup>
	C terminus	Missense	AG <sup>2039</sup> T→AAT	Ser <sup>680</sup> Asn	No change	(218–221)
Inactivating mutations						
	EC domain Exon 6	Missense	AT <sup>479</sup> T→ACT	Ile <sup>160</sup> Thr	Inactivating	(222)
	EC domain Exon 7	Missense	GC <sup>566</sup> A→GTA	Ala <sup>189</sup> Val	Inactivating	(220)
	EC domain Exon 7	Missense	AA <sup>572</sup> T→ATA	Asn <sup>191</sup> Ile	Inactivating	(223, 224)
	EC domain Exon 9	Missense	GA <sup>671</sup> T→GTT	Asp <sup>224</sup> Val	Inactivating	(225)
	TM2	Missense	G <sup>1255</sup> CC→ACC	Ala <sup>419</sup> Thr	Inactivating	<sup>e</sup>
	IL3	Missense	C <sup>1717</sup> GC→TGC	Arg <sup>573</sup> Cys	Inactivating	(222)
	TM6	Missense	C <sup>1801</sup> TC→GTC	Leu <sup>601</sup> Val	Inactivating	(225)
Activating mutation <sup>f</sup>						
	IL3	Missense	GA <sup>1700</sup> C→GGC	Asp <sup>567</sup> Gly	Activating	(226)

<sup>a</sup> EC, Extracellular; TM, transmembrane segment; IL, intracellular loop; EL, extracellular loop.

<sup>b</sup> Not tested.

<sup>c</sup> J. W. M. Martens, S. Lumbroso, A. Richter-Unruh, H. G. Brunner, A. P. N. Themmen, and Ch. Sultan, unpublished.

<sup>d</sup> Rare polymorphism.

<sup>e</sup> E. Docherty, P. Pakarinen, A. Tiitinen, A. Kiilavuori, I. Huhtaniemi, S. Forrest, and K. Aittomäki, unpublished.

<sup>f</sup> The constitutive activity of this mutation has been disputed; see text.

<sup>g</sup> A. Richter-Unruh, J. W. M. Martens, M. Verhoef-Post, W. A. Kors, G. H. G. Sinnecker, A. L. Boehmer, S. L. S. Drop, S. P. A. Toledo, H. G. Brunner, and A. P. N. Themmen, unpublished.

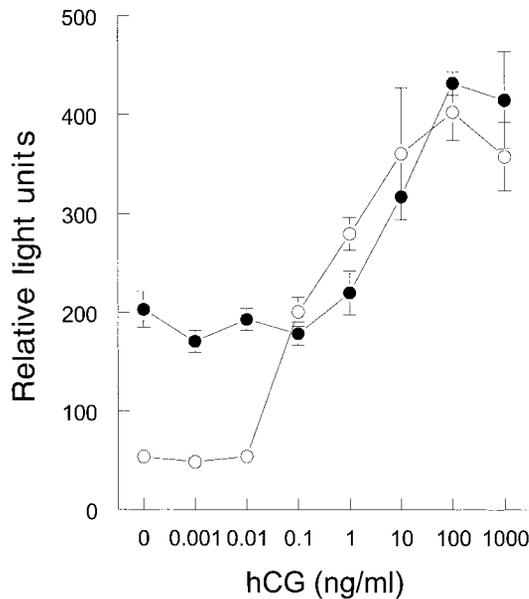


FIG. 7. Mutant Met<sup>571</sup>Ile LH receptor increases basal cAMP-responsive reporter activity. HEK293 cells were cotransfected with a cAMP-responsive luciferase reporter plasmid (202), a  $\beta$ -galactosidase reporter plasmid driven by a constitutive promoter together with the expression plasmid pSG5 containing the human WT LH receptor cDNA (open circles) or a human LH receptor cDNA containing the Met<sup>571</sup>Ile mutation (closed circles). Cells were incubated with the indicated concentrations of hCG for 4 h. The luciferase activity in the cell lysates is presented after normalization for  $\beta$ -galactosidase activity as a measure of transfection efficiency. Expression with the WT LH receptor-expression construct results in a clear response to hCG with low levels of reporter activity in the absence of hormone. The Met<sup>571</sup>Ile LH receptor shows similar response to hCG but with a substantial increase in basal cAMP-responsive reporter activity. [Courtesy of Dr. M.Verhoef-Post (unpublished).]

Interestingly, the latter mutation exhibits incomplete penetrance, since one of four carriers of the Met<sup>398</sup>Thr allele was unaffected (191), indicating that other factors may affect the FMPP phenotype.

Some of the amino acid changes found in the LH receptor in FMPP patients involve a major change in amino acid type (Table 3), e.g., Asp<sup>578</sup>Gly involving change from a large charged to a small uncharged amino acid residue, while in other patients, more conserved alterations were found, e.g., the changes from isoleucine to leucine at codons 542 and 575. All activating LH receptor mutations are situated in the cytoplasmic halves of the transmembrane segments or in the third intracellular loop (Fig. 5). In some cases, the amino acid change may involve increased or activating interaction with the Gs protein, while other residues may be important for interactions between the different transmembrane segments. Recently, Abell *et al.* (236) showed that a synthetic peptide containing a part of the cytoplasmic half of TM6 including the Asp<sup>578</sup>Gly mutation, has Gs stimulatory activity, whereas a peptide containing the WT residue does not. A TM6 peptide containing the isoleucine-to-leucine mutation at codon 575 had a similar effect. These results suggest direct interactions between some of the changed amino acids and the Gs protein, although the results are also consistent with a theoretical model of this part of the LH receptor in which the activating mutations perturb specific interactions of TM6 with TM5 and

TM7 that are critical for stabilizing the inactive state of the receptor (237). Recently, another molecular model was built of the LH receptor, which has been used to compare several different activated and inactivated LH receptor mutants (238). The model indicates that in activated mutants a crevice is opened that is formed by IL-2 and -3 and the cytosolic extensions of TM3, -5, and -6. This crevice, which may allow G protein interaction with otherwise buried amino acid residues, is closed in inactive LH receptor mutants (238). The LH receptor conformations with a closed or opened crevice, respectively, may represent the R and R\* states, which have been proposed in models for other G protein-coupled receptors, such as the constitutively active forms of the  $\beta_2$ -adrenergic receptor (239).

The relationship between cAMP as a second messenger of hCG binding to the LH receptor and the level of stimulation of testosterone production has been addressed mostly in *in vitro* studies with rodent Leydig cells. Leydig cell testosterone production proved to be much more sensitive to LH or hCG than hormone binding or cAMP levels would suggest. However, careful examination of adenylyl cyclase activity, cAMP levels, protein kinase A activation, in addition to specific inhibitory cAMP analogs, showed that as a result of intracellular amplification by the signaling cascade from hormone binding to cholesterol side-chain cleavage enzyme activity, very small increases in cAMP can give rise to large increases in steroid hormone production (e.g., see Refs. 240–244). Thus, the size of the change in the *in vitro* basal activity of the mutant LH receptors may correlate with the severity of precocious Leydig cell activation in the patient *in vivo*. Boys that carry the Asp<sup>578</sup>Tyr mutation show an early onset of precocious puberty at the age of 1 yr (208, 215, 245), and the activating effect of the tyrosine substitution was indeed much stronger than observed with the corresponding glycine mutant (206). The effect of the type of amino acid substitution at codon Asp<sup>578</sup> appears to be related more to the bulkiness of the amino acid than its charge or hydrophobicity (216, 246).

Activating LH receptor mutations appear to have no phenotype in the female, which may be explained by low or absent LH receptor expression in prepubertal girls. In addition, expression of the LH receptor would occur mostly in theca cells that surround follicles growing independently of FSH. However, since these follicles probably do not express high levels of the aromatase enzyme, thecal androgens produced under the influence of the activated LH receptor are not sufficiently aromatized to induce puberty. It is also possible that FSH-evoked paracrine influences from granulosa cells are needed before the theca cells are capable of active androgen production in response to LH stimulation (247). In adult cycling women, the exact timing of the ovulatory LH peak is an important feature of correct ovarian function, and expression of an activated LH receptor might have deleterious effects on this well regulated system. However, a detailed clinical examination of a female carrier of an Asp<sup>578</sup>Gly LH receptor mutation, also the mother of a boy with FMPP, revealed no infertility or other problems (248). Probably, the mutation did not activate the receptor beyond the prepubertal level, and the negative feedback systems that regulate ovarian function were intact (248). Although the activated

LH receptors show increased basal activity, the *in vitro* dose-response relationships (Fig. 7) indicate that they would still respond to the high concentrations of LH that are needed to trigger ovulation.

### B. Inactivating mutations of the LH receptor

A special form of complete male pseudohermaphroditism was described in 1976 in a 35-yr-old 46,XY woman characterized by high LH levels, normal FSH, and extremely low testosterone (249). LH responded well to GnRH challenge, whereas FSH increased only marginally. Testosterone increased after ACTH challenge but was completely unresponsive to hCG. The subject had female external genitalia and two abdominal testes with epididymides and vasa deferentia, but absent Müllerian structures. Upon microscopic examination, the testes were found to contain seminiferous tubules with normal appearing Sertoli cells, occasional immature germ cells, but, notably, no Leydig cells (249). Leydig cell hypoplasia (LCH) or Leydig cell agenesis, as this syndrome was named, was reported on several occasions in prepubertal, adolescent, and adult males (250–254), also in familial fashion (255–257). Prompted by the lack of hCG responsiveness, noted in all cases, and by the absence of LH or hCG binding sites in membrane preparations of removed testis tissue (258–260), it was hypothesized that Leydig cell precursors failed to develop, or that Leydig cell differentiation did not occur, as a consequence of aberrant LH receptor expression or function. Two types of LCH have been proposed (261, 262). The severe form, as described above, is characterized by complete 46,XY male pseudohermaphroditism, low testosterone and high LH levels, total lack of responsiveness to LH/hCG challenge, and absent development of secondary male sex characteristics. There is a notable lack of breast development, which is the clearest phenotypic difference between this condition and androgen insensitivity (testicular feminization) due to inactivating mutations in the androgen receptor gene. The milder forms of LCH display a broader array of phenotypic expression, ranging from micropenis (261) to severe hypospadias (198). In fact, LCH may present as a disorder of sex differentiation and virilization caused by absent or low testosterone production, ranging from very mild undervirilization to complete pseudohermaphroditism, and the relative severity of the phenotype may depend on the degree of responsiveness to LH/hCG (see also below).

The poor or totally lacking responsiveness to LH/hCG has led to the hypothesis that loss-of-function mutations in the LH receptor gene may be the underlying cause of LCH. Many different types of mutations may cause full inactivation of function of the LH receptor gene product. Deletions may remove large parts of the LH receptor gene and cause complete absence of any LH receptor protein. Otherwise, nonsense mutations or frameshift-inducing base insertions or deletions cause premature truncations of the LH receptor protein and loss of its function. In fact, the first report describing an LH receptor gene mutation in a LCH kindred concerned a missense mutation, Ala<sup>593</sup>Pro, in TM6 near the extracellular side of the plasma membrane (199) (Table 3). The homozygous Ala<sup>593</sup>Pro mutation was found in two

46,XY pseudohermaphrodite adult siblings, born from consanguineous parents, who presented with female external genitalia, primary amenorrhea, and lack of breast development. Their parents were heterozygous for the mutation. Sections of testicular tissue showed hyalinized seminiferous tubules with almost total lack of germ cells and very few, immature type Leydig cells in the interstitium. Transient expression of the mutated LH receptor *in vitro* revealed a low number of hCG binding sites with normal high affinity. However, when hCG-induced cAMP production was determined, no effect was detected with the mutant LH receptor, even at very high hCG concentrations.

Subsequently, an identical homozygous mutation was found in a 46,XX sister of the 46,XY siblings (200). She presented with a relatively mild phenotype: amenorrhea with normally developed primary and secondary sex characteristics, increased LH and FSH, and low levels of estradiol and progesterone that were unresponsive to hCG treatment, confirming the inactivating mutation in the LH receptor gene. Histological examination of an ovarian biopsy sample revealed all stages of follicular development, including primordial follicles and preantral and antral follicles with a well developed theca cell layer, but no preovulatory follicles or corpora lutea. In one ovary, a large cyst was present, presumably a remnant of a nonovulated follicle. Clinical examination revealed small uterus, normal-sized vagina with hyposecretory function and thin walls, and decreased bone mass, all indicative of low estrogen levels. These observations strongly support the view that LH is essential for ovulation and sufficient estrogen production, while follicular development is initially autonomous, and at later stages dependent on intact FSH action. Other similar cases have also been described, all siblings of 46,XY complete male pseudohermaphrodites (196, 198, 201).

Additional mutations of the LH receptor, causing LCH in 46,XY patients or amenorrhea in 46,XX patients, have now been reported (Table 3). As expected, some of these involve deletions or nonsense mutations. Accordingly, a deletion in exon 8 causes complete LH receptor dysfunction, both in terms of absence of binding and absence of signal transduction (195). Since exon 8 encodes a part of the extracellular domain, such absence of hormone binding is expected. Partial inactivation of LH receptor function was found in a patient with a homozygous deletion of exon 10 (24). The removal of the amino acids encoded by exon 10 from the LH receptor protein results in inhibited receptor transport to the plasma membrane and partial obliteration of LH receptor function (23). In addition, nonsense mutations have been found in different regions of the transmembrane domain (TM4: Trp<sup>491\*</sup>; TM5: Cys<sup>545\*</sup>; and IL3: Arg<sup>554\*</sup>) (A. Richter-Unruh *et al.*, unpublished; 197, 198). These mutations cause truncation of the LH receptor protein and corresponding absence of at least part of IL-3 and TM6 and -7, which are important regions for G protein coupling. Indeed, expression of the Cys<sup>545\*</sup> LH receptor mutant revealed complete absence of hormone-induced cAMP production and also very low hormone binding, probably as a result of misfolding of the receptor during expression (197). A smaller deletion of Leu<sup>608</sup>Val<sup>609</sup> (201) in TM7 suppressed severely but incompletely the LH receptor function, as evidenced by a 30-fold

lower number of plasma membrane LH binding sites after transient expression of the mutant LH receptor, and only 1.5- to 2.5-fold increase in cAMP production upon hCG stimulation (201).

Inhibiting mutations have also been identified in the extracellular domain of the LH receptor. In a compound heterozygous subject with LCH (other allele: Cys<sup>554\*</sup>), a 27- or 33-bp insertion (see below) was found between codons 18 and 19 in exon 1 (A. Richter-Unruh, *et al.*, unpublished; 192). This region contains an imperfect leucine-triplet repeat encoding: LQLLKLLLLLQ < insertion site > PPLPRA. A polymorphism in the LH receptor gene exists in which CTGCAG (LQ) is inserted at the same position, without deleterious effects on receptor function (186–188, 263–265). The partial gene duplication at this site is probably the result of unequal crossing over (266) and encodes LLKLLLLLQ (27 bp) or the same sequence with one additional LQ unit (33 bp), since it is unknown whether the allele in which the insertion took place contained the additional polymorphic sequence. The insertion site is located immediately upstream of the proposed signal peptide cleavage site and may therefore interfere with protein transport to the plasma membrane. *In vitro* expression of the insertion mutant showed complete absence of LH receptor function (192).

The first LH receptor missense mutation in the extracellular domain was found in a patient with incomplete LCH (193). This Cys<sup>131</sup>Arg amino acid substitution in exon 5 caused a 95% decrease in hormone binding and a limited response of adenylyl cyclase to hCG at high hormone concentrations. About one third of the interstitial testicular cells expressed both LH receptor and P450c17 (17-hydroxylase) as evidenced by immunohistochemical staining, indicating some residual effect of LH or hCG on the precursor Leydig cells in this case (193). A homozygous missense mutation Phe<sup>194</sup>Val was found in exon 7 of the LH receptor gene (194). This mutation was located in a stretch of five amino acids (AlaPhe<sup>194</sup>AsnGlyThr) that is perfectly conserved in the family of glycoprotein hormone receptors. The corresponding Ala<sup>189</sup> of this sequence in the FSH receptor protein has been found to be mutated in patients with ovarian FSH resistance (see below; Ref. 220), but functional studies of this mutation have not yet been reported (194). Located much closer to the first transmembrane domain and encoded by exon 11, two amino acid changes have been found, Cys<sup>343</sup>Ser (J. W. M. Martens, S. Lumbroso, A. Richter-Unruh, H. G. Brunner, A. P. N. Themmen, and Ch. Sultan, personal communication) and Glu<sup>354</sup>Lys (196), which cause complete inactivation of LH receptor function. Both Cys<sup>343</sup> and Glu<sup>354</sup> are conserved throughout the family of glycoprotein hormone receptors. The equivalent Glu<sup>358</sup> in the rat LH receptor has been extensively mutated, and it was found that changing this amino acid to Lys causes low expression and inhibition of signal transduction (267, 268).

Since quite a number of mutations have been identified in the LH receptor gene, a comparison can be made between the extent of the phenotype of LCH patients and the residual, if any, activity of their LH receptor alleles, similar to the effect of activating amino acid substitutions in FMPP. Presence of the Ala<sup>593</sup>Pro mutation on both alleles or compound heterozygosity of the Cys<sup>343</sup>Ser and Cys<sup>543</sup>Arg mutations, all of

which completely inactivate the LH receptor, is associated with complete pseudohermaphroditism (199) (J. W. M. Martens, S. Lumbroso, A. Richter-Unruh, H. G. Brunner, A. P. N. Themmen, and Ch. Sultan, personal communication). Truncation of the LH receptor protein in patients homozygous for a nonsense mutation causes a similar phenotype (197, 198). A carrier of a homozygous mutant LH receptor that is severely but not completely affected in its activity, such as the Cys<sup>131</sup>Arg LH receptor mutant, has a micropenis with hypospadias (193). In contrast, patients homozygous for mutations that cause even less complete impairment of LH receptor function, such as Ser<sup>616</sup>Tyr (198) and Ile<sup>625</sup>Lys (202), show a mild phenotype (micropenis). Interestingly, the same Ser<sup>616</sup>Tyr mutation in combination with a deletion of exon 8 on the other LH receptor allele results in a much more severe phenotype (perineoscrotal hypospadias) in line with the expected lower residual receptor activity (195). These correlations of patient phenotype with receptor behavior *in vitro* emphasize that there is no clear distinction between complete and partial feminization of external genitalia in patients with LCH as proposed previously (262, 269, 270). It will be of great interest to study the phenotype of 46,XX siblings of patients with a mild form of LCH, to determine whether reduced, but not totally absent, LH signal transduction also causes problems with ovarian function.

Not much is known about the molecular effects of the mutations on LH receptor function. Although in some cases G protein coupling itself may be affected, such as in cases of receptor truncation in which the G protein coupling domain is absent, other causes of decreased receptor activity may be caused by lack of transport from the endoplasmic reticulum or the Golgi apparatus, accompanied by incorrect processing such as N-linked glycosylation and/or palmitoylation. Some studies have started to address these details of the receptor function. Deletion of Leu<sup>608</sup>Val<sup>609</sup> in TM7 causes 80% of the LH receptor molecules to be retained inside the cell, compared with 40% of the WT receptors in this study (201). However, although the receptors expressed at the cell surface bound hCG with similar affinity as the WT LH receptor, their ability to activate cAMP production was severely reduced (201). A comparison of the effect of the Ser<sup>616</sup>Tyr, Ile<sup>625</sup>Lys and Ala<sup>593</sup>Pro mutations on LH receptor function showed very different results (202). All three mutant receptors showed much decreased cell surface expression, and their signaling capacities were compared with the WT LH receptor expressed at similar receptor densities. The Ala<sup>593</sup>Pro LH receptor completely lacked hormone-dependent signaling activity and was expressed at a very low level (200, 202). The Ser<sup>616</sup>Tyr LH receptor hardly displayed signaling, and the affected receptor activity in the patient appeared to be caused chiefly by the low level of expression. In contrast, although expressed at a higher level, the Ile<sup>625</sup>Lys LH receptor exhibited severely reduced, but not absent, signaling (202). In most other reports of inactivating mutations in the LH receptor, comparisons have been made between level of hormone binding and receptor activation after *in vitro* transfection in a suitable cell line, without investigating the mechanisms responsible for reduced expression at the plasma membrane (192, 193, 195, 197, 198).

In males, the phenotype of impaired LH/hCG signaling is

more severe than in women, showing that LH/hCG is essential for correct sex differentiation and that it is mandatory for any reproductive function at all. Nevertheless, even in the complete absence of LH/hCG signaling, some autonomous Leydig cell function remains, as is exemplified by the presence of epididymides and vasa deferentia in a LCH patient with complete absence of LH receptor function (199). At the time of fetal testicular differentiation, around 8 weeks of fetal life, Leydig cells start to differentiate and to express the steroidogenic enzymes necessary for androgen production (58, 59, 271). Probably, Leydig cells produce some androgens at this time during development, although they do not yet appear to express LH receptor and are independent of LH/hCG. A similar situation has been found in the *hpg* (272) and common  $\alpha$ -subunit *null* mutant mice (128) (see below), which display normal male fetal sex differentiation in the absence of LH. In the human, however, after this hCG-independent activity, Leydig cells do express LH receptors and go through differentiation and proliferation stages that parallel serum concentrations of hCG (59, 271). In some patients who have been diagnosed with LCH, no LH receptor gene mutations have been found, despite DNA sequencing of all 11 exons of the gene (A. Richter-Unruh *et al.*, unpublished; 273). Other regions of the gene that regulate correct splicing of LH receptor pre-mRNA or the level of expression of the LH receptor protein may be changed in these patients. These promoter/enhancer and intronic DNA sequences are difficult to investigate in the large LH receptor gene. On the other hand, diagnosis of LCH is not always unambiguous, since other possible causes of pseudohermaphroditism such as androgen receptor insensitivity and steroidogenic enzyme alterations have to be excluded before LCH can be established.

Several polymorphisms have been identified in the LH receptor gene. Base changes in intron 1 and exons 8 and 11 appear to be silent. The other three polymorphic sites cause a change in the protein product of the gene: insertion of LeuGln at codon 18, Asn<sup>291</sup>Ser, and Asn<sup>312</sup>Ser (Table 3). However, for none of the polymorphic sites, a modification of LH receptor function or linkage to phenotype has been described (Table 3).

### C. Inactivating FSH receptor mutations

Few mutations have so far been identified in the FSH receptor gene (Table 3 and Fig. 6). The paucity of FSH receptor mutations found in patients may indicate that the phenotype(s) caused by them may be less clear than the effects of LH receptor mutations and therefore escape our attention. Alternatively, a selection mechanism may be operative against FSH receptor gene mutations, perhaps based on a strong dominant antifertility effect that precludes the inheritance of the faulty allele. FSH has an important role in the ovary in follicular maturation and in maintenance of granulosa cell estrogen production. In the male, FSH regulates Sertoli cell proliferation and differentiation in the immature testis and is proposed to participate in the regulation of spermatogenesis, assuring that it is qualitatively and quantitatively normal. Thus, loss-of-function mutations in the FSH receptor might result in small testes with impaired spermatogenesis in the male, while in women a phenotype

may be expected to be characterized by low estrogen production and infertility as a result of absent follicular maturation, such as is the case in premature ovarian failure or resistant ovary syndrome. Conversely, a male carrier of an activating FSH receptor variant may develop more testicular Sertoli cells and have large testes (megalotestis), with no other clinical abnormalities, while a female carrier might present with overstimulation of granulosa cell growth causing ovarian malignancies, a high chance of dizygotic twinning, and premature menopause as a result of increased selection of growing follicles accompanied by enhanced rate of primordial follicular recruitment. In line with these notions, the candidate syndromes in which FSH receptor mutational analysis has been carried out include premature ovarian failure, gonadal dysgenesis, resistant ovary syndrome, hypergonadotropic hypogonadism, PCOS (219, 274–276), granulosa cell or Sertoli cell tumors (277–280), and males with absent or low and aberrant sperm counts with high FSH levels (221), with megalotestis (224), or with idiopathic male infertility (218). However, in none of these conditions have mutations in the FSH receptor gene been found. These studies did reveal three polymorphisms in exon 10 that cause amino acid alterations (Table 3). However, the altered FSH receptor protein was fully active, and no linkage with any of the syndromes studied was noticed (218–221).

In the first successful search for loss-of-function FSH receptor mutations, advantage was taken of the considerable enrichment of mutations for certain recessively inherited disorders in Finland (281). A Finnish population-based study of hypergonadotropic ovarian dysgenesis revealed upon linkage analysis a locus termed ODG1 that was associated in a recessive inheritance pattern with the syndrome (282). Subsequently, the locus was mapped to chromosome 2p (220) that contained both the LH receptor and FSH receptor genes, at 2p21 and 2p21–16, respectively (7, 8). On the basis of phenotype of the patients (absence of follicular maturation) and that no male pseudohermaphroditism was found in the families (which would be a sign of LH receptor inactivation), the FSH receptor gene was chosen as the candidate gene. Sequencing of the coding regions of the FSH receptor gene resulted in identification of a missense Ala<sup>189</sup>Val mutation that segregated perfectly with the phenotype (220). As noted above, Ala<sup>189</sup> is the first amino acid in a perfectly conserved stretch of five amino acids in the glycoprotein hormone receptors (Ala<sup>189</sup>PheAsnGlyThr) in which also an inactivating mutation of the LH receptor has been identified (194). The presence of a Val at position 189 may interfere with the efficiency of glycosylation, resulting in impaired receptor trafficking and folding accompanied by a decrease in binding (21). In fact, the bulk of FSH receptor immunoreactivity in cells transfected with the mutated receptor cDNA appears to sequester within the cells (P. Pakarinen, A. Rannikko, P. Manna, I. Beau, E. Milgrom, M. Misrahi, and I. Huhtaniemi, unpublished). Scatchard analysis and cAMP stimulation studies *in vitro* showed that the Ala<sup>189</sup>Val FSH receptor mutant has normal binding affinity, and severely reduced plasma membrane expression and signal transduction (Fig. 8) (220). In fact, taking into account the reduced level of plasma membrane FSH receptors, the cAMP stimulation per receptor was roughly normal, but quantitatively insufficient since the bulk of the receptors were sequestered inside the

cell. The Ala<sup>189</sup>PheAsnGlyThr FSH receptor sequence also contains a consensus N-linked glycosylation signal, which was found to be mutated (heterozygous Asn<sup>191</sup>Ile) in a healthy fertile woman (223, 224). *In vitro* expression of the FSH receptor mutant revealed almost complete inactivity, confirming the importance of this region for the receptor function (224).

A more detailed description of the phenotype of homozygous female carriers of the Ala<sup>189</sup>Val FSH receptor mutant allele (283) showed that these patients were clinically similar to other patients with ovarian dysgenesis, with totally absent or poor development of secondary sex characteristics and high serum levels of FSH and LH. The notable difference was the presence of ovarian follicles in almost all cases with verified FSH receptor mutation, consistent with the FSH independence of primordial follicle recruitment and early follicular growth and development. In contrast, total absence of all follicles, including those in primordial stage, was observed in the cases where the FSH receptor mutation could not be detected. Thus, the FSH receptor mutation phenotype is distinct from the common form of ovarian dysgenesis as found in Turner's syndrome with streak ovaries and absence of growing follicles (283).

In men, this particular FSH receptor mutation has a less clear phenotype. A total of five homozygous males were identified and studied in Finnish families with the Ala<sup>189</sup>Val FSH receptor mutation. All men were found to be normally masculinized with normal circulating testosterone, normal or slightly elevated LH, moderately elevated FSH, and slightly to severely reduced testicular volume (164). Two of the men had fathered two children each. However, all of the five men studied had abnormal semen parameters ranging from severe or moderate oligozoospermia to normal sperm concentration with a low volume and teratozoospermia in

one individual (164). Conspicuously, none of them was azoospermic. These observations lead to the conclusion that FSH contributes to testicular size and qualitatively and quantitatively normal spermatogenesis. However, in the presence of normal androgen production, fertility is possible in the absence of FSH action and unlike suggested previously (discussed in Refs. 284 and 285), FSH action is not compulsory for the pubertal onset of spermatogenesis.

The Ala<sup>189</sup>Val FSH receptor mutation seems to be another member of the Finnish heritage of genetic diseases and is unlikely to be found in other populations (219, 276, 286, 287). Recently, two pairs of compound heterozygous FSH receptor mutations were described from France in women with primary or secondary amenorrhea, normal pubertal development, and follicular development up to the antral stage (222, 225). Two of the mutations, Ile<sup>160</sup>Thr and Asp<sup>224</sup>Val, both present in the extracellular domain (exons 6 and 9, respectively), caused almost completely impaired FSH binding. In accordance, cells expressing these receptor mutants showed no or marginal cAMP response to FSH stimulation. The other two mutations, Arg<sup>573</sup>Cys (IL 3) and Leu<sup>601</sup>Val (TM6), caused less complete receptor inhibition, displaying clear ligand binding and a residual 12–24% cAMP response to FSH, as compared with WT receptor. Localization of receptor protein by confocal immunofluorescent microscopy confirmed these findings, showing that the completely inactive receptor protein was sequestered inside cells, whereas both the WT and incompletely inactivated receptors were present on the plasma membrane (Fig. 9). It seems, therefore, that the degree of FSH receptor inactivation by mutations is largely determined by the degree of receptor sequestration inside the cell. However, a very recent study has shown that an Ala<sup>419</sup>Thr mutation in the second transmembrane loop of FSH receptor specifically abolishes signal transduction without marked effect on ligand binding (E. Docherty, P. Pakarinen, A. Titiinen, A. Kiilavuori, I. Huhtaniemi, S. Forrest, and K. Aittonmäki, unpublished observation).

Although the FSH receptor mutations described to date are few, the description of the phenotype in relation to the severity of effect of the mutation on residual FSH receptor activity may also indicate a genotype-phenotype relationship, as found for the LH receptor mutations. In patients with the lowest remaining FSH receptor activity, hypergonadotropic primary amenorrhea with hypoplastic ovaries is found, while in carriers of less affected mutations, there is secondary amenorrhea with normal sized ovaries and follicular development up to the antral stage, underlining the essential role of FSH in growth and development of the ovarian follicles (222, 225, 283). Even the plasma estradiol and inhibin B levels appeared to correlate with levels of receptor inactivation, and ovaries of patients with the milder forms of mutations may respond to high-dose FSH stimulation. Hence, the molecular diagnosis of these rare patients may help in design of rational treatment for their infertility.

#### D. Activating FSH receptor mutation

To date, a single activating mutation in the FSH receptor has been identified (Table 3) (226). A hypophysectomized male under treatment with testosterone had normal sper-

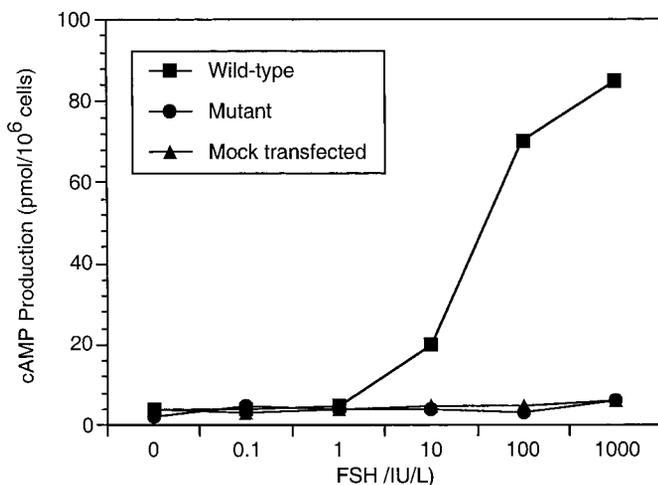


FIG. 8. Ala<sup>189</sup>Val mutant FSH receptor does not function in signal transduction. FSH-stimulated cAMP production of MSC-1 cells transfected with the FSH receptor expression construct. Squares denote wild type, circles represent Ala<sup>189</sup>Val FSH receptor mutant, and triangles depict mock-transfected controls. Each batch of cells was co-transfected with a plasmid expressing bacterial luciferase gene under a powerful viral promoter, to control for transfection efficiency. The cAMP production was equalized to a constant amount of luciferase expression and calculated per 10<sup>6</sup> transfected cells after a 3-h incubation. [Courtesy of Dr. P. Pakarinen (unpublished).]

matogenesis in spite of undetectable gonadotropins (224, 226). Usually, androgen treatment alone is not sufficient to support spermatogenesis in the absence of gonadotropins. Screening of the transmembrane domain-encoding exon 10 of the FSH receptor gene resulted in identification of a heterozygous Asp<sup>567</sup>Gly mutation located in the third intracellular loop. Mutation of the equivalent Asp<sup>564</sup>Gly in the LH receptor causes constitutive activity in FMPP patients (206). However, the proof of constitutive activity of the Asp<sup>567</sup>Gly FSH receptor *in vitro* did not appear as straightforward as it was in the case of activating LH receptor mutants. In standard transient transfection experiments, the mutant FSH receptor behaved similarly to the WT version, both in the presence and absence of FSH, although careful examination of basal receptor activity in experiments employing smaller

amounts of transfected DNA, revealed a 1.5-fold increase in cAMP production (226). However, the constitutive activity of the Asp<sup>567</sup>Gly FSH receptor was disputed in two separate studies on the role of TM5, TM6, and the intervening third intracellular loop in receptor activation (27, 288). Thus, the role of the FSH receptor mutant in the spermatogenic response to testosterone in the hypophysectomized patient remains unresolved. The FSH receptor is not generally resistant to activating amino acid changes. Construction of the Leu<sup>460</sup>Arg mutation in TM3 of the FSH receptor, which is the equivalent of the Leu<sup>457</sup>Arg LH receptor mutation identified in a sporadic FMPP patient (207), resulted in significant constitutive activity (235).

Nevertheless, the relatively mild effects on spermatogenesis of complete absence of FSH receptor activation both in human and mouse (220, 289, 290) raises the question of whether constitutive FSH receptor activity alone could explain the phenotype of the hypophysectomized male. In relation to the patient described in this report, it should also be stated that his circulating testosterone concentration in the absence of replacement therapy was 4.9–7.7 nmol/liter, *i.e.*, 5- to 8-fold above the normal castrate range (226). The phenotype detected may therefore not be representative of the role of FSH alone in the maintenance of spermatogenesis in the absence of LH/testosterone action. Further identification of other activating FSH receptor mutations in patients is necessary for resolution of this apparent discrepancy.

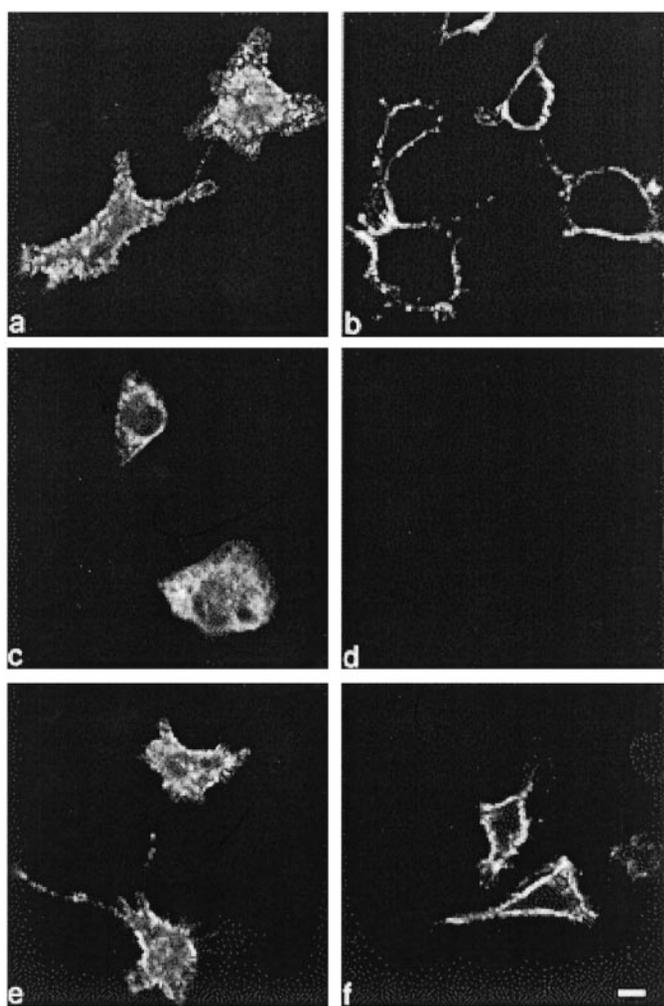


FIG. 9. Confocal microscopy of WT and mutated FSH receptor immunofluorescence localization in intact and permeabilized cells. Cell surface expression of WT and mutated FSH receptors. COS-7 cells were transfected with expression vectors encoding either the WT (a and b) or the mutated Ile<sup>160</sup>Thr (c and d) or Arg<sup>573</sup>Cys (e and f) receptors. Permeabilized (a, c, and e) or nonpermeabilized (b, d, and f) cells were incubated with the monoclonal FSHR 323 antibody. Confocal microscopy was used to study the cellular distribution of receptors. [Reproduced with permission from I. Beau *et al.*: *J Clin Invest* 102:1352–1359, 1998 (222). © Journal of Clinical Investigation.]

## VI. Animal Models of Disrupted Gonadotropin Function

The animal models for overexpression of the gonadotropin genes, as well as targeted disruption of the gonadotropin subunit and receptor genes, have been of great importance. Their phenotypes have corroborated the findings made in humans, helping to decipher the effects of a specific mutation in a patient whose phenotype may not always be purely due to a single genetic change. In addition, in those cases where a human mutation has not yet been identified, the animal phenotypes produced will help in prediction of the effect of such mutations in the human.

### A. Gonadotropin overexpression

Both LH and FSH overexpressing transgenic mice have been produced. These are good models for human conditions with elevated gonadotropin secretion, which have been considered to cause both infertility and gonadal tumors (291–293).

Risma *et al.* (294) produced LH overexpressing mice by expressing, under control of the bovine  $\alpha$ -subunit promoter, the bovine LH $\beta$ -subunit containing the 29-amino acid C-terminal extension of the hCG $\beta$  subunit gene. This resulted in LH with a long half-life and about 10-fold increase in plasma concentration. However, the elevation was only seen in female mice, apparently due to a functional negative feedback loop between the testes and the transgene expression. The female mice were infertile, ovulated infrequently, maintained a prolonged luteal phase, and developed pathological ovarian changes such as marked enlargement, multiple cysts, and granulosa and theca-interna cell tumors. In addition, a

subset of the mice displayed renal abnormalities. The ovarian tumorigenesis supports some other transgenic models on tumor promoter effects of high gonadotropin levels (295–297). This feature may be related to the structural relationship of gonadotropins with cystine-knot growth factors, including nerve growth factor, transforming growth factor- $\beta$ , and platelet-derived growth factor- $\alpha$  (6). This would possibly entail activation of other signal transduction pathways than that employing cAMP, as has been recently demonstrated with an activating LH receptor mutation detected in Leydig cell tumors; the IP3 pathway was preferentially stimulated by the mutated LH receptor (217). On the other hand, chronically elevated cAMP levels may also provide a tumorigenic signal, as seems to be the case in the function of constitutively activated TSH receptors in toxic thyroid nodules (298).

The elevated LH levels appeared to prolong the life span of corpora lutea in pseudopregnancy-like fashion. The development of multiple cysts and increased LH/FSH and androgen/estrogen ratios are akin to changes seen in human PCOS. The LH $\beta$  overexpressing mouse thus clearly demonstrates that pathologies of ovarian function are associated with chronically elevated LH levels, which to a certain extent resemble analogous conditions in the human, *i.e.*, the PCOS and postmenopausal ovarian stromal tumors.

In males, the transgene did not increase LH levels but, nevertheless, for unknown reasons, reduced their fertility and testis size. It remains to be seen what phenotypic expression chronically elevated gonadotropin levels would cause in the male. It also is unclear how well these animals with pharmacologically elevated gonadotropin levels are able to simulate human conditions with much lower elevation in gonadotropin concentrations, or mild constitutive activation of the LH receptor function through mutation.

Kumar *et al.* (290, 299–302) have produced mice with both absent and enhanced FSH action and studied their specific effects on mouse phenotype, as well as explored their contribution to gonadal tumorigenesis of inhibin-deficient mice. FSH overexpressing male mice had normal testicular differentiation and spermatogenesis. Nevertheless, they were infertile, possibly due to some behavioral effects, functional incompetence of the sperm, or abnormal secretory products of the enlarged seminal vesicles (302). Interestingly, these mice presented with elevated testosterone production and enlarged seminal vesicles secondary to elevated androgens, demonstrating that supraphysiological FSH levels somehow stimulated Leydig cell function, possibly through a Sertoli cell paracrine factor (303). Whether a gain-of-function mutation of the FSH receptor gene would produce a similar phenotype in human remains to be seen. With the present knowledge this seems unlikely, since men with pituitary adenomas secreting large amounts of FSH have no testicular phenotype (304).

The transgenic FSH overexpressing females also were infertile, with highly hemorrhagic and cystic ovaries and elevated serum testosterone, estradiol, and progesterone. The latter may have caused the kidney and urinary tract abnormalities observed in these animals which, interestingly, were also found in LH-overproducing mice (294). No gonadal tumors were found in these mice, indicating that FSH alone is not oncogenic. The infertility was due to disrupted fol-

liculogenesis and development of ovarian cysts. Thus, they mimicked the human ovaries observed in hyperstimulation and PCOS. Likewise, women with elevated serum FSH levels, in conditions such as postmenopausal ovarian cancer (305), ovarian hyperstimulation syndrome (306), and with FSH-secreting pituitary adenomas (307), present with cystic and hemorrhagic ovaries. Interestingly, the female phenotypes of the FSH and LH overexpressing mice are very similar (see above). The human equivalent to this condition, *i.e.*, activating FSH receptor mutation in women, remains to be characterized.

The same authors (297) have created inhibin-deficient mice that develop gonadal sex cord-stromal tumors and cancer cachexia-like syndrome. These mice have been cross-bred into the FSH-overexpressing and FSH-deficient genetic backgrounds, which has provided the opportunity to study the role of this gonadotropin as a contributing factor in gonadal tumorigenesis (302). Since gonadotropins have also been suggested to play a role in human ovarian tumorigenesis (291, 308), these double mutant mice are a useful model for this human malignancy. The tumorigenesis was delayed and the cachexia-like syndrome was prevented in the FSH/inhibin-deficient mice, apparently because of lower activin A levels. It was concluded that FSH is not a direct causative factor of gonadal tumors, but is an important trophic modifying factor. A similar role for LH has been proposed on the basis of another transgenic mouse model that expresses the SV40 T-antigen under inhibin  $\alpha$ -subunit promoter (295, 296, 309–311). These mice also develop ovarian and testicular somatic cell tumors, whose growth is dependent on LH. It is therefore feasible that gonadal tumorigenesis could be associated with activating mutations of gonadotropin receptor genes, but, obviously, such cases would be extremely rare.

### B. Targeted disruption of gonadotropin genes

A classical, naturally occurring knockout of gonadotropin secretion is the hypogonadotropic hpg mouse (312), due to a long deletion in the GnRH gene. GnRH synthesis and secretion are totally abolished in these mice, and there is a consequent near-total deficiency of FSH and LH. This mouse mutant has been extensively used as a model with which to study the phenotypic expression of hypogonadotropic hypogonadism and for its experimental treatments.

Targeted disruption (knockout) of the  $\alpha$ -subunit (128) and FSH $\beta$  (290) genes have been produced, but that of LH $\beta$  has not yet been reported. Genetic disruption of the  $\alpha$ -subunit gene caused, as expected, hypogonadal and hypothyroid phenotype resulting in dwarfism. Thyroid development of the animals was arrested in late gestation, and gonadal development was arrested several weeks after birth. GnRH neuron migration, development of secondary sex organs, and gonadal development during the fetal and neonatal periods were normal.

Hypothyroidism of the mice was an expected finding, and a more intriguing finding concerned effects of lack of gonadotropins. It was found that in both sexes, the sexual differentiation proceeded normally until birth. This finding strengthens the contention that gonadotropin action, at least in the rodent, is not needed *in utero*. Earlier studies have

demonstrated that, whereas female sexual differentiation seems to occur without influence of the fetal ovary, the male sex organ differentiation is critically dependent on the two hormones produced by the fetal testis, *i.e.*, testosterone and AMH. In adult testis, testosterone production is critically dependent on LH action. It seems that neither of the pituitary gonadotropins, secreted already *in utero*, is needed for fetal testicular activity. In fact, it was shown recently that in rat fetuses, the testicular testosterone surge on days 17–18 of fetal life precedes the appearance of LH in circulation (313), and male sexual differentiation of hpg mice is also normal despite near-total lack of gonadotropins (312). A great number of paracrine factors, ineffective in adult testis, are able to stimulate fetal testicular steroidogenesis (313). Hence, the rodent fetal testes seem to be able to produce testosterone and AMH without gonadotropins, as was clearly demonstrated by the common  $\alpha$ -subunit null mutant mice (128). Male sexual differentiation seems to occur normally even in mice with disrupted thyroid-specific enhancer-binding protein (T/ebp) (314), born without the pituitary gland (P. Parkarinen, F. El-Gehani, S. Kimura, L. J. Pelliniemi, and I. Huttaniemi, unpublished data). The situation in humans may be somewhat different, due to the presence of hCG, known to be able to stimulate fetal testes (59). Since the single LH-deficient human male so far described was normally masculinized at birth (118), hCG was apparently sufficient to stimulate his testicular steroidogenesis *in utero*. However, also in the human there is some LH/hCG-independent fetal Leydig cell function, as indicated by the presence of epididymides and vasa deferentia in the patients with complete LCH (see Section V.B).

The FSH-deficient female mice produced by disruption of the FSH $\beta$  gene with the embryonic stem cell technique (290) are infertile due to a block in folliculogenesis before antral follicle formation. The phenotype of these mice is very similar to a respective model with disrupted FSH receptor function (289, 315) and the human cases with inactivating mutation in the FSH $\beta$  subunit (123, 124, 126) or the FSH receptor (220) genes. All female mice were infertile, and upon histological examination, their ovaries were small and thin, lacking corpora lutea and failing to show follicular development beyond the preantral stage. As a sign of ovulatory competence, PMSG/hCG treatment and mating of the knockout mice resulted in normal rate of superovulation and two-cell embryos. Unlike males, the females had elevated LH levels, apparently due to sex differences in feedback regulation. All data on genetic inactivation of FSH action thus agree on the crucial role of FSH in female fertility, due to its action during the final stages of follicular maturation and antral follicular formation.

In males, much milder phenotypic effects were seen. The knockout males were normally masculinized and fertile, although their testes were reduced in size. Again, the phenotype of the males was very similar to the phenotype of the FSH receptor knockout males (289, 315) and of men with FSH receptor defect (164). The apparent discrepancy with the two men with confirmed inactivating mutations in the FSH $\beta$  gene were discussed above. In the FSH $\beta$  knockout mice, the total seminiferous tubule volume was reduced, apparently due to ineffective proliferation of Sertoli cells in the prepubertal age

because of lack of FSH (316). The Leydig cell number, as well as LH, testosterone, and accessory sex gland weights were normal, indicating that the putative FSH-dependent Sertoli cell factor, suggested to stimulate Leydig cells (303), is physiologically of minor importance. In keeping with the suppressed spermatogenesis, although adequate for fertility, was the finding of 75% lower epididymal sperm numbers, lower proportion of motile sperm, but normal viability. Hence, this model clearly demonstrates that FSH is needed for normal testicular size and quantitatively and qualitatively normal spermatogenesis, but not for spermatogenesis or male fertility *per se*.

### C. Targeted disruption of gonadotropin receptor genes

In contrast to the many animal models that have been developed to investigate the direct role of gonadotropins, the only mouse model directed toward gonadotropin receptor function is the FSH receptor *null* mutant mouse (289, 315). To date, no mice with disrupted LH receptor (or LH) function have been reported. Likewise, the models for constitutive LH or FSH activation are missing. The FSH receptor *null* mutant mice in the two existing reports were developed using homologous recombination strategy with a mouse genomic DNA construct in which exon 1 of the FSH receptor was replaced by a marker gene. The absence of FSH receptor was verified by Northern blotting and FSH binding experiments. Female FSH receptor *null* mice were infertile and showed thin uteri caused by low estrogen production by the small ovaries, with blockade of follicular development at the preantral stage. No Graafian follicles or corpora lutea could be identified.

Male FSH receptor *null* mice were fertile, but had small testes and decreased testosterone levels. Semen analysis showed a decrease in the number and motility of sperm, and a relative increase in aberrant spermatozoa, such as bent tails and cytoplasmic droplets. The decrease in testis size appeared to be caused by a decrease in seminiferous tubule volume, although a decrease in tubule length, which would be caused by a decrease in Sertoli cell number, was not excluded. FSH levels were increased in both male (3-fold) and female animals (15-fold), accompanied by a significant enlargement of the anterior lobe of the pituitary gland with a high number of FSH producing cells.

In many respects, the FSH receptor *null* mutant mice appear to be a complete phenocopy of the human patients with inactivating FSH receptor mutation described above (164, 220, 283): incomplete suppression of spermatogenesis with increased proportion of aberrant forms of spermatozoa and a blockade of follicular development, but intact primordial follicle recruitment. However, some intriguing and unexplained observations were made in the mice that have not been described in human patients. First, the inhibin-mediated feedback appears to be altered in FSH receptor *null* mutant mice. FSH was increased strongly in females, although no change in immunoreactive inhibin was found. In the males, however, inhibin was decreased with only a slight elevation of FSH. Furthermore, heterozygous females had unchanged FSH levels, but showed an intermediate phenotype, indicating that the expression level of the FSH receptor

in these animals is not under strict regulation, but rather is dependent on gene dosage. Heterozygous males also displayed an intermediate phenotype with respect to the effect on FSH and testosterone levels. Interestingly, in the FSH *null* mutant mouse model, such a difference between heterozygous and WT animals has not been noted (290). Lastly, in spite of the absent antral follicular development, anovulation, and decreased estrogen levels, vaginal smears of the mice still showed a periodic estrous cycle with a recognizable estrus every 4 days (289). This was not observed in another FSH receptor knockout model, where all homozygous mutant females were anestrus, with imperforate vaginas (315).

### VII. Future Directions

Human mutations for most of the gonadotropin ligand and receptor genes are already known. Of the possible permutations, the largest number of cases have been reported for the activating and inactivating LH receptor mutations, and their phenotypic expression is now relatively well characterized. Only one male with inactivating LH $\beta$  mutation has so far been reported (118). Although the phenotype of this subject is clear and is apparently in line with the known actions of LH at the different times of development, additional cases with similar mutation would strengthen our knowledge about LH action. It would also be intriguing to study women with similar mutation, which should elucidate the role of LH and LH receptor, if any, in the fetal period of female development. Likewise, mouse models of disrupted LH synthesis, as well as activating and inactivating LH receptor function, still remain to be reported. There is a transgenic mouse overexpressing LH, but in this particular strain, only the females are hypergonadotropic (294). Therefore, the phenotype of male mice with LH overproduction still remains to be characterized.

As for the consequences of mutations in FSH and FSH receptor genes, much less is still known. Three females and two males with mutated FSH $\beta$  gene have been reported (123–127, 162). The female cases all display the expected lack of follicular development that was successfully treated with gonadotropins. The findings are identical to the inactivating FSH receptor mutations and the FSH $\beta$  and FSH receptor *null* mice, and therefore the role of FSH in the female can be considered quite well explored. However, there is a clear discrepancy between the consequences of FSH receptor or FSH $\beta$  mutations in men (125, 127, 162, 164). Five men reported with the FSH receptor mutation have at least some spermatogenic activity, whereas the two men with disrupted FSH $\beta$  function are azoospermic. It is apparent that more men with both mutations must be studied before the final word about the absolute or relative necessity of FSH for spermatogenesis is said. This question is of practical importance in view of the ongoing trials of FSH treatment in idiopathic male infertility and the prospects of FSH elimination as a strategy of male contraception.

Some polymorphisms have been detected in genes of the gonadotropins and their receptors. Their impact on pituitary-gonadal function is still largely unexplored. Somewhat more

is known about a common polymorphism detected in the LH $\beta$  gene (see above, Section IV.C), which seems both to predispose and protect its carriers from various pathologies of pituitary-gonadal function, such as PCOS, infertility, and breast carcinoma. The phenotypic expression of this polymorphism seems to vary between different populations, which may be a general phenomenon concerning polymorphisms. It is likely that additional polymorphisms will be found both in genes of the gonadotropins and their receptors, and they are likely to provide further explanations for the wide individual variability observed in reproductive functions.

The finding of activating TSH receptor mutations in thyroid adenomas suggested that such mutations in the LH and FSH receptors might have similar tumorigenic effects. Constitutive activation of the cAMP pathway by activating mutations in G $_s\alpha$  [gsp mutation (317)] has been found to be oncogenic in human ovarian stromal and testicular Leydig cell tumors (318), but no association has been indicated between FMPP and Leydig cell adenoma, suggesting that increased cAMP levels are not necessarily oncogenic. Recently, an activating LH receptor mutation (D<sup>578</sup>H) was identified in Leydig cell adenoma specimens from three boys (217). This particular LH receptor mutation has the special characteristic, not found in other mutants, that it not only causes high levels of cAMP in the absence of ligand, but also causes constitutive coupling to the IP $_3$  pathway, suggesting that this coupling, by itself or through synergism with the cAMP pathway, is essential to the tumorigenic activity of this mutation. However, the increased activation of the IP $_3$  pathway may also be symptomatic for persistent activation of alternate G protein-mediated signal transduction pathways that have been shown to activate proliferation, *e.g.*, through activation by  $\beta$ - $\gamma$  subunits (319–321). Possibly, a different mode of G protein activation may also be operative in the oncogenic action of the gsp mutation and explain why increased cAMP levels induced by mutant LH receptors do not cause Leydig cell adenomas, whereas the gsp mutation does. By and large, it will be interesting to address the possible specific roles and interactions of the different signal transduction systems in the overall actions of gonadotropins.

Recently, an FMPP patient with testicular seminoma was reported (322). The occurrence of these relatively rare disorders could be attributed to chance but could also be the effect of continuous stimulation by increased levels of androgens. The significance of the finding of Leydig cell adenoma and testicular seminoma in FMPP patients may become more apparent when more patients have been described. Although quite extensive studies have been carried out, no FSH receptor mutations, activating or inactivating, have been identified in granulosa cell or Sertoli cell tumors (277–280). The tumor-promoting potential of high FSH and LH levels has also been suggested by the inhibin peptide knockout models (297), the SV 40 T-antigen-expressing transgenic mice (295), and mice overproducing LH (294). The potential tumorigenicity of gonadotropins and their molecular mechanisms need to be addressed in more detail.

The recent discovery of gonadotropin receptor expression in extragonadal tissues (86) still remains without explana-

tion. All phenotypes observed with respect to human gonadotropin receptor mutations are related to specific gonadal expression, suggesting that extragonadal gonadotropin effects are unlikely to be of major physiological significance. Possibly, extragonadal LH receptor expression is caused by illegitimate or "leaky" transcription, which is detected by the very sensitive PCR methods that are often applied in these studies. The LH receptor knockout mouse model will probably be pivotal in solving this controversy. Mouse transgenic models will also be important in elucidating the importance of the multiple mRNA splice variants that are known to exist for the gonadotropin receptor genes (323–335).

Although the physiology and pathophysiology of gonadotropin function was previously characterized by classical physiological and biochemical research methods, novel information brought by recent molecular approaches has elucidated totally new aspects of these regulatory mechanisms. All is not yet known; on one hand, the research of importance of the genetic variability of gonadotropin action and, on the other hand, novel genetically modified animal models, will undoubtedly unravel totally new features of gonadotropin functions.

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