New Natural Inactivating Mutations of the Follicle-Stimulating Hormone Receptor: Correlations between Receptor Function and Phenotype

P. Touraine*, I. Beau*, A. Gougeon, G. Meduri, A. Desroches, C. Pichard, M. Detoeuf, B. Paniel, M. Prieur, J-R Zorn, E. Milgrom, F. Kuttenn, and M. Misrahi

Department of Endocrinology and Reproductive Medicine
Hôpital Necker (P.T., C.P., M.D., F.K.)
Institut Fédératif de Recherche (IFR-NEM)
75743 Paris Cédex 15, France

INSERM U 135, Hormones, Gènes et Reproduction et Laboratoire d'Hormonologie et Biologie Moléculaire
Hôpital de Bicêtre
Assistance Publique-Hôpitaux de Paris et Institut Fédératif de Recherche IFR21 (I.B, G.M., A.D., E.M., M.M.)
94275 Le Kremlin Bicêtre Cedex, France

INSERM U 407, Faculté Médecine Lyon-Sud (A.G.)
69600 Oullins, France

Department of Gynecology and Obstetrics (B.P.)
Hôpital Intercommunal de Créteil
94010 Creteil, France

Department of Cytogenetics
Hôpital Necker-Enfants Malades (M.P.)
75743 Paris Cedex 15, France

Department of Gynecology and Obstetrics
Hôpital Cochin (J-R Z.)
75014 Paris, France

Premature ovarian failure occurs in almost 1% of women under age 40. Molecular alterations of the FSH receptor (FSHR) have recently been described. A first homozygous mutation of the FSHR was identified in Finland. More recently, we described two new mutations of the FSHR in a woman presenting a partial FSH-resistance syndrome (patient 1). We now report new molecular alterations of the FSHR in another woman (patient 2) who presented at the age of 19 with primary amenorrhea contrasting with normal pubertal development. She had high plasma FSH, and numerous ovarian follicles up to 3 mm in size were evidenced by ultrasonography. Histological and immunohistochemical examination of ovarian biopsies revealed the presence of a normal follicular development up to the antral stage and disruption at further stages.

DNA sequencing showed two heterozygous mutations: Asp224Val in the extracellular domain and Leu601Val in the third extracellular loop of FSHR. Cells transfected with expression vectors encoding the wild type or the mutated Leu601Val receptors bound hormone with similar affinity, whereas binding was barely detectable with the Asp224Val mutant. Confocal microscopy showed the latter to have an impaired targeting to the cell membrane. This was confirmed by its accumulation as a mannose-rich precursor. Adenylate cyclase stimulation by FSH of the Leu601Val mutant receptor showed a 12 ± 3% residual activity, whereas in patient 1 a 24 ± 4% residual activity was detected for the Arg573Cys mutant receptor. These results are in keeping with the fact that estradiol and inhibin B levels were higher in patient 1 and that stimulation with recombinant FSH did not increase follicular size, estradiol, or inhibin B levels in patient 2 in contrast to what was observed for patient 1. Thus, differences in

0888-8809/99/$3.00/0
Molecular Endocrinology
Copyright © 1999 by The Endocrine Society

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

the residual activity of mutated FSHR led to differences in the clinical, biological, and histological phenotypes of the patient. (Molecular Endocrinology 13: 1844–1854, 1999)

INTRODUCTION

Normal pubertal development and fertility depend on the interplay of hypothalamic, pituitary, and gonadal factors. Various genetic defects of the hypothalamic-pituitary-gonadal axis that cause hypogonadism have been identified (reviews in Refs. 1 and 2). FSH and its receptor play a pivotal role in follicular development and in female fertility. This receptor belongs to a highly homologous subgroup of G protein-coupled receptors that also includes the LH and TSH receptors (review in Ref. 2). The recent description of mutations of the FSH receptor has provided insights into the role of FSH at different steps of follicular maturation.

The first mutation that was identified was the Ala189Val homozygous substitution detected in a highly consanguinous Finnish population (3). It was associated with primary amenorrhea, streak ovaries containing primordial and primary follicles but without any further follicular development (4). This observation indicated that the initiation of folliculogenesis was independent of FSH action. A similar conclusion has been very recently reached in mice with targeted disruption of the FSH receptor gene and which present a block in folliculogenesis at the preantral stage (5). On the other hand, it is well known that FSH is necessary for the development of preovulatory follicules (6, 7). Between these stages, the role of FSH for preantral or early antral follicles is still under discussion due to the lack of appropriate experimental models.

Recently we have described a novel phenotype associated with partial loss-of-function mutations of the FSH receptor (8). In this case the patient had primosecondary amenorrhea, high gonadotropin levels (especially FSH), and normal sized ovaries. Ultrasonography, ovarian histology, and immunocytochemistry showed a normal follicular development up to a small antral stage (follicles of 5 mm) and then a disruption at further stages. We now report a second such case associated with two different mutations. The patient presented primary amenorrhea and her antral follicles developed only to a size of 3 mm. She was a compound heterozygote bearing an Asp224Val or a Leu601Val mutation on each allele. As reported in the previous patient, transfection studies showed altered but not suppressed function of the mutated receptors. However the residual function was more limited than in the previous case. There was thus a correlation between receptor activity measured *in vitro* and the *in vivo* findings, *i.e.* the phenotype and the most advanced stage to which follicular development could proceed.

The propositus was a 19 yr-old Caucasian woman who consulted for primary amenorrhea. Puberty had occurred at the age of 13, with normal development of secondary sex characteristics. The patient was the second daughter of two nonconsanguinous parents. Her 21-yr-old sister described menstrual irregularity since her first menstruations at age 14. The patient's height was 174 cm and her weight 73 kg. Plasma FSH and LH were high, 63 and 26 IU/liter, respectively [normal (N): 4–8]. Plasma E_2 levels were low: 40–80 pmol/liter (N: 70–1000), and testosterone plasma level was 1.7 nmol/liter (N: 0.7–2.1 nmol/liter), Δ4-androstenedione was 8.4 nmol/liter (N: $<$5 nmol/liter) and dehydroepiandrosterone was 48.1 nmol/liter (N: 7–40 nmol/liter). Sex hormone binding globulin was at a normal low level: 34 nmol/liter (N: 30–69 nmol/liter). Plasma concentration of inhibin B was low, 30 pg/ml (N: 60–175 pg/ml). No antithyroid or antiovary autoantibody could be detected. The karyotype was normal: 46,XX. Pelvic ultrasonography showed a normal uterus and normal sized ovaries. Ten to 12 follicles of 3 mm were detected, regularly spread in each ovary. Under coelioscopy, two biopsies were performed on each ovary. The patient received an oral estrogen-progestin treatment (ethinyl-estradiol, 50 μg, and norgestrel, 500 μg) for 3 months. At the end of this treatment, plasma LH and FSH were low (1 IU/liter). An ovarian stimulation by recombinant FSH (Puregon, Organon, Puteaux, France) was performed with doses increased every 3–4 days until a cumulative dose of 5625 IU had been obtained. It was monitored by hormone assays and pelvic ultrasonographies.

The study was approved by the review boards of the different institutions. Informed consent was obtained from the patient and her family.

RESULTS

Sequencing of the FSH Receptor (FSHR) Gene

The sequencing of the complete coding region of the human FSHR gene in the patient revealed two heterozygous substitutions (Fig. 1): a substitution of an adenine for a thymidine in exon 9 yielded an Asp224Val substitution in the extracellular domain of the receptor. The second substitution of a cytosine for a guanine in exon 10 yielded a Leu601Val substitution in the third extracellular loop of the receptor. Each parent of the propositus was heterozygous for one of the mutations. The father carried the Asp224Val and the mother the Leu601Val substitution. The sister was heterozygous for the Leu601Val mutated allele.

Functional Study of the Mutated Receptors

Cells transfected with either the wild-type or the mutated Leu601Val receptors bound FSH with a similar

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

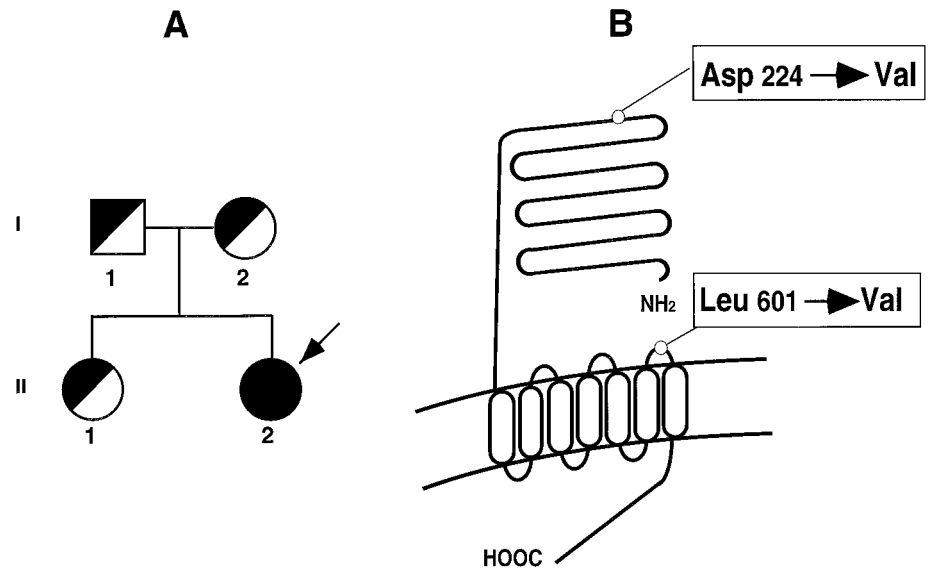

Fig. 1. Pedigree of the Family (A) and Location (B) of the two Mutations Detected in the Patient

In panel A, the propositus is indicated by an *arrow*. *Solid symbols* denote affected subjects, *half-solid symbols* denote unaffected heterozygotes, *circles* indicate females and *squares* indicate males. In panel B, the two heterozygotic mutations are indicated in the extracellular domain and in the third extracellular loop of the FSHR.

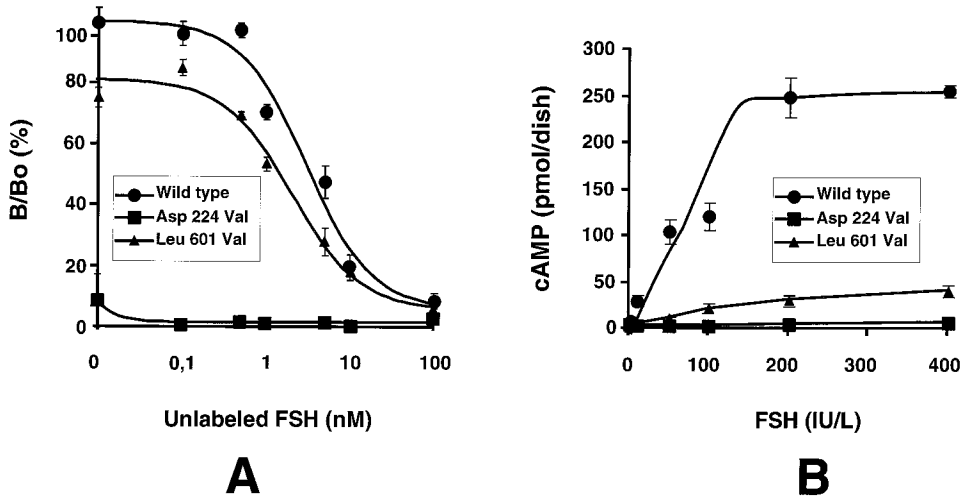

Fig. 2. Functional Studies of the Wild-Type and Mutant FSHRs

COS-7 cells were transfected with expression vectors encoding wild-type or mutated receptors. Panel A shows ligand binding. The cells were incubated with ^{125}I-labeled FSH in the absence or in the presence of increasing concentrations of unlabeled FSH (see *Materials and Methods*). The dissociation constant (K_D) was 4.1 ± 1.6 nM for the wild-type and 2.6 ± 0.4 nM for the Leu601Val mutant receptors, respectively. Each *point* represents the mean (±SEM) of triplicate determinations. Three experiments were performed with similar results. The concentrations of binding sites of the wild-type and the Leu601Val mutant receptors are indicated in the legend of Fig. 6. Panel B shows FSH-induced cyclase activation of receptors. Transfected cells were incubated for 45 min with increasing concentrations of FSH, and the accumulation of cAMP was measured (see *Materials and Methods*). Three experiments were performed with similar results. *Circles* correspond to the wild type, *squares* to the Asp224Val, and *triangles* to the Leu601Val FSHR mutants.

affinity (Fig. 2A). By contrast, FSH binding to the Asp224Val receptor was barely detectable.

FSH-induced adenylate cyclase stimulation was then studied in COS-7 cells transfected with expression vectors encoding the wild-type or the mutated receptors (Fig. 2B). The Leu601Val mutant did not cause stimulation of adenylate cyclase at low FSH concentrations. However, for very high FSH concentrations, a limited accumulation of cAMP was observed. There was no hormone-induced synthesis of cAMP in cells expressing the Asp224Val mutant receptor.

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

Table 1. Natural Loss of Function Mutations of the FSH Receptor: Correlation between Receptor Function and the Phenotype

	Finnish Patients[a]	Patient 2	Patient 1[b]
Pubertal development	± Delayed	Normal	Normal
Amenorrhea	Primary	Primary	Secondary
E_2 (pmol/liter)			
Basal	30	40–80	70–150
After FSH stimulation	NA	40–80	240
Inhibin B (pg/ml)			
Basal	NA	30	50
After FSH stimulation	NA	30	125
Ultrasonography:			
Follicle size (mm)			
Basal	NA	2-3	4-5
After FSH stimulation	NA	2–3	5–8.3
Follicular development	Primordial and primary follicles	Antral follicles	Antral follicles
In vitro activity of FSHR mutants (adenylate cyclase stimulation, % of wild-type receptor)	Nonsignificant	12 ± 3% Leu601Val	24 ± 4% Arg573Cys

NA, Not available.
[a] Ref. 4.
[b] Ref. 8.

Altered Surface Targeting and Processing of the Asp224Val Receptor Mutant

Impaired FSH binding to cells expressing the Asp224Val mutant receptor could have been due either to a modification of the binding site or to an alteration in receptor cellular trafficking. To distinguish between these two possibilities, cells were transfected with the wild-type or the mutated receptors, and receptor distribution was studied (Fig. 3). Transfected cells were incubated with an antibody directed against the extracellular domain of the receptor. When the cells were permeabilized with saponin before the incubation with the antibody, a strong intracellular staining was observed in all cases. When the cells were not permeabilized, labeling was observed on the cell surface for cells expressing the wild-type receptor or the Leu601Val mutant, but no labeling was observed for cells expressing the Asp224Val mutant receptor.

In experimental mutagenesis studies, intracellular trapping of mutated receptors has often been observed to be associated with an altered processing of the glycoprotein (9). We thus studied the glycosylation of the Asp224Val mutant receptor as compared with the wild-type receptor. For that purpose, the receptor was immunopurified from transfected cells and submitted to deglycosylation by endoglycosidase H (specific for high-mannose precursor moïeties of glycoproteins) or *N*-glycanase F (which removes all N-linked glycosaccharides). Receptors were analyzed by Western blot using a monoclonal antireceptor antibody.

As shown in Fig. 4, two species corresponding to the wild-type receptor were detected as previously described (10). The highest molecular mass species of approximately 87 kDa was resistant to endoglycosidase H but sensitive to *N*-glycanase F and resolved into an approximately 75 kDa species. It corresponds to the mature receptor, which has undergone a complete glycosylation and which is expressed at the cell surface (10). The approximately 81 kDa species was sensitive to both *N*-glycanase F and endoglycosidase H and resolved into an approximately 75 kDa species. It thus corresponds to a protein with high-mannose moïeties, which has been shown to be a precursor of the FSHR (10). Such a precursor accumulates in the endoplasmic reticulum (11).

In cells expressing the Asp224Val mutant, only the precursor was observed, and no mature receptor carrying complex carbohydrates could be detected (Fig. 4B).

Histological and Immunohistochemical Studies of the Ovary

Histological examination of ovarian biopsies showed a normal number of healthy primordial and primary follicles (Fig. 5A). Secondary follicles also displayed a normal morphology (Fig. 5B). Three antral follicles were found in the biopsies: the two smaller (0.22 and 0.28 mm) were apparently normal (not shown), whereas the larger (diameter: 0.8–1 mm) was clearly degenerating (Fig. 5C). It had an irregular antrum containing remnants of granulosa cell layers. The basal lamina was thick and irregular and the theca interna was hypertrophied (100–150 μm).

Immunocytochemical studies were undertaken on this follicle. They showed a strong staining of thecal cells for side chain cleavage enzyme ($P450_{scc}$) (not shown) and for 17α hydroxylase ($P450_{c17}$) (Fig. 5D). The theca was also stained for 3β-hydroxysteroid dehydrogenase, but the labeling was markedly weaker

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

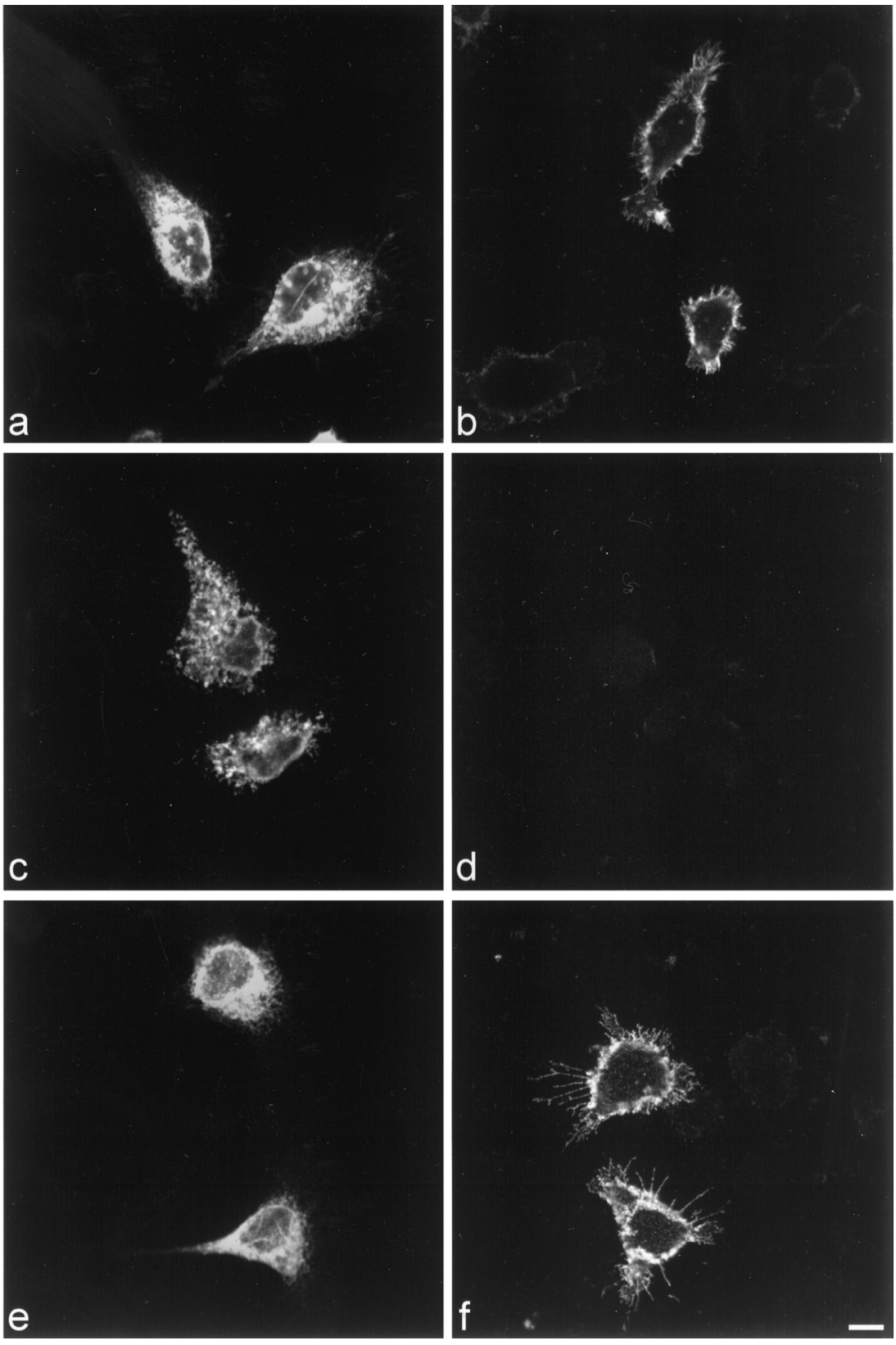

Fig. 3. Cell Surface Expression of Wild-Type and Mutated FSHRs

COS-7 cells were transfected with expression vectors encoding either the wild- type (a and b) or the mutated Asp224Val (c and d) or Leu601Val (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.

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

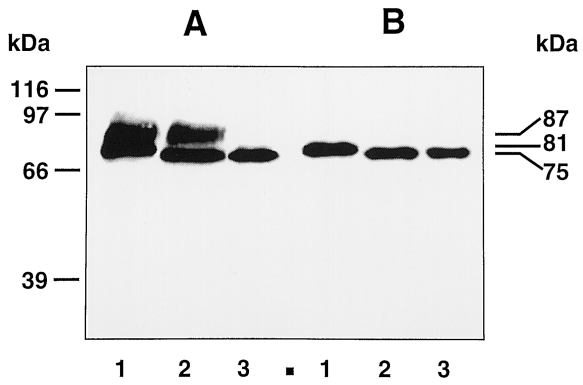

Fig. 4. Western Blots of Wild-Type and Asp224Val Receptor Mutant Expressed in COS-7 Cells

COS-7 cells were transfected with the expression vectors encoding the wild-type (A) or the Asp224Val receptor mutant (B). The receptor was then immunopurified and treated by endoglycosidase H (2) or N-glycanase F (3) or not treated by enzymes (1). Immunoblot experiments were performed as described in *Materials and Methods*.

(Fig. 5E) and thus contrasted with that observed in the previously studied patient 1 (7). No immunostaining could be observed with the antiaromatase antibody (not shown).

Correlation of Receptor Function with Clinical and Biological Findings in Patients with Partial Ovarian Failure and FSHR Mutations

We have previously reported another patient (patient 1) bearing two heterozygous mutations of the FSHR gene (8). We compared the activity of her transfected receptors with that of the present case (patient 2). We then related these observations to the effects of FSH *in vivo* in the two patients.

The biological activity of the four mutated FSHRs was compared with that of the wild-type receptor (Fig. 6). Similar transfection efficiencies were assessed using a β-galactosidase assay. This experiment was reproduced four times with similar results. The mutations yielded receptors with residual activities of 24 $\pm$ 4% for the Arg573Asp (patient 1) and 12 $\pm$ 3% for the Leu601Val (patient 2) receptor mutants, respectively, after a maximal stimulation by FSH. Activities of the mutated receptors Ile160Thr (patient 1) and Asp224Val (patient 2) were more severely altered, 9 $\pm$ 2% and 4 $\pm$ 2% of the wild-type receptor, respectively. Residual activity was therefore higher in the receptor mutants carried by patient 1 as compared with patient 2. This could be related to the fact that patient 1 had antral follicles developing up to 5 mm whereas patient 2 had follicles developing only to 3 mm at ultrasonography. Furthermore, plasma estradiol and inhibin B levels were 70–150 pmol/liter and 50 pg/ml in patient 1 and 40–80 pmol/liter and 30 pg/ml in patient 2. Patient 2, therefore, showed a lower sensitivity to endogenous FSH than patient 1 (Table 1).

The difference was even more marked when exogenous FSH was administrated *in vivo* to both patients. Recombinant FSH was injected in increasing doses to a final total dose of 5625 U. In Patient 1, this stimulation increased the follicular size up to 8.3 mm, whereas no change in follicular size was observed in patient 2. Plasma estradiol and inhibin B levels also markedly increased from 35 to 240 pmol/liter and from 50 to 125 pg/ml, respectively, in patient 1, whereas no significant change was observed in patient 2 under stimulation with the same increasing doses of recombinant FSH (Table 1). For ethical reasons it was decided not to further increase the dose of FSH.

There was thus a correlation between the FSH-stimulated *in vitro* activity of the mutated receptors found in the two patients and their *in vivo* response to endogenous and exogenous FSH.

DISCUSSION

We report here two novel mutations of the FSHR gene in a woman (patient 2) presenting with primary amenorrhea, low ovarian estrogen secretion, and follicular growth up to the early antral stage with a maximal diameter of 3 mm at ultrasonography. This patient was a compound heterozygote, having inherited a different mutated allele from each parent: Asp224Val in exon 3 from her father and Leu601Val in exon 10 from her mother. This is the second patient observed presenting with premature ovarian failure (POF) due to partial loss of function mutations of the FSHR gene.

POF concerns almost 1% of women under age 40 (12). It has long been suspected that POF could be due, in some cases, to an ovarian resistance to gonadotropin stimulation, since a few patients with amenorrhea but also presence of follicles at ovarian histology have been reported (13). This FSH resistance ovary syndrome is better understood now that inactivating mutations of the FSHR gene have been described. A first homozygous mutation (Ala189Val) was identified in a Finnish population of ovarian dysgenesis (3), with primary amenorrhea and delayed puberty in most cases (4). Histological study of the ovaries in some of these patients showed a streak or hypoplastic aspect and the presence of primordial and primary follicles with impaired follicular development at further stages. The mutation dramatically impaired receptor function. This mutation has not been detected in patients outside of Finland who display the same phenotype (1, 8, 14). More recently, we reported the case of a patient (patient 1) who was a compound heterozygote for two inactivating mutations of the FSHR (8). The phenotype of this patient was different from the one described in the Finnish population. Indeed, patient 1 presented with secondary amenorrhea, normal sized ovaries including antral follicles up to 5 mm. The mutations impaired, but did not abolish, FSHR function.

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

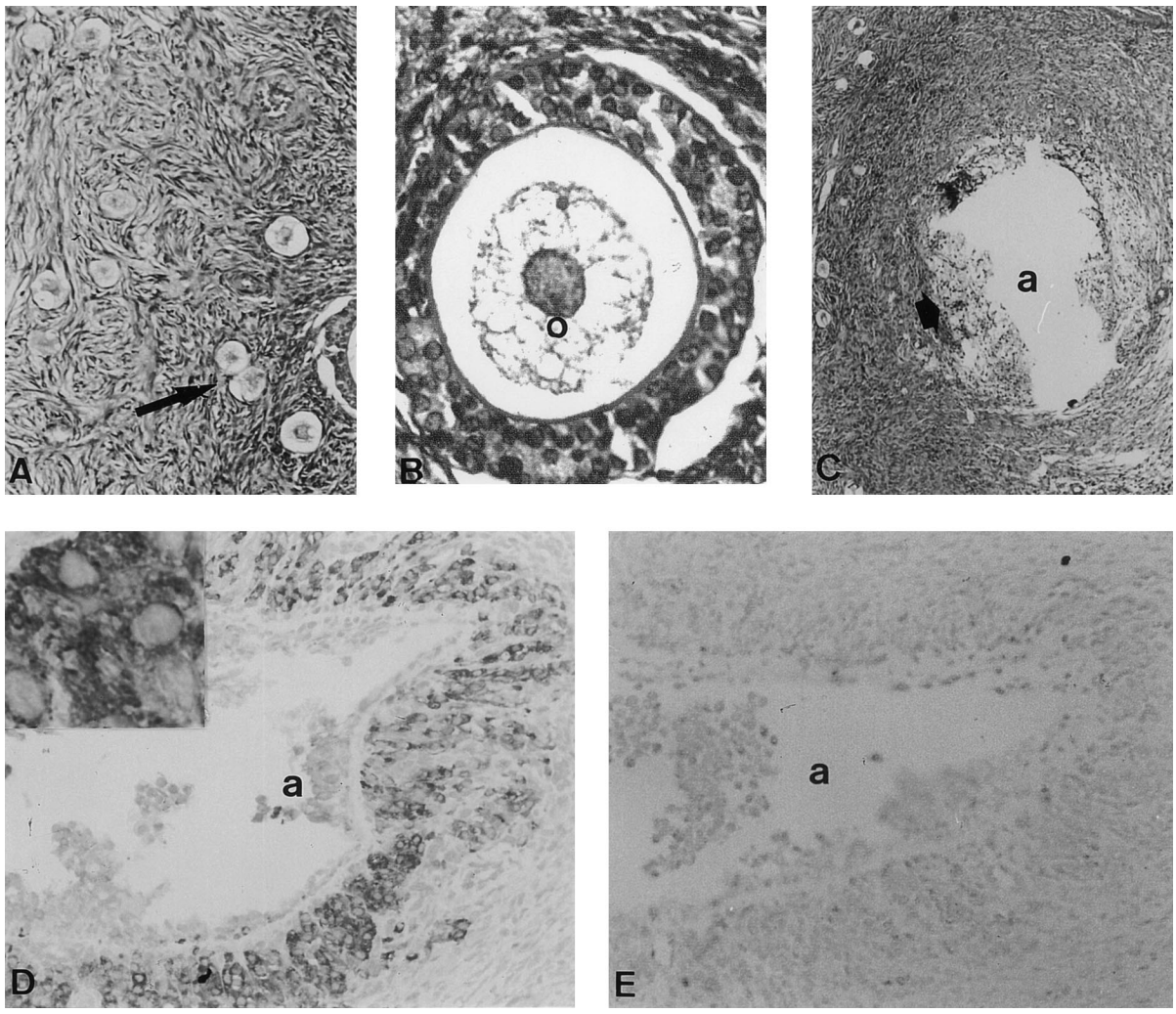

Fig. 5. Ovarian Histology and Immunocytochemistry

A, Section of the ovarian cortex comprising several primordial and primary follicles (*arrow*). Hematoxylin-eosin stain (×100). B, Small secondary follicle: a central oocyte is surrounded by four layers of granulosa cells. Hematoxylin-eosin stain (×300). C, Degenerating antral follicle (0.8 mm diameter) showing an irregularly shaped antrum and a disrupted granulosa layer. Hematoxylin-eosin stain (×40). D, Section of the same antral follicle immunostained for P450c17: the cells of the theca interna are intensely labeled (×200). *Inset*, Higher magnification of the immunoreactive cells. E, Section of the same follicle immunostained for 3β-HSD: the disposition and the number of stained cells are the same but the immunolabeling is very weak. BL, Basal lamina; a, antrum; G, granulosa cells; TI, theca interna; o, oocyte.

By analogy with the molecular defects found in the related LH or TSH receptors (reviews in Refs. 2 and 15), we postulated that such partial cases may be more frequently observed than complete loss of FSHR function. Indeed, patient 2 was also found to have partial impairment of FSHR function. Clinically, this patient had a more severe phenotype with primary amenorrhea. She had smaller follicles than those of patient 1 at ultrasonography and a complete absence of response to recombinant FSH at a total dose of 5625 IU, which elicited in patient 1 a progressive increase in follicular growth (from 5 to 8.3 mm), and plasma concentrations of estradiol (from 35 to 240 pmol/liter) and inhibin B (from 50 to 125 pg/ml).

Functional analysis of the mutated receptors revealed a correlation with the phenotypes observed. Indeed, in the Finnish report, the homozygous extracellular mutation of the FSHR yielded a receptor with no significant response to hormone *in vitro* (3). Patient 1 was a compound heterozygote; one of the two mutations, the Arg573Cys mutation, yielded a receptor with a residual 24 $\pm$ 4% activity when compared with the wild-type receptor. In the case of patient 2, one of the two mutant receptors, the Leu601Val mutant, had 12 $\pm$ 3% residual activity when compared with the wild-type receptor. In both patients the other mutant receptors were almost completely inactive.

Interestingly, in both patients 1 and 2, follicular development occurred up to the antral stage, but ultra-

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

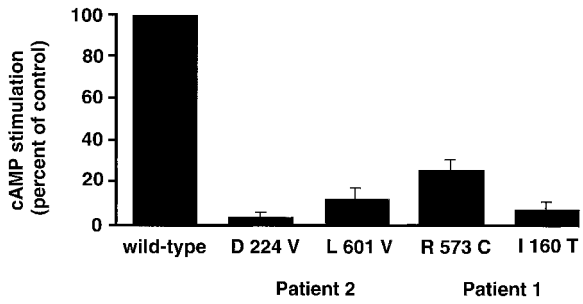

Fig. 6. Comparison of the Biological Activities of the Wild-Type and the Mutated FSHRs

The biological activities of the different receptors were determined by transfecting the corresponding expression vectors in COS-7 cells and measuring the receptor-mediated cyclase stimulation with 400 IU/liter of Metrodine as described in *Materials and Methods*. The results are expressed as the percent of adenylate cyclase stimulation observed with the wild-type receptor (mean ± SEM of four different experiments): 24 ± 4% for the Arg 573 Cys, 12 ± 3% for the Leu601Val, 9 ± 2% for the Ile160Thr, and 4 ± 2% for the Asp224Val receptor mutants, respectively. For the Ala189Val receptor mutant, detected in the Finnish population, there was no FSH-induced cyclase stimulation (3). The number of binding sites per cell was 13,500 ± 2,000 for the Arg573Cys mutant, 10,000 ± 900 for the Leu601Val mutant, and 14,000 ± 3,300 for the wild-type receptor. For the Asp224Val and the Ile160Thr receptor mutants, deficient in hormone binding, the B_{max} could not be measured.

sonographic examination showed that it proceeded up to a follicular size of 5 mm in patient 1 and only 3 mm in patient 2. The fact that FSH stimulation was able to increase follicular size as well as estradiol, and inhibin B secretion in patient 1 indicated that ovarian follicles of this patient were partly responsive to high concentrations of FSH. In Patient 2, the same maximal dose of recombinant FSH was ineffective, and this can be explained by a more pronounced alteration of receptor function associated with a less important initial follicular development.

FSH effects on follicular growth and maturation differ markedly at various developmental stages. A body of evidence indicates that in mice and rats initiation of follicular growth, when a resting follicle enters the growth phase, does not require FSH (16, 17). The absence of FSHR gene expression in nongrowing human follicles confirms this point (18). Evidence points to the need of FSH for further follicular development between the primary and the antral stage. In rat models, hypophysectomy or GnRH antagonist administration leads to an almost complete absence of follicular growth beyond the secondary stage (19). The same pattern has been obtained in xenografts of human ovarian tissues in hypogonadal SCID/hpg mice, (20). Finally, in mice bearing a homozygous invalidation of either the FSHβ (21) or the FSHR (5) gene, development was blocked before the antral stage. In humans, clinical observations also suggest FSH independence during the first steps of follicular development. In situations where low levels of circulating gonadotropins are present, *i.e.* prepubertally or in women with hypogonadotropic hypogonadism or with mutations of FSHβ, morphological examination of the ovaries revealed the existence of small preantral follicles but few antral follicles, indicating that gonadotropin deficiency is associated mainly with a disruption of the final stages of preovulatory folliculogenesis (6, 22–25).

The arrest of follicular development at a relatively precise stage in each of our two patients suggests that a different FSH or receptor activity is necessary to promote the maturation of antral follicles in each case. Growth may be arrested at various stages depending on how severely FSHR function is impaired.

A different mechanism has been proposed in preovulatory follicles. A threshold concentration of FSH must be reached. But once started, the final follicular development proceeds up to the ultimate stages, even if the concentration of FSH decreases (7, 26).

Immunohistochemical studies of the ovaries of patient 2 revealed only a faint staining for 3β-hydroxysteroid dehydrogenase (3β-HSD). This observation is also in agreement with a more pronounced defect in follicular development than in patient 1, in whom theca cells were found to express this enzyme. It has been shown during preovulatory follicle maturation in pigs that maximal expression of theca 3β-HSD was observed only 4 days after maximal expression of P450 aromatase and P450c17 (27). This led to the proposal that 3β-HSD is rate limiting for the overall follicular steroidogenesis by limiting the substrate for P450c17.

The cellular expression of the mutated receptors highlights the crucial role of two residues in FSHR function. The Asp224Val mutation (patient 2) yielded a receptor that was not expressed at the cell membrane. A similar situation was found for the Ile160Thr receptor mutant (patient 1). The study of the glycosylation of the mutated receptors indicates that they carry mannose-rich carbohydrates and have thus not reached the Golgi apparatus. This impairment of receptor cell trafficking may be due to an altered receptor conformation, which impedes the further progression of the receptor in other cellular compartments. The accumulation of misfolded proteins triggers the ER unfolded response inducing the selective synthesis of endoplasmic reticulum chaperones that bind to misfolded exportable proteins (28–30). This accumulation, alternatively, may be due to the lack of interaction of the mutated receptor with a specific chaperone necessary for proper receptor folding (31).

The Leu601Val substitution was characterized by an impairment in signal transduction, without any change in its affinity for FSH. It thus highlights the role of the third extracellular loop in FSHR transduction. However, mutation of the same leucine 601 [numbered Leu583 by Ryu *et al.* (32)] into an alanine improved hormone binding affinity by 4- to 6-fold, indicating an inverse relationship with cAMP stimulation. This observation led to the suggestion that leucine 601 interacted with the ectodomain and constrained hormone

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

binding in the wild-type receptor (32). We could not confirm this hypothesis since repeated experiments in our study failed to detect a significant difference in FSH binding affinities of the wild-type and of the mutated receptor. Leucine 601 may thus be involved in maintaining the proper conformation of exoloop 3 and of the adjacent transmembrane helices 6 and 7. The latter have been shown to be important in signal transduction (33).

In conclusion, novel mutations of the FSHR have been identified that lead to a novel clinical phenotype. There was a correlation between the residual activity of the mutated receptors and the phenotype. These new observations offer a model to understand the role of FSH during the early steps of follicular development.

MATERIALS AND METHODS

DNA Sequencing

DNA was extracted from peripheral blood leukocytes. The 10 exons of the human FSHR gene were amplified by PCR and sequenced on both strands using previously described primers (3, 34). Genomic sequencing was performed using a *Taq* dideoxyterminator cycle sequencing kit and a 373 A automated sequencer (PE Applied Biosystems, Foster City, CA).

Construction of Expression Vectors Encoding Mutated FSHRs

The human FSHR cDNA cloned into the pSG5 expression vector has been described elsewhere (8). The mutations were introduced into the pSG5-FSHR plasmid vector by oligonucleotide-mediated mutagenesis using PCR.

The Leu601Val substitution was engineered with two mutagenic primers: a direct primer A1: 5′-AGGTGCCCGTCATCACTGTGTCCAA-3′ and a reverse primer B1: 5′-CACAGTGATGACGGGCACCTTGAGG-3′ starting, respectively, at positions 1793 and 1813 of the cDNA sequence (+1 corresponds to the first nucleotide of the initiation codon) (35). The mutated base is *underlined*.

Two other primers were used. Primer C1: GCTGCTCATTGCATCAGTTGATATCCATAC; and primer D1: GAGGGACAAGTATGTAAGTGGAACCACTGG, starting at positions 1245 and 2036, respectively, of the cDNA sequence. A fragment of 821 bp containing the mutation was constructed in two pieces. The first fragment was obtained by PCR using oligonucleotides A1 and D1. The second fragment was obtained using oligonucleotides B1 and C1. The full-length product of 821 bp was obtained by hybridization of the two fragments and PCR amplification using primers C1 and D1. After digestion with PflMI (there are two PflMI sites located at distances of 413 and 298 bp on either side of the mutation), the fragment was ligated into the pSG5-hFSHR vector, which had been previously digested with PflMI. The Asp224Val substitution was generated using a similar strategy. Two mutagenic primers were used: a direct primer A2 (5′-GTCATTCTAGTTATTTCAAGAACAAGGATC-3′) and a reverse primer B2 (5′- CCTTGTTCTTGAAATAACTAGAATGACTGG-3′) starting at position 467 and 492 of the cDNA sequence, respectively (the mutated base is *underlined*). The two other primers were C2 (GGACCTGGAGAAAATAGAGATCTCTCAGAA) and D2 (GACCCCTAGCCTGAGTCATATAATCAACTT) and started at positions 210 and 929 of the cDNA sequence, respectively.

The full-length fragment of 719 bp containing the Asp224Val mutation was digested with *Afl*II and Bsu36 I (the restriction sites were located 141 bp upstream and 242 bp, respectively, downstream from the mutation), purified, and ligated to the pSG5-FSHR vector digested with the same restriction enzymes.

All constructs were verified by double-strand sequencing.

Study of FSH Binding to the Wild-Type and Mutated Receptors

COS-7 cells were transfected with the wild-type and the mutated receptors using Superfect (Qiagen, Chatsworth, CA) as previously described (8). Forty-eight hours later, cells were incubated for 1 h at 30 C with 400,000 cpm/ml of radioiodinated FSH (Amersham Pharmacia Biotech, Arlington Heights, IL; specific activity, 135 μCi/μg) as previously described (8). The incubations were performed in the absence or in the presence of increasing concentrations of unlabeled recombinant FSH. Nonspecific binding was determined in samples containing an excess (10 μg/ml) of unlabeled FSH and subtracted from the total binding. All experiments were performed twice with triplicate samples. Transfection efficiencies were estimated by cotransfecting pRSV-βgal and measuring β-galactosidase activity in the cells. Similar efficiencies were observed when expression vectors encoding either the wild-type or the mutated FSHRs were used.

cAMP Assay

cAMP was measured as previously described (8) after 45 min incubation of transfected cells with varying concentrations of FSH (10^{-11} to 10^{-7} M) (Metrodine, Serono Laboratories, Inc.).

Immunofluorescence and Confocal Microscopy

Indirect immunofluorescence studies were performed on COS-7 cells transiently expressing wild-type or mutated FSHR using the monoclonal FSHR 323 antibody (8). This antibody, which recognizes an epitope located in the extracellular domain of the FSHR (10), allows the study of receptor expression at the cell membrane. For this purpose, intact cells or permeabilized cells were incubated with the antibody as previously described (8). A Cy3-labeled rabbit antimouse IgG (Sigma Chemical Co., St Louis, MO) was used as a secondary antibody. Immunofluorescence was then analyzed with an Axiovert 135M microscope (Carl Zeiss, Thornwood, NY) in conjunction with a confocal LSM 410 laser scanning unit (Carl Zeiss) (8, 36).

Immunoblotting of FSHR

COS-7 cells were transfected as described above. They were scraped 48 h later in PBS containing protease inhibitors. After centrifugation at 800 × *g* for 10 min at 4 C, the pellet of cells was suspended in solubilization buffer as previously described (10). The receptor was then immunopurified from membrane extracts using the FSHR323 antibody coupled to Affi-Gel 10. The purified FSHR was quantified using a specific immunoenzymatic assay (10). The receptor (300 fmol) was then deglycosylated with *N*-glycanase F or endoglycosidase H as previously described (10, 37).

The immunopurified human (h) FSHR was electrophoresed on a 8% SDS polycrylamide gel under reducing and denaturing conditions. Proteins were electrotransferred to nitrocellulose and detected using the FSHR323 antibody as previously described (10, 37).

Histological and Immunohistochemical Study

The ovarian biopsy from patient 2 was fixed in buffered formol and embedded in paraffin. Some sections were

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

stained with conventional histological stains (hematoxylin-eosin, Masson's trichrome) for optical microscopy while the other sections were processed for immunohistochemistry.

The expression of steroidogenic enzymes was studied on paraffin sections after deparaffinization and antigen retrieval in 0.01 M citrate buffer, pH 6, in a 800 W microwave oven at full power for three cycles of 5 min as described (38).

The following polyclonal rabbit antibodies were used: anti-P450-side chain cleavage enzyme ($P450_{scc}$) (39) (dilution 1:3000), anti-3β-HSD (40) (dilution 1:3000), anti-17α hydroxylase ($P450_{c17\alpha}$) (41) (dilution 1:5000), and antiaromatase ($P450_{arom}$) (42) (Hauptman-Woodward Medical Research Institute, Inc., Buffalo, NY) (dilution 1:3000). They were incubated overnight with the sections at 4 C in a humid chamber. Endogenous peroxidase was quenched with 3% H_2O_2 in PBS (pH 7.4) for 5 min. The bound Igs were revealed with an antirabbit biotinylated antibody and with peroxidase-labeled streptavidin (LSAB2 immunostaining kit, DAKO Corp., Carpinteria, CA) used according to manufacturer's instructions. Aminoethylcarbazole (Sigma Chemical Co.) was used as a chromogen and Meyer's hematoxylin was used as a nuclear counterstain. Replacement of the primary antibody with preimmune rabbit Igs at the appropriate dilution resulted in the absence of immunolabeling. Polyclonal anti-Von Willebrandt factor antibody (DAKO Corp.) was used to define thecal vascularization.

Acknowledgments

We thank P. Leclerc (Service commun de microscopie confocale, IFR21) for his help in confocal microscopy analysis. The rabbit polyclonal antibody anti-$P450_{scc}$ was a kind gift from Professor Israel Hanukoglu, Departement of Hormone Research, Weizmann Institute of Science, Rehovot, Israel. The anti-3βHSD rabbit polyclonal antibody was a gift from Professor Van Luu-The, Department of Molecular Endocrinology, Laval University School of Medicine, Quebec, Quebec, Canada.

Received May 19, 1999. Revision received July 12, 1999. Accepted July 22, 1999.

Address requests for reprints to: Professor Micheline Misrahi, INSERM U. 135, Hôpital Bicêtre-3ème niveau, 78, rue du Général Leclerc, 94275 Le Kremlin Bicêtre Cedex, France. E-mail: micheline.misrahi@bct.ap-hop-paris.fr.

This work was supported by the INSERM, the Faculté de Médecine Paris Sud, the Délégation à la Recherche Clinique (P.H.R.C no. AOM 96133) Assistance Publique-Hôpitaux de Paris, and the Association de Recherche sur le Cancer.

* Isabelle Beau and Philippe Touraine contributed equally to this work and should be viewed as first authors of this paper.

REFERENCES

1. Simoni M, Gromoll J, Nieschlag E 1997 The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr Rev 18:739–773
2. Misrahi M, Beau I, Méduri G, Bouvattier C, Atger M, Loosfelt H, Ghinéa N, Vu Hai MT, Bougnères P, Milgrom E 1998 Gonadotropin receptors and the control of gonadal steroidogenesis: physiology and pathology. Baillières Clin Endocrinol Metab 12:35–66
3. Aittomäki K, Dieguez Lucena JL, Pakarinen P, Sistonen P, Tapanainen J, Gromoll J, Kaskikari R, Sankila EM, Lehväslaiho H, Reyes Engel A, Nieschlag E, Huhtaniemi I, de la Chapelle A 1995 Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. Cell 82:959–968
4. Aittomäki K, Herva R, Stenman UH, Juntunen K, Ylöstalo O, Hovatta O, de la Chapelle A 1996 Clinical features of primary ovarian failure caused by a point mutation in the follicle stimulating hormone receptor gene. J Clin Endocrinol Metab 81:3722–3726
5. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P 1998 Impairing follicle stimulating hormone (FSH) signaling *in vivo*: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci USA 95:13612–13617
6. Gougeon A 1996 Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 17:121–155
7. Zeleznik AJ 1993 Dynamics follicular growth a physiologic perspective. In: Adashi EY, Leung CK (eds) The Ovary. Raven Press, New York, pp 41–55
8. Beau I, Touraine P, Meduri G, Gougeon A, Desroches A, Matuchansky C, Milgrom E, Kuttenn F, Misrahi M 1998 A novel phenotype related to partial loss of function mutations of the follicle stimulating hormone receptor. J Clin Invest 102:1352–1359
9. Buteau H, Pezet A, Ferrag F, Perrot-Applanat M, Kelly PA, Edery M 1998 N-glycosylation of the prolactin receptor is not required for activation of gene transcription but is crucial for its cell surface targeting. Mol Endocrinol 12:544–555
10. Vannier B, Loosfelt H, Méduri G, Pichon C, Milgrom E 1996 Anti-human FSH receptor monoclonal antibodies: immunochemical and immunocyto-chemical characterization of the receptor. Biochemistry 35:1358–1366
11. Misrahi M, Ghinéa N, Sar S, Saunier B, Jolivet A, Loosfelt H, Cerutti M, Devauchelle G, Milgrom E 1994 Processing of the precursors of the human TSH receptor in various eucaryotic cells (human thyrocytes, transfected L cells and baculovirus infected insect cells). Eur J Biochem 222:711–719
12. Anasti JN 1998 Premature ovarian failure: an update. Fertil Steril 70:1–15
13. Jones GS, de Moraes-Ruehesen M 1969 A new syndrome of amenorrhea in association with hypergonadotropism and apparently normal ovarian follicular apparatus. Am J Obstet Gynecol 104:597–600
14. Conway E, Höppner W, Gromoll J, Simoni M, Conway GS 1997 Mutations of the FSH receptor gene are rare in familial and sporadic premature ovarian failure. J Endocrinol 152 [Suppl]:P257
15. Misrahi M, Milgrom E 1997 The TSH receptor. In: Weetman AP, Grossman A (eds) Pharmacotherapeutics of the Thyroid Gland. Springer-Verlag, Berlin, vol 128:33–73
16. Halpin DM, Jones A, Fink G, Charlton HM 1986 Postnatal ovarian follicle development in hypogonadal (*hpg*) and normal mice and associated changes in the hypothalamic-pituitary ovarian axis. J Reprod Fertil 77:287–296
17. Nakano R, Mizuno T, Katayama K, Tojo S 1975 Growth of ovarian follicles in rats in the absence of gonadotrophins. J Reprod Fertil 45:545–546
18. Oktay K, Briggs D, Gosden RG 1997 Ontogeny of follicle stimulating hormone receptor gene expression in isolated human ovarian follicles. J Clin Endocrinol Metab 82:3748–3751
19. McGee EA, Perlas E, LaPolt PS, Tsafriri A, Hsueh AJW 1997 Follicle-Stimulating hormone enhances the development of preantral follicles in juvenile rats. Biol Reprod 57:990–998
20. Oktay K, Newton H, Mullan J, Gosden RG 1998 Development of human primordial follicles to antral stages in *SCID/hpg* mice stimulated with follicle stimulating hormone. Hum Reprod 13:1133–1138

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024

21. Kumar TR, Wang Y, Lu N, Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201–204
22. Rabin D, Spitz I, Bercovici B, Bell J, Laufer A, Benveniste R, Polishuk W 1972 Isolated deficiency of follicle-stimulating hormone. N Engl J Med 287:1313–1317
23. Rabinowitz D, Benveniste R, Lindner J, Lorber D, Daniell J 1979 Isolated follicle-stimulating hormone deficiency revisited. N Engl J Med 300:126–128
24. Matthews CH, Borgato S, Beck-Peccoz P, Adams M, Tone Y, Gambino G, Casagrande S, Tedeschini G, Benedetti A, Chatterjee VKK 1993 Primary amenorrhea and infertility due to a mutation in the β-subunit of follicle stimulating hormone. Nat Genet 5:83–86
25. Goldenberg RL, Powell RD, Rosen SW, Marshall JR, Ross GT 1976 Ovarian morphology in women with anosmia and hypogonadotropic hypogonadism. Am J Obstet Gynecol 126:91–94
26. Hillier SG 1994 Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. Hum Reprod 9:188–191
27. Garrett WM, Guthrie HD 1997 Steroidogenic expression during preovulatory follicle maturation in pigs. Biol Reprod 56:1424–1431
28. Cox JS, Shamu CE, Walter P 1993 Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell 73:1197–1206
29. Mori K, Ma W, Gething MJ, Sambrook J 1993 A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus. Cell 74:743–756
30. Medeiros-Neto G, Kim PS, Yoo SE, Vono J, Targovnik HM, Camargo R, Hossain SA, Arvan P 1996 Congenital hypothyroid goiter with deficient thyroglobulin identification of an endoplasmic reticulum storage disease with induction of molecular chaperones. J Clin Invest 98: 2838–2844
31. Rozell TG, Davis DP, Chai Y, Segaloff DL 1998 Association of the gonadotropin receptor precursors with the protein folding chaperone calnexin. Endocrinology 139: 1588–1593
32. Ryu K, Gilchrist RL, Tung CS, Ji I, Ji TH 1998 High affinity hormone binding to the extracellular N-terminal exodomain of the follicle-stimulating hormone receptor is critically modulated by exoloop 3. J Biol Chem 273: 28953–28958
33. Fernandez LM, Puett D 1996 Identification of aminoacid residues in transmembrane helices VI and VII and of the lutropin/choriogonadotropin receptor involved in signaling. Biochemistry 35:3986–3993
34. Gromoll J, Pekel E, Nieschlag E. 1996 The structure and organization of the human follicle-stimulating hormone receptor gene. Genomics 35:308–311
35. Minegish T, Nakamura K, Takakura Y, Ibuki Y, Igarashi M 1991 Cloning and sequencing of human FSH receptor cDNA. Biochem Biophys Res Commun 175: 1125–1130
36. Beau I, Groyer-Picard MT, Le Bivic A, Vannier B, Loosfelt H, Milgrom E, Misrahi M 1998 The basolateral localization signal of the FSH receptor. J Biol Chem 273: 18610–18616
37. Beau I, Misrahi M, Gross B, Vannier B, Loosfelt H, Vu Hai MT, Pichon C, Milgrom E 1997 Basolateral localization and transcytosis of gonadotropin and thyrostimulin receptors expressed in MDCK cells. J Biol Chem 272: 5241–5248
38. Meduri G, Charnaux N, Loosfelt H, Jolivet A, Spyratos F, Brailly S, Milgrom E 1997 LH/hCG receptors in breast cancer. Cancer Res 57:857–864
39. Hanukoglu I, Suh BS, Himmelhoch S, Amsterdam A 1990 Induction and mitochondrial localization of cytochrome P450 scc system enzymes in normal and transformed ovarian granulosa cells. J Cell Biol 111: 1373–1381
40. Dupont E, Luu The V, Labrie F, Pelletier G 1990 Light microscopic immunocytochemical localization of 3β-hydroxy-5-ene-steroid dehydrogenase/Δ5-Δ4 isomerase in the gonads and adrenal glands of the guinea pig. Endocrinology 126:2906–2909
41. Kominami S, Shinzawa K, Takemori S 1983 Immunochemical studies on cytochrome P450 in adrenal microsomes. Biochim Biophys Acta 755:163–169
42. Kitawaki J, Yoshida N, Osawa Y 1989 An enzyme-linked immunoabsorbent assay for quantitation of aromatase cytochrome P-450. Endocrinology 124:1417–1423

Downloaded from https://academic.oup.com/mend/article/13/11/1844/2747732 by guest on 18 April 2024