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REVIEW

Multiple facets of follicle-stimulating hormone receptor function

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Abstract Follicle-stimulating hormone (FSH) is a glycoprotein hormone produced by the anterior pituitary gland. This gonadotropin plays an essential role in reproduction. Its receptor (FSHR) belongs to the superfamily of G protein-coupled receptors (GPCR), specifically the family of rhodopsin-like receptors. Agonist binding to the FSHR triggers the rapid activation of multiple signaling cascades, mainly the cAMP–adenylyl cyclase–protein kinase A cascade, that impact diverse biological effects of FSH in the gonads. As in other G protein-coupled receptors, the several cytoplasmic domains of the FSHR are involved in signal transduction and termination of the FSH signal. Here we summarize some recent information on the signaling cascades activated by FSH as well as on the role of the intracytoplasmic domains of the FSHR in coupling to membrane and cytosolic proteins linked to key biological functions regulated by the FSH–FSHR system.

Keywords Follicle-stimulating hormone · Follicle-stimulating hormone receptor · Signal transduction · Granulosa cells · G protein-coupled receptors

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Introduction

Growth of ovarian follicles, granulosa cell differentiation, ovulation, and luteinization are regulated by the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Pituitary gonadotropins are synthesized and secreted by specialized cells (gonadotrophs) of the anterior pituitary gland in response to the pulsatile stimulus of gonadotropin-releasing hormone, a decapeptide produced by the anterior hypothalamus [1]. In the ovary, FSH binds to specific receptors located on the cell surface of small granulosa cells present in immature preantral follicles. Follicle-stimulating hormone provokes specific, time-related changes in granulosa cell gene expression, promoting proliferation, differentiation, antrum formation, and oocyte maturation. Upon stimulation by this gonadotropin, the follicle grows and differentiates into antral preovulatory follicles, from which a mature, fertilizable oocyte is released in response to the LH stimulus [2]. In the male, LH and FSH regulate androgen production and spermatogenesis through complex interactions with the Leydig cells and Sertoli cells, respectively. Follicle-stimulating hormone stimulates Sertoli cell proliferation and along with LH and testosterone, is required for maintenance of qualitatively and quantitatively normal spermatogenesis [3–5]. Therefore, abnormalities in the structure of FSH or its receptor (FSHR) resulting from mutations in their corresponding genes lead to phenotypes characterized by hypogonadism and infertility in women and by subfertility in men [6].

This review concentrates on the mechanisms through which the interaction of the FSHR with its ligand leads to a biological response as well as on the role of different structural determinants present in the intracellular regions of the receptor important for the function and interaction of

this receptor with cytosolic and membrane proteins and effectors.

Follicle-stimulating hormone and follicle-stimulating hormone receptor

Pituitary gonadotropins belong to a family of closely related glycoproteins, comprised of FSH, LH, choriogonadotropin (CG) hormone, and thyroid-stimulating hormone (TSH), which are synthesized in different cell types (gonadotrophs or thyrotrophs). As with other members of the glycoprotein hormone family, FSH is a heterodimer consisting of a common α-subunit non-covalently linked with a β-subunit, the latter which is structurally unique in its peptide sequence for each member of the glycoprotein hormone family [3, 7–12]. Although the structural and functional specificity of each hormone resides in the β-subunit, there is evidence suggesting that both subunits contact the receptor [13]. The gonadotropin is decorated with N-linked oligosaccharides attached to αAsn52 and αAsn78, and βAsn7 and βAsn24 [1, 3, 5, 12, 14, 15]. Oligosaccharides in glycoprotein hormones play important roles including folding, secretion, and maintenance of heterodimer stability; they also determine the metabolic clearance rate of the hormone and influence on the activation of its cognate receptor [10]. As do other glycoprotein hormones, FSH exhibits a wide variability in oligosaccharide composition, which constitutes the main chemical basis for the observation of isoforms formation and which may partially explain some differential effects of the hormone observed in in vitro systems [12, 16–18]. It should be appreciated by the reader that charge differences (isoforms) arise from heterogeneity of sialic acid capping of terminal galactose residues, in the case of FSH. However, the presence or absence of that charge is dependent on the glycosylation site structural heterogeneity (i.e., one or two branches). Thus isoforms arise not only from charge differences due to sialic acid, but also because of glycoform structural variation. One striking example has been recently reported. Glycosylation of the primate FSH β-subunit exhibits a particular glycoform which possesses only α-subunit oligosaccharides and that is more active in vitro than the tetraglycosylated variant [15, 19].

The initial event in glycoprotein hormone action begins with binding of hormone to highly specific receptors located in the cell surface membrane of the target cell. Glycoprotein hormone receptors belong to the superfamily of G protein-coupled receptors (GPCR), specifically the family of rhodopsin-like receptors (family A) [20]. These receptors consist of a single polypeptide chain of variable length that threads back and forth across the lipid bilayer seven times forming characteristic α-helical transmembrane domains (TM) connected by alternating extracellular and intracellular loops (iL), with an extracellular amino-terminal domain and an intracellular carboxyl-terminal segment (Ctail) (Fig. 1) [21]. The human (h) FSHR consist of 695 amino acids, with the first 17 amino acids encoding a signal sequence [1, 3]. The homology between different mammalian FSHRs is high, reaching almost 90% in the TMs, 85% in the extracellular domain, and 80% in the Ctail [4]. Compared to the LH and TSH receptors, the homology of the hFSHR in the TMs is ∼69%, and drops to ∼40% and 25% in the extracellular domain and the Ctail, respectively [3]. Glycoprotein hormone receptors are also related to each other by the presence of a large extracellular domain or ectodomain, containing several leucine-rich repeats as well as by the homologous structure of their corresponding ligands (see above) [8, 22]. The reported crystal structure of a single chain form of FSH in complex with the extracellular domain of the human (h) FSHR ($FSHR_{ECD}$) [13, 23, 24] demonstrated the formation of weakly associating $FSHR_{ECD}$ dimers with each receptor molecule occupied by one molecule of ligand. This association among FSHR molecules may have potential implications for the mechanisms mediating receptor activation and intracellular signaling.

Binding of FSH to the FSHR occurs in the large extracellular NH_2-terminal domain (Fig. 1), where the participation of the leucine-rich repeat regions are essential to determine ligand selectivity [3, 25, 26]. In fact, mutations in these regions lead to reduction in the binding of agonist to the receptor [26–28]. It is not known how the signal from the large extracellular domain liganded complex is transmitted to the transmembrane domains. It is envisioned that activation of the liganded receptor involves a series of conformational changes that derive from binding of FSH, perhaps including contact with the crevice provided by the juxtaposition of the extracellular loops of the transmembrane domains allowing reception of the ectodomain-bound agonist [29, 30]; changes in the extracellular loops in turn propagate through the TM helices and modify the conformation of intracellular domains of the receptor allowing exposure of particular motifs or sequences important for G protein coupling and effector activation, as well as for the interaction of the receptor with proteins that eventually define the signal transduction pathway(s) that will be activated in response to agonist and/or the fate of the activated receptor [3, 31–34]. It has been suggested that the extracellular loop 3 of the hFSHR, particularly the amino acid residues Leu584, Ile585, and Lys590, interact with the α-subunit of FSH and contributes to determine the signaling response, specifically cAMP and/or inositol phosphate production [29–31]; in addition, the NH_2-terminal end of the exoloop 1, specifically residues 405–409 are also important for cAMP signaling [35]. Thus the TM

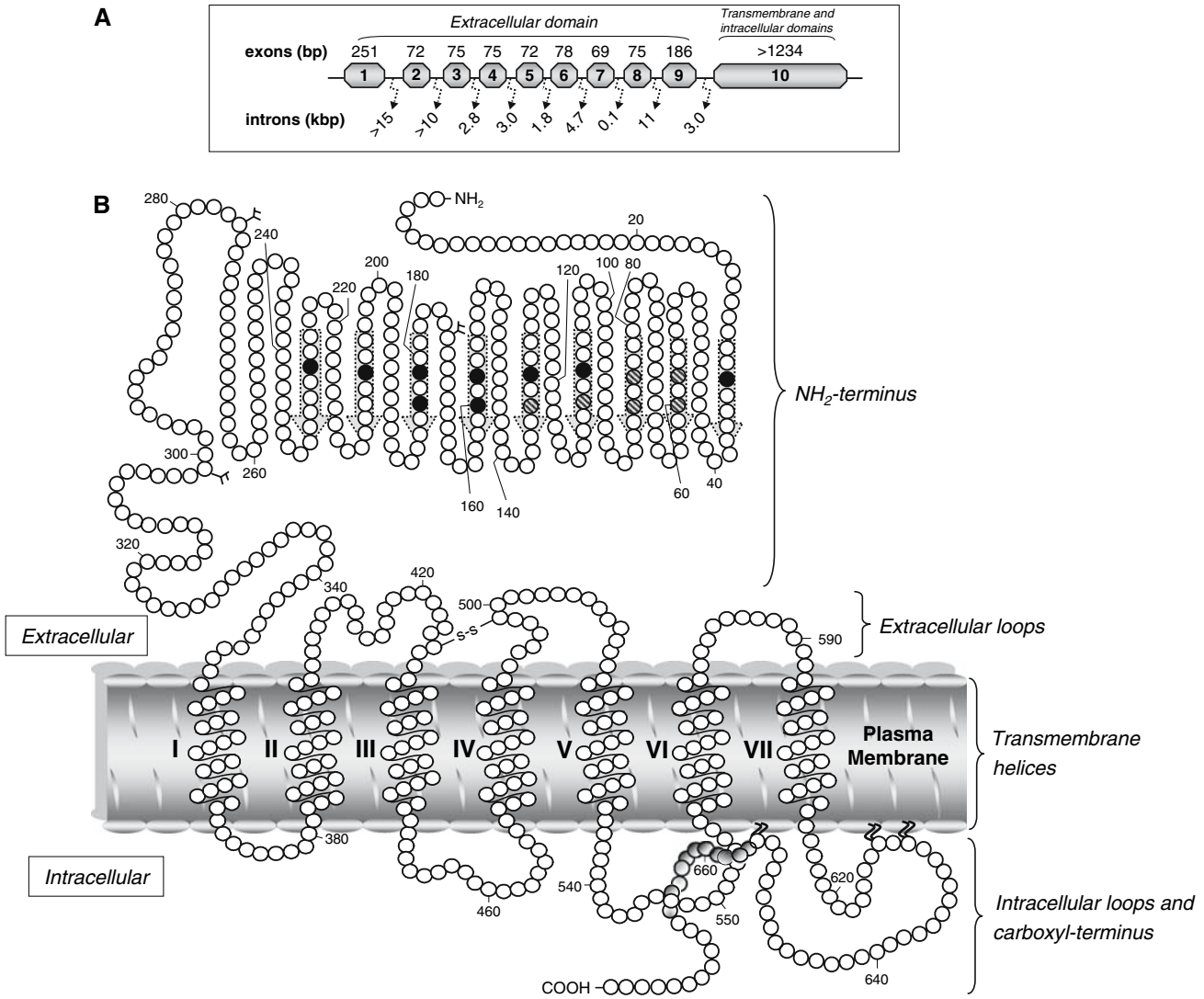

Fig. 1 (**a**) Structural organization of the human FSHR gene. Exons 1–10 are represented by octagons. Numbers above each octagon indicate the number of base pairs of the corresponding exon. Intron length is indicated by the numbers between exons in kbp. (**b**) Schematic representation of the human FSHR protein. Amino acid residues are represented by circles. As other GPCRs, the FSHR consists of a single polypeptide chain of variable length, that threads back and forth across the lipid bilayer seven times forming characteristic α-helical transmembrane domains (I–VII) connected by alternating extracellular and intracellular loops, with an extracellular amino-terminal domain and an intracellular carboxyl-terminal segment. Consecutive leucine-rich repeats are indicated by discontinuously lined gray arrows. Leucine and isoleucine residues are represented by black and hatched circles, respectively. Putative N-linked carbohydrates are represented by branched lines. The palmitoylated Cys627, Cys629, and Cys655 residues anchoring the carboxyl-terminus to the plasma membrane are indicated by the zigzagging parallel lines

helices and their corresponding connecting loops are responsible for the signal generation.

Activation of multiple signaling pathways by the FSH/FSHR complex

Upon agonist binding, the activated FSHR stimulates a number of intracellular signaling pathways. In the classical, linear signaling cascade, occupancy of the FSHR causes activation of the heterotrimeric G_s protein, followed by dissociation into two molecules, the α-subunit and the β/γ heterodimer. The α-subunit stimulates the effector adenylyl cyclase with the consequent increase in the synthesis of the second messenger cAMP, activation of protein kinase A (PKA), phosphorylation of a number of transcriptional regulators, including the cAMP regulatory element-binding protein (CREB), and activation of transcription (Fig. 2) [36–38]. The β/γ heterodimer activates phospholipase C but this enzyme is also activated by alternate $G_{s\alpha}$ subunits as well

[39]. Increasing evidence indicates that downstream of the adenylyl cyclase/cAMP/protein kinase A signaling pathway, activation of the FSH receptor by agonist also triggers activation of multiple intracellular signaling cascades as well as some PKA-independent pathways [37, 40–46]. Further, high doses of FSH also stimulate formation of inositol-phosphate turnover [41], and some glycosylation-modified FSH analogs inhibit PKA-mediated signaling [47], suggesting a potential association of the FSHR with other G proteins.

A number of signal transduction pathways operate in the granulosa cells during follicular development (Fig. 2). Engagement of multiple signaling pathways is necessary in order to ensure proliferation and proper differentiation of the maturing follicular cells and the oocyte. These pathways are activated by endocrine signals, mainly FSH and LH, as well as by locally produced factors, mainly insulin-like growth factor-1 (IGF-1), sex steroids, and members of the transforming growth factor-β (TGF-β) family and the Wnt/Frizzled family [45], which act through auto-, para-, or intracrine mechanisms in a time and cell differentiation stage-specific manner. Overall, FSH is a key-determining factor in granulosa cell differentiation prior to ovulation. In fact, absence of FSH or its receptor leads to arrest in follicular growth and infertility [6].

Although activation of PKA has been considered critical for FSH-mediated function in all mammalian species

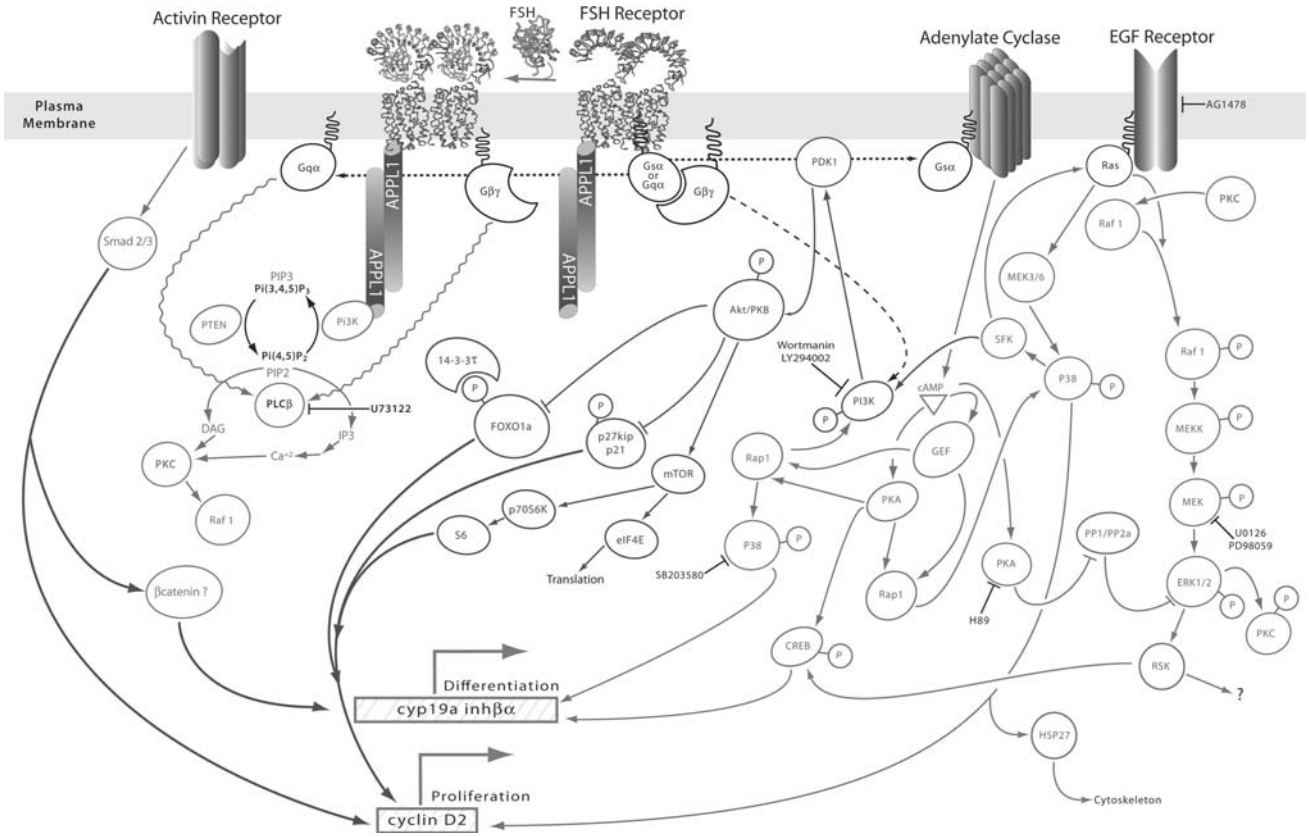

Fig. 2 Signal transduction pathways triggered upon agonist occupancy of FSH receptor dimers [131]. There are many gaps in our knowledge of how FSH signals in granulosa cells and differences between laboratories that complicate. Protein kinase A (PKA) activation of CREB is the most well studied pathway [36, 38]. FSH-induced differentiation functions of granulosa cells (LH receptor, aromatase, 17β-estradiol production) are blocked by dominant negative PKB, wortmannin and H89 demonstrating that both the PKA and PKB pathways are essential for FSH induced differentiation of granulosa cells [54]. Other genes including inhibin (inh)-α, and 3β-hydroxysteroid dehydrogenase are also amplified by constitutively active PKB [2]. In contrast, PKB has no effect on cyclin D2 or PCNA (proliferating cell nuclear antigen, which is an auxiliary protein to DNA polymerase) [54]. It has been reported that PKB activation can occur in the presence of PKA inhibitor H89 or with a cAMP analog that activates a GEF [43]. Whether differentiated functions occur through a GEF mediated PKB activation is not clear, since cAMP analogs that activate GEFs do not stimulate the differentiated functions demonstrating that activation of both pathways is not sufficient for differentiated functions and indicating that a further complexity exists perhaps related with a spatial or temporal component [54]. Another possibility is that the Gβ/γ dimer might directly stimulate Pi3K [55]. Recently, it has been demonstrated that the sarc family of kinases (SFK) modulate ERK activity through an FSH dependent mechanism [46]. That study provides an overarching mechanism for FSH action which touches on several pathways. Sustained activation of the ERK pathway via PKC has been proposed to elicit a tonic inhibition of Cyp19a expression [53] opposing transient increases of expression seen following FSH treatment. The mechanism of activation of PKC likely involves the production of inositol phosphates via the activation of phopholipase C

studied so far, there are a number of effects that cannot be simply explained by activation of the classic cAMP/PKA/CREB pathway. Rather, distinct kinase cascades have been shown to be activated by FSH in a cell context dependent manner (Fig. 2). In a first scenario, cAMP-activated PKA not only activates directly the transcription factor CREB and other transcriptional regulators to trigger specific gene expression (i.e., cyp19A) but also acts on other downstream signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway [38, 48–51]. In granulosa cells from different species, FSH-provoked phosphorylation and activation of the RAF/MEK1,2/ERK1,2 cascade downstream the cAMP/PKA pathway has been described [38, 49, 51–53]. Follicle-stimulating hormone also activates the p38MAPK through a PKA-mediated MEK3,6 pathway [38, 48]. Activation of these MAPKs pathways following FSH stimulation may be involved in the differential regulation of steroidogenesis, cell proliferation and survival, and cytoskeletal reorganization in maturing granulosa cells.

A second scenario is presented by several signaling cascades that integrate IGF-1-linked pathways to FSH/FSHR-activated, PKA-independent pathways [43, 45]. In this setting, both FSH and IGF-1 share common downstream targets in maturing granulosa cells, in which cAMP plays a central role. cAMP acting through cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) has been shown to indirectly activate (through the small GTP-binding protein Rap1) p38MAPK as well as the phosphatidylinositol-dependent kinase (Pi3K) cascade through Ras-like small GTPases, leading to activation of phosphoinositide-dependent kinases (PDK), protein kinase B (PKB/Akt), and serum and glucocorticoid-induced kinase (SGK, a kinase that is not activated by IGF-1) [43, 45, 54, 55]. The latter kinases phosphorylate transcription factors, including members of the Forkhead family (e.g., FOXO1a) thereby impeding transcriptional activation of genes associated with apoptosis [45, 46]. The rapid activation of SGK and PKB/Akt that follows FSH stimulation, inhibits apoptosis and promotes cell survival through the induction of the cell cycle regulators, cyclin D2 and cyclin E in proliferating granulosa cells [45]. The concomitant participation of IGF-1 in the developing follicle enhances FSH effects by promoting energy utilization and cell survival. A third, more recently described PKA-independent scenario, is mediated by the rapid activation of membrane-recruited Rous sarcoma oncogene (SRC) tyrosine kinases (SFKs) and Ras in response to FSH, leading to activation of ERK1/2, p38MAPK, and PKB, and subsequently to phosphorylation and down regulation of the proapoptotic protein FOXO1a [46]. In addition, FSH-stimulated Ras activation and ERK1/2 phosphorylation also involves activation of the epidermal growth factor (EGF) receptor directly by SFKs or other factors that bind the EGF receptor; this alternate pathway leads to activation of Ras and MAPKs [46]. Through FSH-promoted activation of all the above-described signaling pathways, FSH is able to control in a time-related fashion expression of specific genes that, in turn, maintain cell survival and influence differentiation of granulosa cells until formation of the preovulatory follicle is completed.

The importance of intracellular calcium-mediated signaling has been well demonstrated in many biological systems. Nevertheless, in the case of the FSHR, little attention has been paid to this potentially important pathway. In this regard, it has been shown that FSH exposure of swine granulosa cells or rat Sertoli cells results in a rapid increase in intracellular Ca^{2+} concentrations and that this increase may occur in a cAMP-dependent and -independent manner [44, 56–60]. In the first scenario, cAMP appears to increase intracellular calcium by promoting calcium mobilization from intracellular stores, an effect that could be mediated by cAMP stimulation of phospholipase-Cε, whereas in the second, different G proteins (e.g., $G_{\alpha q/11}$ protein) or the β/γ heterodimer of $G_{\alpha s}$ might be involved [41]. Further, in some models, cAMP-independent Ca^{2+} influx requires the presence of extracellular calcium [59, 61]. In this vein, it has been recently shown that FSH-promoted Ca^{2+} influx in rat Sertoli cells is mediated by a distinct $G_{\alpha h}$/PLC-δ1 pathway and that this effect is dependent on intracellular PLC-δ1-mediated generation of inositol 1,4,5 triphosphate (IP3) but independent of intracellular Ca^{2+} release [44]. Whatever the mechanism(s) involved in FSH-dependent Ca^{2+} influx, Ca^{2+}-mediated signaling seems to be important for functional differentiation of granulosa cells as suggested by recent data showing that intracellular Ca^{2+} positively modulates FSH-driven transactivation of the cytochrome $P450_{SCC}$ gene (cyp11A) in porcine granulosa cells [61], that Ca^{2+}/calmodulin-dependent protein kinase IV (CaMK IV) is involved in basal cyp11A gene expression in cultured granulosa-lutein cells [62], and that fertility is markedly reduced in CaMK IV-deficient female mice [63].

To add complexity to the potential role of intracellular Ca^{2+}-mediated signaling in FSH target cells, it has been demonstrated that expression of an alternatively spliced growth factor type FSHR variant originally identified in the sheep ovary, mediates Ca^{2+} influx upon FSH binding [64, 65]. This particular receptor variant is composed of 259 amino acid residues and possesses a single transmembrane domain, a large NH_2-terminus, and a short intracellular Ctail [64]. In immortalized porcine granulosa cells lacking the wild-type (Wt) FSHR, this receptor variant is connected with FSH-provoked activation of MAPK signaling in a PKC/Ca^{2+}-dependent manner [40, 66], which potentially relates this receptor with proliferative actions stimulated by FSH.

The synthesis of estrogens by the granulosa cells is regulated by FSH and the presence of estradiol receptors in maturing granulosa cells indicates that estrogen action is important in follicular maturation. In granulosa cells, estrogens enhance cell function by controlling cellular energy flow, glucose metabolism, and cell survival [37]. Considering the effects derived from the concerted actions of FSH and estradiol on granulosa cell growth and differentiation [67, 68] as well as the existence of cross talk between signaling pathways triggered by activation of G protein-coupled receptors and sex steroid hormone receptors [69, 70], we recently explored the effects of FSH on the transcriptional activation of estrogen receptor responsive genes employing L cells stably expressing the FSHR and both α- and β-estrogen receptors, in which several estrogen-responsive reporter genes were transiently expressed [71, 72]. We found that FSH activated transcription of different estrogen-responsive elements-containing reporters, and that the combination of FSH and estradiol resulted in a synergistic effect on transactivation as well as on cell proliferation (Fig. 3a and b). The effect of FSH on estrogen receptor-dependent transactivation involved the cAMP/PKA- and to a lesser extent the MAPK-mediated signaling, but not the Pi3K/Akt pathway (Fig. 4). Over expression of CREB-binding protein (CBP), but not SRC-1, enhanced FSH-induced transactivation of

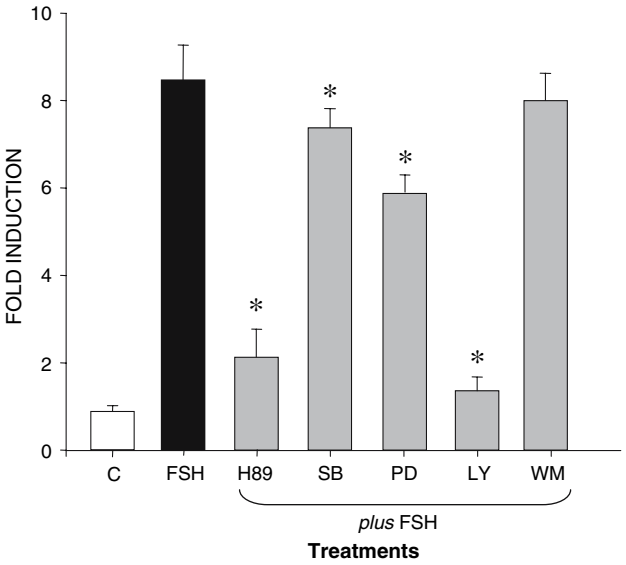

Fig. 4 Activation of the 3X-ERE-TATA-Luc reporter in L cells stably expressing the human FSHR and exposed to FSH (50 ng/ml) for 24 h in the presence or absence of kinase inhibitors [H89 (PKA inhibitor) and SB203580 (SB; p38 inhibitor), 10 μM; PD98059 (PD; ERK1/2 inhibitor) and LY294002 (LY; Pi3K inhibitor), 25 μM; and Wortmannin (WM; Pi3K inhibitor), 100 nM]. In contrast to Wortmannin, LY294002 strongly inhibited FSH-mediated transactivation of the reporter gene due to its ability to bind the estrogen receptor [71]. Reprinted from Ref. 72, with permission from Elsevier

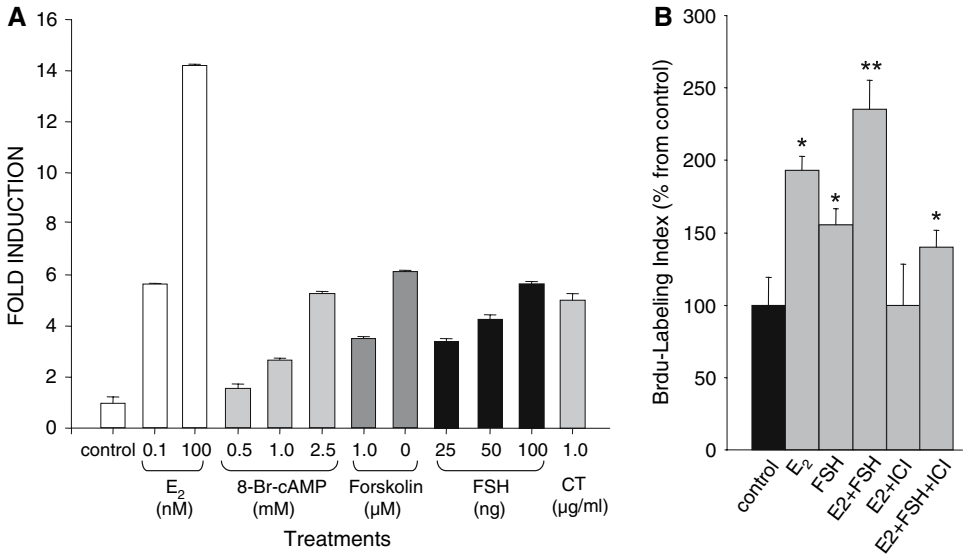

Fig. 3 (**a**) Activation of the 3X-ERE-TATA-Luc reporter by estradiol (E2) or activators of the Gsα-adenylyl cyclase-PKA signaling cascade [8-Br-cAMP, forskolin, cholera toxin (CT) and FSH] in L cells stably expressing the human FSHR. Cells were transfected with the reporter plasmid and treated for 24 h with vehicle (control) or activators at the indicted doses. The data shown represent the mean ± SEM from three independent experiments performed in triplicate. Reprinted from Ref. 72, with permission from Elsevier. (**b**) Proliferation of L cells stably expressing the human FSHR and exposed to vehicle (control), 17β-estradiol (E_2), and E_2 plus FSH for 8 h in the absence or presence of the antiestrogen ICI182,720 (ICI). Cell proliferation was determined by Bromodeoxyuridine (BrdU)-labeling for 4 h, with the BrdU-labeling index expressed relative to the group treated with vehicle. Data are the mean ± SEM of BrdU positive stained cells over 200 counted cells. * $P < 0.05$ vs. E2 + ICI182,720; ** $P < 0.05$ vs. all other experimental groups after normalization for 200 counted cells but before setting the control value to 100%

estrogen-responsive genes. Since the estrogenic-like effect of FSH was not blocked by antiestrogens, it is possible that FSH-stimulated, cAMP-activated CBP cooperates with estrogen receptors on genes that contain estrogen-responsive elements through mechanisms involving the participation of other proteins and/or basal transcription factors (e.g., CREB), which in turn may mediate the transcriptional response of estrogen-sensitive reporter genes to FSH stimulation. Based on these data, we proposed that transactivation of estrogen response element-containing promoters by FSH may represent a potential site for the integration of two distinct signal transduction pathways that may additionally contribute to cell growth, proliferation, and differentiation in the ovary (Fig. 5). This may particularly occur in small growing follicles, where FSH may impact cell proliferation through its estrogenic-like effects when expression of the aromatase enzyme is still absent [45, 73]. Nevertheless, further studies employing natural target cells for both FSH and estrogens are necessary to define more precisely the importance of this potential cross talk between FSH- and estradiol-mediated signal transduction pathways.

Correlates of the structure of the intracellular domains of the FSHR and intracellular signaling

As mentioned above, the intracellular domains of GPCRs are involved in a number of effects, including G protein coupling and association with other proteins that ultimately lead to activation of specific signaling cascades or that define the fate of the activated receptor. Mutagenesis studies of several GPCRs have indicated that several cytoplasmic domains of these receptors, particularly the iL2, the juxtamembrane portions of the iL3, and the Ctail, are involved in a number of receptor functions, including signal

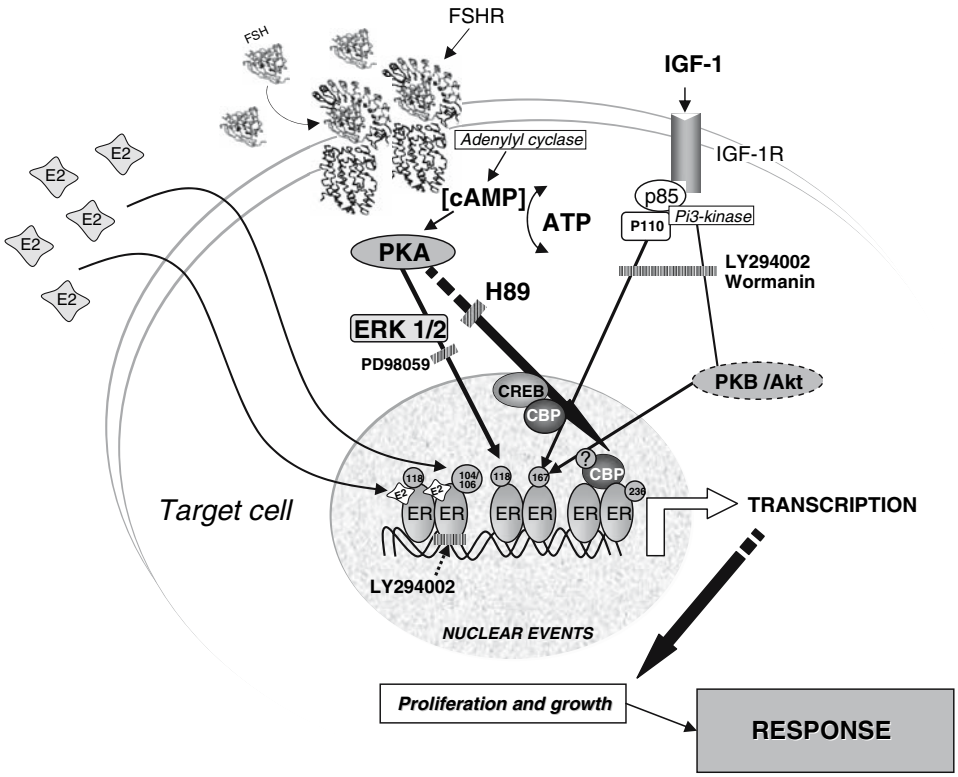

Fig. 5 Schematic of the proposed mechanism through which FSH may exert estrogen-like effects [72]. Activation of the FSHR by its cognate ligand may induce transcriptional activation of estrogen-sensitive genes through a PKA-triggered signaling pathway (blocked by H89), using also to a lesser extent the ERK1/2 (blocked by PD98059) and p38 (blocked by SB203580) pathways. Based on overexpression studies (see text), we propose that the stimulatory and/or synergistic effects of FSH on the transcriptional activation of estrogen-sensitive genes and cell proliferation is mediated by modification of coactivator proteins [e.g., the cAMP response element-binding protein-binding protein (CBP)], which in turn may contribute to cAMP-dependent estrogen receptor (ER) activation. Presumably, CBP cooperates with the estrogen receptor on genes that contain estrogen-responsive elements through mechanisms involving the participation of other proteins and/or basal transcription factors [e.g., cAMP response element-binding protein (CREB)], which in turn mediate the transcriptional response of estrogen-sensitive genes to FSH stimulation. The small gray circles represent amino acid residues of the estrogen receptor which may potentially be phosphorylated by estradiol (E2), ERK1/2, and other kinases (Pi3K and PKB/Akt) activated by insulin-like growth factor-1 (IGF-1). p85 and p110 are the regulatory and catalytic subunits of Pi3K, respectively. LY294002 may bind to the estrogen receptor and behave as an antiestrogen [71]

transduction [21]. The iL1 of the FSHR encompasses 13 amino acid residues (including those residues in the juxtamembrane portions of the TM1 and TM2) with 100% amino acid sequence homology between the human and the rat receptors [3, 5]. In the rat FSHR, the serine and threonine residues of this loop are involved in agonist-induced cAMP-independent receptor phosphorylation as well as in agonist-induced uncoupling and arrestin 3-associated receptor internalization (Fig. 6) [74–76]. Interesting, mutation of all serine and threonine residues also resulted in constitutive activation of the receptor, whereas replacement of Thr370 (Thr378 in the hFSHR) prevented cell surface expression [75]. As observed previously [77–80], single mutations may result in the production of trafficking-defective proteins or in constitutively activated conformers. In the particular case of the constitutively active rat FSHR iL1 mutant, the structural alteration may disturb necessary interhelical and/or intracellular loop interactions necessary for dampening of basal activity and maintenance of a predominantly inactive conformation of the receptor. Recent studies employing a yeast two-hybrid screen of an ovarian cDNA library, identified APPL1 (adaptor protein containing PH domain, PTB domain, and leucine zipper motif [81]) as an interactor partner with the FSHR through its iL1 [33, 34]; this adaptor protein interacts with several signaling factors [82, 83] and may apparently coordinate intracellular signaling by a complex involving the FSHR, PKB, and FOXO1a, leading to antiapoptotic effects (see above). More specifically, APPL1 appears to interact with Lys376, Leu377, and Phe382 at the iL1, since replacement of these residues with alanine prevents full interaction of the receptor with the adaptor [34]. Potentially productive interactions between the iL1 of the FSHR, APPL1, and Pi3K are shown in Fig. 2.

The importance of the iL2 in signal transduction has been documented for a number of GPCRs [21]. In fact, peptides mimicking the iL2 sequence of some GPCRs, may efficiently block receptor-G protein coupling [84, 85]. In particular, the highly conserved GPCR Glu/Asp-Arg-Tyr motif (E/DRY motif, permutated to ERW in the FSH receptor) has been implicated in G protein interaction and receptor activation [86]. Mutation of the central Arg has resulted in either partial or complete abolition of agonist-stimulated signal transduction [87–94] or in variable degrees of constitutive activation of the altered receptor [95, 96]. Other residues, such as hydrophobic leucines located in the middle of the iL2 [97, 98] and residues present in the carboxyl-terminal end [99–101] of several GPCRs, including the LH and TSH receptors [87, 94], have been also implicated in G protein activation. Several residues in the iL2 of the hFSHR exert distinct roles in function (Fig. 6) [102, 103]. Threonine 453 is a potentially phosphorylatable residue [76] and its replacement by Ala virtually abolished agonist-stimulated second messenger production without affecting plasma membrane expression of the mutant receptor [102]; it is possible that this

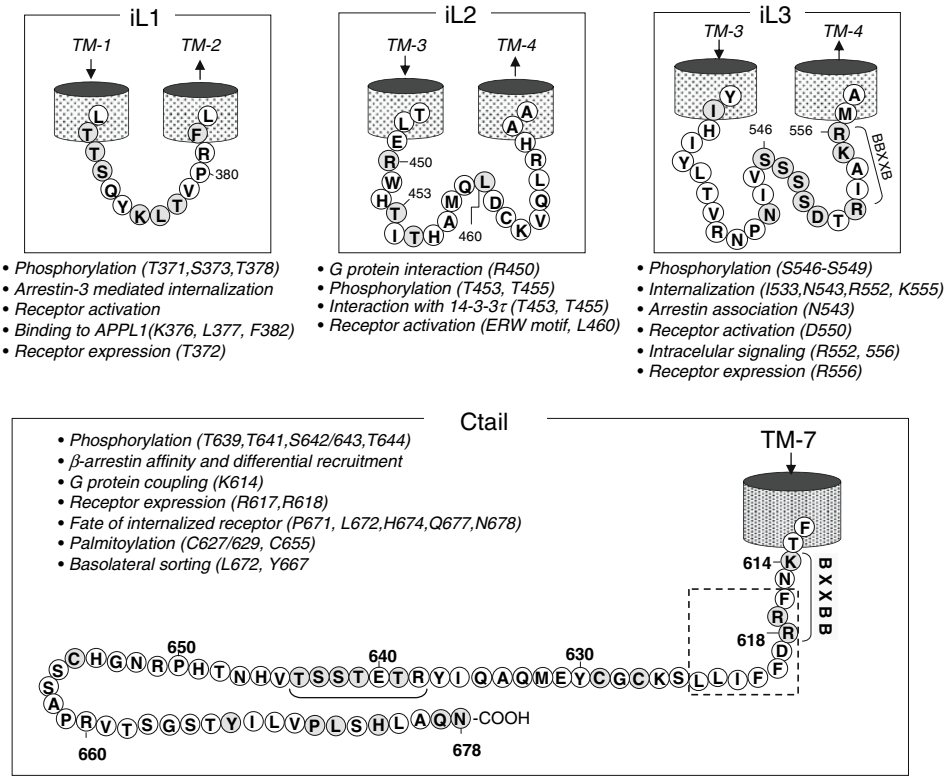

Fig. 6 Schematic of the first (iL1), second (iL2), and third (iL3) intracellular loops, and the carboxyl-terminus (Ctail) of the human FSH receptor. Amino acid residues presumably involved in interaction with membrane proteins or in which substitutions may potentially affect receptor function (legends below or above sequences) are shaded in gray. Brackets in the carboxyl-terminal end of the iL3 and the NH_2-terminal end of the Ctail delimit the BBXXB motif reversed, which has been shown to be associated with G protein activation in other GPCRs [99]. The discontinuous line square in the NH_2-terminal end of the Ctail delimits the $F(X)_6LL$ motif required for receptor transport to the cell surface membrane [130]

particular residue may be important to form the active structure of the FSHR via interaction with other iL2 residues, as suggested for bovine rhodopsin [104] in which replacement of those NH_2-terminal iL2 amino acid residues expected to cluster on the same face forming the active structure of rhodopsin, prevented G protein activation. Replacement of Leu460 by Ala, Asp, or Pro led to variable degrees of constitutive activation of the hFSHR and altered responses to agonist stimulation [102], suggesting that disruption of the bonds stabilized by the hydrophobic Leu460 may either promote varying degrees of receptor isomerization to an active, unconstraint state or compromise receptor/G_s-protein activation, depending on the nature of the replacing amino acid residue. These findings also suggest that in this FSHR region, the iL2 of the receptor may actually act as a conformational switch for allowing the receptor to achieve an active conformation upon agonist stimulation, rather than as a G protein recognition domain. Finally, agonist-stimulated second messenger production was abolished by changing the hydrophilic Arg450 (central to the ERW motif) to Ala or His (a basically charged residue with a more rigid side chain), whereas the conservative substitution Arg → Lys did not affect FSH-stimulated intracellular signaling [102]. These observations are in agreement with studies on the related LH receptor, in which similar substitutions impaired the ability of the mutants to mediate intracellular signaling [94, 105], and suggest that a flexible, positively charged residue at this position is apparently required in both gonadotropin receptors to allow the iL2 to interact properly with other receptor domains and lead to an active receptor configuration upon stimulation by agonist. More recently, mutational and computational modeling have established that integrity of this particular motif is required for productive interaction of the LH receptor with the G_s protein, rather than for intramolecular structural changes that lead to receptor activation [106].

The 14-3-3 proteins play important roles in intracellular signaling, cell division, and apoptosis, using a variety of mechanisms, including prevention of the interaction between BAD and the death-inhibitory protein Bcl2 [107, 108]. They may also act as a scaffold to bring proteins together, as in the case of Raf and PKC [109], and as a sequestrant to bring proteins into inappropriate compartments, thereby inhibiting function. Recently, Cohen and colleagues [110] employed a linked construct of the iL1 and iL2 (iL1–iL2 bait) to detect FSHR interacting proteins. This study identified a specific interaction between the FSHR iL-2 and the adapter protein 14-3-3τ, an interaction that was corroborated by showing that the adaptor protein coimmunoprecipitated with the full-length FSHR in a FSH and time-dependent manner. The FSHR iL2 sequence identified as target motif comprises five threonine residues in positions 442, 444, 447, 453, and 455, located in the NH_2-terminal end of the loop, which could serve as phosphate acceptors and thus as phosphothreonine-based motifs for the interaction [111]. Interaction between the FSHR and this adapter may be one of the mechanisms by which FSH inhibits apoptosis, since this gonadotropin induces phosphorylation of FOXO1a [46] and this transcription factor may be potentially sequestered by 14-3-3.

Several structural determinants present in the carboxyl-terminal end of the FSHR iL3 appear to be involved in receptor-G protein interactions and receptor function. In particular, this loop is considered pivotal in attenuation of agonist-stimulated, receptor-mediated intracellular signaling via internalization [74, 76, 112, 113]. Naturally occurring point mutations and studies employing synthetic peptide strategies have provided some information on the potential role of this intracytoplasmic region in FSHR-mediated signal transduction. Naturally occurring mutations involving the conserved Arg556 (Fig. 6) led to decreased agonist-stimulated second messenger production [114], while mutations in the conserved aspartate (at position 550) provoked constitutive activation of the hFSHR probably by disrupting the helix capping structure that helps to maintain the receptor in an inactive conformation [115–117]. Studies using synthetic peptides homologous to the rat FSHR 551–555 iL3 bearing the BBXXB motif reversed (BXXBB, where B is a basic amino acid) involved in G protein activation of several cell surface membrane receptors [99, 118–121], showed that the peptide promoted guanine nucleotide exchange in testes light membranes and reduced FSH-stimulated cAMP and estrogen production from primary Sertoli cell cultures [122, 123]. The role of this BXXBB motif present in the juxtamembrane region of the hFSHR on receptor function has been recently analyzed in more detail [103, 113, 124]. Although replacement of all three basic residues by alanine abolished plasma membrane expression of the triple mutant receptor, the functional role of each basic residue appeared to differ depending on its position within the minimal motif. Lysine in position 555 apparently participates in the mechanisms subserving ligand-stimulated turnover of the receptor since its replacement with Ala increased its turnover due to increased ubiquitination [113, 124], whereas Arg552 and Arg556 appear to participate in the interaction of the receptor with the G_s protein [103, 124]. More recent studies in our laboratory have shown that an Arg556Ala hFSHR mutant is poorly expressed at the plasma membrane and exert strong dominant-negative effects upon Wt FSHR expression (Ulloa-Aguirre, unpublished). The effect of replacing Arg552 in the hFSHR clearly differ from the scenario prevailing in the other glycoprotein receptors since replacement of the basic K566 in the hLH receptor or K621 in the hTSH receptor (corresponding to Arg552 in

the hFSHR), had no effect on agonist-provoked cAMP accumulation [116, 125]. In addition, in the human FSH and TSH receptors, simultaneous substitution of all three basic residues completely abolished agonist-stimulated cAMP production and severely impaired intracellular trafficking and membrane expression of the modified receptors [87, 124], whereas in the rat LH receptor (92% homology with the human counterpart in the serpentine region of the receptor) simultaneous replacement of two out of the three cationic residues present in this motif had no functional consequence [126].

Several structural and functional features characterize the carboxyl-terminal domain of FSHR (Fig. 6). This domain is rich in serine and threonine residues which are potential sites for phosphorylation [127], and plays important roles in defining differential recruitment of β-arrestins, β-arrestin-dependent activation of the ERK signaling cascade, and the fate of the receptor after agonist-induced internalization [127–129]. The hFSHR carboxyl-terminal domain also contains the minimal BBXXB motif reversed (also involved in G protein coupling of other receptors [99]) in its juxtamembrane region located in the segment encompassed by residues 650–653 [103, 124] (Fig. 6). The last two residues of the BXXBB motif (Arg617 and Arg618) and the preceding Phe616 constitute the NH_2-terminal end of the highly conserved $F(X)_6LL$ motif required for receptor transport to the cell membrane [130]. The BXXBB motif in this location appears to be more important for receptor expression than for G protein coupling, as indicated by mutagenesis studies [124]. Substitution of Lys614 with Ala in the hFSHR reduced both ligand binding and membrane localization of the receptor to $\sim$60% of the levels exhibited by the Wt receptor; consequently, cAMP production was also reduced. Replacement of Arg617 or Arg618 further reduced the ligand binding capacity and membrane expression of the mutant receptor, and virtually abolished FSH-stimulated cAMP production. Thus, mutations in these latter basic residues may provoke conformational changes in the NH_2-terminal end of the Ctail, which includes the above-mentioned $F(X)_6LL$ motif (at Phe616, Leu623, and Leu624), and thereby impair both receptor trafficking and cell membrane localization of the receptor. Nevertheless, the finding that a fraction of these mutants were still detected as mature receptor forms [124] suggests that this motif may be also involved in receptor-G protein coupling. Recent studies from our laboratory have shown that similar to the Arg556Ala mutant hFSHR, these expression-deficient Ctail mutant hFSHRs exert dominant negative effects on wild-type receptor transport to the cell surface membrane (Ulloa-Aguirre A, unpublished). This observation is interesting and takes special significance when considering recent evidence for oligomer formation of full-length FSHRs in situ (see below) [131].

The Ctail of the hFSHR exhibits three cysteine residues (at positions 627, 629, and 655) which are potential sites for palmitoylation. Cysteine residues at positions 629 and 655 are highly conserved among FSHRs from several species, whereas Cys627 is only present in the human receptor. In contrast to the LH and TSH receptors, evidence that this FSHR domain is esterified by palmitoylation was lacking until recently. Palmitoylation of GPCRs is a post-translational modification that promotes membrane association, internalization, and/or membrane targeting of proteins [132, 133]. In addition, in some GPCRs this modification contributes to G protein coupling and signal transduction [132]. In the TSH and LH receptors, palmitoylation of their conserved Ctail Cys residues is important for intracellular transport and turnover of the receptors [134–139]. Mutagenesis studies on the Ctail of the hFSHR have shown that all three Ctail cysteine residues are palmitoylated, but that only palmitoylation at Cys629 seems to be functionally relevant. In fact, abrogation of palmitoylation at Cys629 but not at Cys627 or Cys655 severely impaired cell surface membrane expression of the palmitoylation-deficient receptor, without significantly altering the efficiency of the receptor to couple to the G_s protein [103]. It remains to be investigated whether palmitoylation of the FSHR is important to couple the receptor to signaling pathways other than those mediated by cAMP.

Association between FSHRs and implications on signal generation

Although the crystal structure of FSH in complex with the extracellular domain of the hFSHR [13, 23, 24] demonstrated weakly associating $FSHR_{ECD}$ dimers, it is more likely that receptor dimerization occurs through the transmembrane domains. More recently, coimmunoprecipitation experiments have shown that the hFSHR forms oligomers early during receptor biosynthesis, which may be important to allow correct intracellular trafficking of the complex to the plasma membrane [131], as well as for coupling to multiple G proteins [34]. Oligomerization of GPCRs at the endoplasmic reticulum or the plasma membrane may, in fact, explain not only the dominant-negative effects of mutant receptors on Wt receptor expression or function [140–142] but also the selective transactivation effects observed when binding-defective (e.g., FSHRs mutated in their ectodomain), but signaling-competent receptors are coexpressed with binding-competent, but signaling-defective hybrids of the FSHR [143, 144]. The mechanism(s) mediating transactivation of homologous receptors are unknown; although dimerization at the membrane level [13, 23] may explain the positive cooperativity between receptors, the possibility also exists that newly synthesized

receptors may associate at the endoplasmic reticulum and then traffick to the plasma membrane as dimers. Follicle-stimulating hormone receptor dimerization may represent a means to enable not only positive cooperativity among receptor molecules, but also *negative* cooperativity as a quality control checkpoint at the endoplasmic reticulum level.

Conclusions

Despite our increased understanding during the last decade of the structure–function relationship of the FSH–FSHR system and the signaling pathways whereby the activated FSHR leads to biological responses, there are several unanswered questions concerning these aspects that are necessary to clarify for a more effective translation of knowledge on this system to the clinical arena. For example, based on studies in experimental animals (FSHβ and FSHR null mice) it has been proposed that the FSH–FSHR system plays an important role in the development of osteoporosis [145]. Although this is a debatable issue [146, 147], and despite the demonstration that osteoclasts and their precursors possess $G_{i2\alpha}$-coupled FSHRs, which may enhance osteoclast formation [145] and promote bone loss, additional studies are still required to elucidate more precisely the nature of the FSH–FSHR interaction in human bone cells. This is an important issue considering that down-regulation of the FSHR (and thus suppression of the resorptive actions of FSH) is what should be expected in the presence of persistently elevated FSH levels in vivo. In addition, it is important to investigate whether FSH-stimulated activation of the signaling cascade that leads to osteoclast formation is mediated by the 7-TM-spanning FSHR or rather through the alternatively spliced growth factor type FSHR variant [64, 65], which is connected with activation of MAPK signaling. Equally important is to elucidate in more detail the direct role of the FSH–FSHR system on oocyte development [148], the genes that are directly regulated by this system, and the signaling pathways involved. Finally, elucidation of the entire three-dimensional structure of the FSHR is also important since it will allow a better understanding of the mechanisms subserving FSHR activation and to depart from more precise structures for computational modeling and molecular dynamic simulations that mimics more accurately the natural environment of the receptor. This undoubtedly will facilitate the design of agonists and antagonists as well as allosteric modulators potentially useful for the treatment of infertility or in contraception.

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