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Minireview

Follicle-stimulating Hormone Receptor Signaling: Complexity of Interactions and Signal Diversity

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1 **Abstract**

2

3 Follicle-stimulating hormone (FSH) is synthesized in the pituitary by gonadotrope
4 cells. By binding to and interacting with its cognate receptor (FSHR) in the gonads, this
5 gonadotropin plays a key role in the control of gonadal function and reproduction. Upon
6 activation, the FSHR undergoes conformational changes leading to transduction of
7 intracellular signals, including dissociation of G protein complexes into components and
8 activation of several associated interacting partners, which concertedly regulate
9 downstream effectors. The canonical Gs/cAMP/protein kinase A pathway, considered for
10 a long time as the sole effector of FSHR-mediated signaling, is now viewed as one of
11 several mechanisms employed by this receptor to transduce intracellular signals in
12 response to the FSH stimulus. This complex network of signaling pathways allows for a
13 fine-tuning regulation of the gonadotropic stimulus, where activation/inhibition of its
14 multiple components vary depending on the cell context, cell developmental stage, and
15 concentration of associated receptors and corresponding ligands. Activation of these
16 multiple signaling modules eventually converge to the hormone-integrated biological
17 response, including survival, proliferation and differentiation of target cells, synthesis and
18 secretion of paracrine/autocrine regulators and, at the molecular level, functional
19 selectivity and differential gene expression.

20

21 In this minireview, we briefly discuss the complexity of FSHR-mediated intracellular
22 signals activated in response to ligand stimulation. A better understanding of the signaling
23 pathways involved in FSH action might potentially influence the development of new

24 therapeutic strategies for reproductive disorders.

25

26 **Words: 228**

27

28 **Abbreviations:**

29

30 APPL, adaptor protein containing pleckstrin homology domain, phosphotyrosine binding
31 domain, and leucine zipper motif; AREG, amphiregulin or aregulin; CREB, cAMP-
32 response element binding protein; EGFR, epidermal growth factor receptor; EPAC,
33 exchange protein directly activated by cAMP; FOXO1a, forkhead box transcription factor
34 O or forkhead homologue in rhabdomyosarcoma; GPCR, G protein-coupled receptor;
35 IGF-1R, insulin-like growth factor 1 receptor; IP3, inositol 1,4,5 triphosphate; mTOR,
36 mammalian target of rapamycin; LHCGR, luteinizing hormone-chorionic gonadotropic
37 hormone receptor; P70S6K, P70 S6 kinase; PI3K, phosphatidylinositol-3-kinase; PTEN,
38 phosphatase and tensin homolog deleted in chromosome 10; rpS6, ribosomal protein S6;
39 SFK, Src family of protein tyrosine kinases; Sgk, serum- and glucocorticoid-induced
40 kinase; Src, Rous sarcoma oncogene.

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50 Follicle-stimulating hormone or follitropin is synthesized in the anterior pituitary
51 gland by gonadotrope cells. By binding to its cognate receptor in the female and male
52 gonads, this gonadotropin plays a central role in the control of gonadal function and
53 reproduction (1,2). The follicle-stimulating hormone receptor (FSHR) belongs to the highly
54 conserved subfamily of the GPCR superfamily, the so-called *Rhodopsin* family and, more
55 specifically, to the δ -group of this large class of GPCRs (3). As other structurally related
56 glycoprotein hormone receptors [the LHCGR and the TSH receptor (TSHR)], the FSHR
57 is composed of a large NH₂-terminal extracellular domain (ECD), where recognition and
58 binding of its cognate ligand occur. The ECD is structurally linked by the so-called hinge
59 region to the transmembrane domain (TMD), which is composed of seven α -helices
60 connected by alternating intracellular and extracellular loops, and that is involved in the
61 activation and signaling functionality of the receptor. The FSHR ends with a COOH-
62 terminus (or C-tail) at the intracellular side, which contains important functional motifs of
63 the receptor (Fig. 1).

64

65 The FSHR is primarily expressed by the gonads (5). In the testis, this receptor is
66 located at the basolateral Sertoli cell surface, where it supports cell growth and
67 maturation, and thereby spermatogenesis (8); in the ovary, the FSHR is expressed in the
68 granulosa cells where FSH stimulation regulates growth and maturation of ovarian
69 follicles and estrogen production (1). For many years the FSHR was thought to be
70 localized exclusively in the gonads; nevertheless, new evidence from studies in

71 experimental animals and in humans have suggested that this receptor may also be
72 present in extragonadal tissues where FSH may have distinct physiological roles (9).
73 Nonetheless, some of these extragonadal tissues as targets for FSH action have been
74 recently questioned (10), indicating that further studies are still required to validate the
75 existence of extragonadally-expressed FSHRs.

76
77 Studies on other GPCRs as well as on the FSHR, have shed some light on the
78 mechanisms that presumably lead to FSHR activation and signal transduction. It appears
79 that FSH primarily binds to the large ECD and that extracellular loops 1 and 3, which are
80 extracellular projections of the TMD, represent potential secondary gonadotropin binding
81 sites (11). Upon FSH binding, the transmembrane helices (particularly the TMD helices
82 5, 6, 3, and 7) present a series of conformational changes and rearrangements that are
83 transmitted down the intracellular extensions of the α -helices and associated intracellular
84 domains (loops and C-tail; Fig. 1). This reorganization in intracellular domains in turn
85 allows accommodation and activation of a number of effectors, including several receptor-
86 coupled G proteins and other associated proteins and interacting partners, triggering the
87 activation of a number of downstream signaling cascades that will eventually lead to a
88 biological effect. An interesting nuance of this and other GPCRs is that the α -helices
89 conforming the TMD may oscillate between multiple conformations, that will define
90 activation of several or distinct signaling pathways and account for biased signaling (5,12-
91 14). These multiple mechanisms explain the recruitment of manifold signaling molecules
92 to the complex signaling network activated by the FSHR during follicular and Sertoli cell
93 maturation.

94

95 In this review, we provide a brief overview of the multifaceted aspects of FSHR
96 transduction, signaling and biological actions.

97

98 **Signal transduction at the FSHR: signaling pathways, interacting partners, and**
99 **networks.**

100

101 Most investigators agree that the effects of gonadotropins on the differentiated
102 function of their target cells are mainly mediated by the activation of the canonical
103 Gs/adenylyl cyclase/cAMP/PKA pathway, which subsequently leads to CREB
104 phosphorylation and modulation of gene transcription (5,15-20). Nevertheless, it is now
105 clear that this particular signaling cascade is not the only one activated by the action of
106 these hormones. As detailed below, gonadotropins activate additional pathways that are
107 involved in several cellular processes as proliferation and/or differentiation and, at the
108 molecular level, functional selectivity and differential gene expression (1,5,13,14,21-24).

109

110 The FSHR is connected *via* conformational selectivity to a nonlinear and complex
111 signaling network. This network can involve either several G protein subtypes, including
112 the Gs, Gi, and Gq/11 proteins (25-30), interaction with other receptors [e.g. the IGF-1R
113 and the EGFR (24,31-33)] and proteins [e.g. β -arrestins, APPL1, FOXO1a, and 14-3-3 τ]
114 (34-39), or heterodimerization with the LHCGR (26,40-42). These mechanisms have
115 been reported to regulate a number of intertwined signaling pathways, including
116 engagement of distinct kinases (such as PKA, PKC, PI3K, PKB/Akt, p70S6K and ERK1/2)
117 (1,5,14,22,24,27,43,44) (Fig. 2). This complex signaling network allows for a fine-tuning
118 regulation of the gonadotropic stimulus, where activation/inhibition of its multiple

119 components will vary depending on the cell context, developmental stage of the host cells,
120 and concentration of receptors and ligands (1,9,24,43,46-48). Furthermore, preferential
121 activation of distinct signaling modules by the FSHR occurring through stabilization of
122 distinct FSHR conformations in response to binding of particular ligands or receptor
123 mutations may also occur (Figs. 1 and 3) (14,49,50), paving the way for designing drug
124 candidates that may elicit selective signaling at the gonadal level (51,52). Let us briefly
125 dissect the complex signaling network turned on by the activated FSHR.

126
127 *Signaling through G proteins*

128
129 As mentioned previously, FSH binding triggers a sequence of conformational
130 changes within the receptor, which promotes activation of intracellular signaling
131 pathways. The primary transduction effectors described for the FSHR are heterotrimeric
132 G proteins (15,53,54). Upon Gs interaction with intracellular loops 2 and 3 of hormone-
133 bound FSHR on the ERW and BBXXB (B: basic residue and X: non-basic residue) motifs
134 (Fig. 1) (19), adenylate cyclase is stimulated and cAMP-dependent intracellular targets
135 are activated. One main target of cAMP is protein kinase A (PKA) that mediates FSH-
136 dependent signaling including the ERK MAPK cascade that controls Sertoli cell mitotic
137 phase (43). In preantral granulosa cells, FSH-dependent activation of ERK is mediated
138 through PKA-dependent destabilization of a constitutive 100 kDa MEK phospho-tyrosine
139 phosphatase (55), identified recently as dual specificity phosphatase 6 or DUSP6 (56). In
140 fact, activation of phosphatases by PKA appears to be a general mechanism whereby
141 FSH stimulates components not only of MAPK cascades, but also of PI3K-dependent
142 signaling, by indirectly enabling IGF-1R-transduced insulin receptor substrate 1
143 phosphorylation in granulosa cells (57). In these cells, FSH-dependent PKA activation

144 also stimulates p38 MAPK, likely involved in Sgk activation (22) and in modeling
145 granulosa cell shape (58). However, the relationship between FSH/PKA and p38 MAPK
146 phosphorylation has been recently questioned by studies in which expression of a
147 constitutively active form of PKA in granulosa cells was not sufficient alone to activate this
148 particular pathway (59).

149
150
151 Protein kinase A is considered as the master regulator of transcription factors of
152 the cAMP response element-binding protein (CREB)/activating transcription factors (ATF)
153 family (60) (Fig. 2). Nonetheless, in FSH-stimulated Sertoli cells, PKA regulates the
154 activity of other nuclear targets, such as the retinoic acid receptor α (61), an important
155 modulator of male germ cell development. More generally, PKA activity could be involved
156 in global chromatin remodeling, as illustrated by H3 histone phosphorylation in FSH-
157 stimulated granulosa cells (62). Noteworthy, the requirement of CREB itself on FSH-
158 dependent transcriptome also needs to be reconsidered because genome-wide
159 sequencing technologies combined with bioinformatics analyses have recently underlined
160 that FSH-responsive genes in human granulosa cells are notably enriched in binding
161 motifs for GATA family of transcription factors, whereas the frequency of CREB-
162 dependent genes is much scarcer than initially anticipated (63). Scrutinizing FSH-
163 regulated promoter regions at the systems level will certainly bring to light the involvement
164 of unexpected transcriptional regulators and upstream kinases in the future.

165
166 In addition to PKA, FSH-stimulated cAMP rise also activates the exchange proteins
167 directly activated by cAMP or EPACs, that enhance small RAS-like GTPase Rap1 activity
168 (24). EPAC is presumably involved in the activation of PKB/Akt (22), a PI3K target whose

169 activity is counteracted by PTEN to cease Sertoli cell proliferation (64). Likewise, in
170 granulosa cells, Akt neutralizes the anti-proliferative effect of AMP-activated protein
171 kinase (AMPK) (65,66) and promotes the phosphorylation and nuclear exclusion of
172 FOXO1a, one of its prominent substrate that also hampers the G1/S phase transition.
173 Interestingly, recent work has revealed that FOXO1a controls the expression of most
174 FSH-responsive genes in these cells (67). Many of these genes correspond to those
175 involved in autophagy (67,68), and recent studies suggest that FSH might protect
176 granulosa cells from atresia in part by preventing mitophagy (69). The mechanisms
177 involved encompass FOXO1a nuclear exclusion *via* PI3K/Akt/mTOR activation and
178 inhibition of E3 Ubiquitin ligase Parkin recruitment in the outer membrane of damaged
179 mitochondria by PTEN-induced putative kinase 1 (PINK1) (69). Not only Gs- but also β -
180 arrestin-dependent signaling might transduce the anti-atretic protective effect of FSH, as
181 shown recently in granulosa cells (70).

182

183 Both PKA and Akt-dependent pathways cooperatively interact to stimulate the
184 mTOR and p70S6 kinase, ultimately leading to mRNA translation (48,71,72) (73)(Fig. 2).
185 Translational regulation of mRNA in response to FSH has been proposed initially in
186 granulosa cells (71,74). During granulosa cell proliferation, hormone-bound FSHR leads
187 to Gs-dependent and ERK-mediated phosphorylation of TSC2 (tuberin), a mTOR effector
188 that stimulates p70S6K activity, ultimately leading to enhanced cyclin D2 expression
189 (74,75). *Via* the PI3K/Akt mTOR pathway, FSH stimulates the expression of hypoxia-
190 inducible factor-1 (HIF-1) mRNA, encoding a transcription factor that regulates the
191 expression of vascular endothelial growth factor (VEGF) (71). Furthermore, activation of

192 FSH-mediated mTOR pathway also induces the expression of some follicular
193 differentiation markers, such as the LHCGR, inhibin- α , P450_{AROM}, or the β II-subunit of
194 PKA. In the testis, cAMP- and PI3K/mTOR-dependent pathways also cooperate in the
195 activation of p70S6K by inducing phosphorylation profiles that differ according to the
196 development stage of Sertoli cells (48,72,73). This developmentally regulated
197 phosphorylation profile of p70S6K is supported by the observation that during the Sertoli
198 cell differentiation phase, Akt is phosphorylated directly by FSHR signaling and not *via*
199 transactivation of the IGF-1R as shown in the mitotic phase (75,76), suggesting different
200 properties of the PI3K-regulated network at both stages (48,72,73). By using these PI3K-
201 dependent signaling mechanisms, FSH may promote rearrangements and post-
202 translational modifications of initiation and elongation factors that form the translational
203 machinery at the 5' untranslated region (UTR) of mRNAs. For example, in Sertoli cells
204 FSH stimulates the phosphorylation of eukaryotic translation initiation factor 4 (eIF4) G
205 and eIF4B, a cofactor of the eIF4A RNA helicase, *via* mTOR and p70S6K respectively.
206 In only a few minutes, all these molecular events induce cap-dependent translation of
207 mRNAs such as VEGF and c-fos (72), as well as Internal Ribosome Entry Site (IRES)-
208 dependent mRNA translation (77). Cell responsiveness to FSH is not limited to the
209 regulation of mRNA transcription and translation, and recent data have proposed that a
210 complex miRNA network orchestrates the stability, and hence the dynamics, of various
211 components of the FSH signaling network (78,79). For example, the stabilization of PTEN
212 would occur through hormone-induced degradation of miRNAs complementary to the
213 PTEN mRNA (80). PTEN is required for Sertoli cells to achieve terminal differentiation

214 (64) and its mRNA is spatially restricted at spermiation in the adluminal region of Sertoli
215 cells (80).

216

217 Like many other GPCRs, the FSHR receptor exhibits some tendency to
218 promiscuity with other G proteins. For example, the FSHR has been reported to interact
219 with Gi when activated by particular glycosylated variants of FSH (25,81,82). Likewise, in
220 Sertoli cells at early stages of maturation, cyclin-D1 expression depends in part on Gi
221 FSHR coupling (43). Further studies are needed to determine whether the FSHR variant
222 expressed at this stage matches with the alternative splicing product of the FSHR
223 identified previously in granulosa cells that stimulates Pertussis toxin-sensitive ERK
224 phosphorylation but is unable to enhance cAMP levels (82). An analogous phenomenon
225 of signaling dichotomy has been observed in cultured rat granulosa cells when the FSHR
226 is occupied simultaneously by FSH and FSHR negative allosteric modulators, which
227 promote persistent FSH-stimulated estradiol biosynthesis in the face of inhibited cAMP
228 production (51). Besides, at supraphysiologic concentrations (>200 ng/ml), FSH also
229 mediates FSHR coupling to Gq/11 (29,83) leading to increased IP3 levels.

230

231 *Role of FSHR associated partners, β -arrestins, Ca^{2+} mobilization, and cross-talk*
232 *with other plasma membrane receptors in FSHR signaling*

233

234 *APPL1, FOXO1a, and 14-3-3 τ* . In addition to signaling through G proteins, the FSHR also
235 directly interacts with a number of partners that elicit particular signaling mechanisms.
236 These partners include, among others, the APPL1 adapter, the above-mentioned

237 FOXO1a, and 14-3-3 τ (34,35,37,38,84-86). The APPL1 adapter is an important regulator
238 of signaling and trafficking events within cells, and is expressed in many tissues, including
239 the testis and the ovary (87). In fact, this adapter interacts with many signaling proteins
240 in a cell type-dependent manner. APPL1 may govern signal specificity and trafficking by
241 interacting with PI3K and Akt (88) that in turn phosphorylates FOXO1a, as well as with
242 the small GTPase Rab5 (89), a mediator of fusion of clathrin-coated vesicles with early
243 endosomes (90). Studies employing a yeast interaction trap, identified APPL1 as an
244 important interacting protein with the FSHR (35,37,38); furthermore, it has been found
245 that linking between this adapter and the FSHR occurs at the intracellular loop (IL) IL1,
246 specifically at Lys393, Leu394 and Phe399 (39) (Fig. 1). In HEK293 cells stably
247 expressing the FSHR, APPL1 and 2 appear to be associated with each other *via* the NH₂-
248 terminus of APPL1 in a FSH-independent manner (35,37,38). Given that the FSHR
249 associates with APPL1 and interacts with FOXO1a (which does not interact with APPL1
250 or 2) (38,66) and that APPL1 interacts with Akt (37), it has been proposed that the
251 interplay between a FSHR-FOXO1a complex with an activated FSHR-APPL1-Akt
252 complex leads to FOXO1a phosphorylation and abrogation of apoptosis (23,35). In fact,
253 as described above, FSH stimulation results in rapid FOXO1a phosphorylation and its
254 exclusion from the nucleus in granulosa cells (66). In the same vein, association of the
255 FSHR with 14-3-3 τ has been mapped to the IL2, overlapping with the canonical G protein
256 binding sites, specifically the ERW motif (Fig. 1) (35). 14-3-3 proteins are involved in a
257 number of biological processes and play an important role in regulating signaling
258 pathways by interacting with phosphorylated signaling proteins (91). In HEK293 cells,
259 overexpression of 14-3-3 τ led to decreased cAMP levels (34) suggesting a role of 14-3-

260 3 τ in FSHR/Gs-mediated signal transduction. The association of APPL1, 14-3-3 τ , and
261 FOXO1a with the FSHR suggests that FSH causes phosphorylated FOXO1a to be
262 sequestered to the cytosol by 14-3-3 τ , with APPL1 facilitating this process.

263
264 *β -arrestins*. Besides evoking ERK1/2 phosphorylation through the Gs pathway, FSHR
265 activates this particular signaling cascade through β -arrestins (43,85), which in addition
266 to their well-recognized role in GPCR desensitization, internalization, and recycling (see
267 below), have also emerged as important players in the regulation of GPCR signaling
268 (13,86). The FSHR has been reported to be phosphorylated by PKA and PKC second
269 messenger-dependent kinases but also by G protein-coupled receptor kinases (GRK) 2,
270 3, 5, and 6 in various cell culture models (36,85,92-95). β -arrestins recruited to GRK2- or
271 GRK5/6-phosphorylated FSHR have been shown to exert distinct intracellular functions
272 in time and space: β -arrestin 1 and 2 binding to GRK2-phosphorylated FSHR leads to the
273 internalization and recycling of the receptor, whereas phosphorylation of the FSHR by
274 GRK5 and 6 is required for β -arrestin-dependent, heterotrimeric G protein-independent
275 ERK signaling, as shown by other GPCRs (13,85,96-99). Concurrently, these findings
276 are consistent with a model of phosphorylation bar code elicited by distinct GRKs that
277 could control the conformation of β -arrestin recruited to the receptor and thereby its ability
278 to interact with specific partners (12,96-100). A well-documented illustration is the
279 temporal encoding of ERK activation: in contrast to G protein-mediated ERK activation,
280 which is rapid and transient, ERK recruitment and signaling activated by β -arrestins is
281 slower (~5-10 min to reach maximum), but protracted ($t_{1/2}$ >1 hour) (85). It has recently
282 been demonstrated that the 5-hydroxytryptamine 2 receptor, β_2 -adrenergic receptor,
283 CXC-chemokine receptor 4, and the FSHR activate the ERK pathway *via* a mechanism

284 involving MEK-dependent β -arrestin 2 phosphorylation at Thr383. Importantly, this
285 agonist-induced phosphorylation of β -arrestin 2 is a necessary step for ERK recruitment
286 to β -arrestin complex and for ERK activation (101). More recently, Gs-dependent and β -
287 arrestin-dependent FSH signaling have been shown to cooperatively activate p70S6K,
288 within a constitutively assembled β -arrestin/p70S6K/rpS6 module (72,102,103); this
289 complex controls the translation of 5' TOP mRNAs, a subset of mRNAs representing
290 almost 20% of cellular mRNA abundance and that encode for ribosomal proteins, poly(A)-
291 binding protein, and factors of the translational machinery (102-104). The co-joined action
292 of Gs- and β -arrestin-transduced signaling on p70S6K contrasts with the parallel action
293 that both effectors have on the ERK MAPK, whose kinetics of phosphorylation differs
294 depending on the transducer (Gs vs β -arrestins)(50,85,103). Finally, the β -arrestin-
295 dependent intracellular signaling may be preferentially activated when the FSHR is
296 stabilized in distinct conformation(s) in response to particular ligands or receptor
297 mutations, in a process known as biased agonism or functional selectivity (Fig. 3), which
298 is discussed below.

299
300 *Ca²⁺ mobilization.* FSH-stimulated Ca²⁺ mobilization in both granulosa and Sertoli cells
301 has been well recognized for a long time and it has been attributed to both rapid influx of
302 Ca²⁺ through T-type Ca²⁺ channels as well as release from intracellular stores (28,30,105-
303 109), where Gi/G β γ /PI3K or Gs- and/or Gq-mediated activation of phospholipase C
304 (PLC) β or other PLC isoforms appear to play an important role. In fact, in rat Sertoli cells,
305 FSHR can signal through the transglutaminase G α h to promote extracellular Ca²⁺
306 mobilization by interacting with PLC δ -1 (28). Moreover, studies in human granulosa cells
307 revealed that in addition to the regulatory roles of APPL1 described above, interaction of

308 this adapter with the FSHR also leads to IP₃ production and release of Ca²⁺ from
309 intracellular stores (39). On the other hand, it has been shown that exposure of swine
310 granulosa cells or rat Sertoli cells to FSH leads to a rapid increase in intracellular Ca²⁺
311 accumulation, which may occur in a cAMP-dependent (*e.g. via* stimulation of PLC ϵ) and
312 -independent (*e.g. via* the Gq/11 $\beta\gamma$ complex) manner (26,30,106,107,110,111).
313 Whatever the mechanism(s) involved in Ca²⁺ mobilization, it seems that Ca²⁺-mediated
314 signaling is important for FSH-regulated functional differentiation of granulosa and Sertoli
315 cells (112-114).

316
317

318 *Cross-talk with other plasma membrane receptors in FSHR signaling.* Among the
319 mechanisms contributing to GPCR signal pleiotropy is receptor cross-talk, which may
320 occur either *via* intracellular effectors or through heterodimerization. In this regard, the
321 tyrosine kinase receptors IGF-1R and EGFR have emerged as important functional
322 partners of the FSHR. Regarding the former, it has been shown that ligand-activated IGF-
323 1R and FSHR act synergistically to enhance steroidogenesis, LHCGR and inhibin- α
324 expression, and cell proliferation in different cell contexts and species, albeit with some
325 differences (33,115,116). In addition, both FSH and locally produced IGF-1 are essential
326 for granulosa cells differentiation, follicle survival and fertility (33,117). Signaling mediated
327 by both receptors converge and overlap in activating distinct signaling cascades
328 downstream of adenylyl cyclase, mainly the PI3K/Akt pathway (22,31). Furthermore,
329 female mice with conditional knockdown of the IGF-1R in granulosa cells or exposed to
330 IGF-1R inhibitor, exhibited defective FSH-stimulated Akt activation and steroidogenesis,
331 indicating the key role played by the IGF-1R on FSHR-regulated cell proliferation and

332 differentiation (22,31). The molecular mechanism(s) proposed for the synergic effects of
333 FSH and IGF-1 at the receptor level include the ability of activated FSHRs to inhibit
334 dephosphorylation of the IGF-1R by protein kinase phosphatases, such as PTP-1B and
335 Src homology 2 domain-containing phosphatase 2 (SHP-2), both expressed in granulosa
336 cells (118-120). Alternatively, the interaction between molecular chaperones and IGF-1R
337 may be enhanced, as suggested by the prevention of *cyp19* and *P450_{scc}* expression in
338 response to FSH by HSP90 (heat-shock protein 90) inhibition (33).

339
340 Another receptor modulating FSH-stimulated granulosa cell proliferation and
341 differentiation in the developing follicle is the EGFR, which participates in phosphorylation
342 of ERK1/2 and p38MAPK in granulosa and cumulus cells of preovulatory follicles (121-
343 123). Thus, in addition to EPAC and Rap1 in promoting p38MAPK phosphorylation (see
344 above), FSH-stimulated induction of EGF-like factors, like AREG, appears as well to play
345 an important role in the PKA-independent activation of MAPK signaling in developing
346 granulosa cells (24,124,125). Additional studies have further indicated that besides
347 promoting activation of the MAPK cascade, AREG and FSH stimulate Akt and FOXO1a
348 phosphorylation through activation of members of the Src family of tyrosine kinases or
349 SFKs (24). As in the case of the IGF-1/IGF-1R pathway described above, it appears that
350 the AREG/EGFR/RAS and FSH/FSHR signaling pathways converge on common
351 downstream targets, mainly ERK1/2, p38MAPK and Akt phosphorylation, leading to up-
352 and downregulation in the expression of different genes that impact on both follicle
353 maturation and granulosa cell differentiation into granulosa-lutein cells exhibiting
354 functional and steroidogenic profiles that differ from those exhibited by maturing
355 granulosa cells (1,123,126-128). The importance of the EGF/EGFR pathway in follicular

356 maturation and ovulation is emphasized by studies in mice showing that the absence of
357 *Areg* results in subfertility and decreased response to exogenous gonadotropin treatment
358 (129).

359

360 In addition to cross-talk with the IGF-1R and EGFR, the FSHR interacts with the
361 structurally-related LHCGR. Although the functional relationship between these two
362 gonadotropin receptors had been well recognized for a long time, it was only recently that
363 functional cross-talk and physical interaction between gonadotropin receptors were
364 reported (26,40,41). Hetero-oligomerization of the FSHR and LHCGR has been
365 demonstrated using BRET, fluorescence correlation spectroscopy, and more recently,
366 photoactivatable-localization microscopy (PALM) (<10 nm resolution) (26,130,131).
367 Biochemical studies in HEK293 cells, showed that when heterodimerized, FSH and
368 LHCG receptors exhibited cross-inhibitory effects on Gs signaling: heterodimerization of
369 LHCGR with FSHR led to attenuation of LH-stimulated cAMP production, whereas when
370 FSHR heterodimerized with LHCGR, FSH-stimulated second messenger production was
371 mitigated (40). More recently, Jonas and colleagues (26) employed PALM to detect
372 physical and functional interaction between the FSH and LHCG receptors in human
373 granulosa-lutein cells. They found that both receptors associate as functionally asymmetric
374 heteromers, and that this association leads to prolonged LH-stimulated Ca²⁺ signaling *via*
375 influx of extracellular calcium in a G α q/11- and G β γ -dependent manner (26).
376 Concurrently, these data demonstrate that gonadotropin receptors, when
377 heterodimerized, may undergo allosteric regulation, with one receptor impacting on cell
378 signaling triggered by the other as previously suggested (42). Cross-talk between

379 gonadotropin receptors may be potentially important for reproductive function, given that
380 *in vivo* both receptors coexist in granulosa cells, particularly during the last stages of
381 follicle development prior to ovulation (1).

382

383 **Signal termination and biased agonism**

384

385 Signal termination at the FSHR is a tightly regulated multistep process known as
386 homologous desensitization. First, GRKs rapidly phosphorylate serine/threonine residues
387 of the FSHR COOH-terminus (5,36,85,93-95). Second, β -arrestins 1 and 2 are recruited
388 and prevent Gs interaction with the core domain of the FSHR through steric hindrance
389 (5,54,94,95,132). Third, the amount of intracellular cAMP decreases and the receptor
390 becomes refractory to further FSH stimulation (13,85,86,133). Following desensitization,
391 the FSHR is rapidly internalized and predominantly resensitized, with a minor fraction of
392 the FSHR being degraded (54,85,134). Interestingly, the FSHR was recently reported to
393 traffic to very early endosomes for its post-endocytic sorting rather than early endosomes
394 like most GPCRs (135-137) (Fig. 2). In contrast with Gs, which leads to full cAMP
395 response with less than 5% of FSHR occupancy, more than 90% of FSHR molecules
396 need to be occupied to achieve maximal β -arrestin recruitment and internalization (54).

397

398 Major advances in GPCR structural biology over the last decade have profoundly
399 changed the conceptual basis of receptor activation and pharmacological efficacy. In
400 particular, it is now appreciated that GPCRs adopt multiple inactive and active
401 conformations that are connected to distinct transduction mechanisms (12,138-147).
402 Accordingly, it has been shown that a given ligand or receptor mutation can modify the
403 stabilized conformation of the receptor-ligand complex, as compared to the wild-type

404 receptor-reference-ligand complex (Fig. 3). This phenomenon is generally referred to as
405 pharmacological bias (12,138,141,144). Practically, this conceptual framework delineates
406 two levels of pharmacological bias. First, ligand bias that are elicited by certain ligands
407 that stabilize a subset of the receptor conformations, leading to an imbalance between
408 the signaling pathways activated when compared to the reference agonist. Second,
409 receptor bias that can occur in the case of mutations [e.g. in the case of FSHR Met512Ile
410 mutation at the second extracellular loop or Ala189Val mutation at the ECD] (7,148,149)
411 or of single nucleotide polymorphisms (e.g. the Asn680Ser polymorphism, Fig. 1)
412 (150,151) at the receptor level, leading to imbalanced or impaired signaling compared to
413 the wild-type receptor (Fig. 3).

414
415 Different classes of small molecules capable of modulating FSHR have been
416 identified (152-157), including three negative allosteric modulators (NAMs) (51,52,158).
417 ADX61623 increased the affinity of FSH binding, yet blocked FSHR-mediated cAMP and
418 progesterone but not estradiol production in rat primary granulosa cells, whereas *in vivo*,
419 it did not affect FSH-induced preovulatory follicle development (51). Two other NAMs,
420 ADX68692 and ADX68693, with structural similarities to ADX61623, were subsequently
421 shown to exhibit biased NAM activities on FSHR in rat primary granulosa cells (52,158):
422 ADX68692 blocked FSHR-promoted cAMP, progesterone and estradiol production
423 whereas ADX68693 inhibited cAMP and progesterone with the same efficacy as
424 ADX68692 but did not block estradiol production. Importantly, ADX68692 but not
425 ADX68693 decreased the number of oocytes recovered in the *ampullae* (52). A positive
426 allosteric modulator (PAM), Org 214444-0, has also been described (159); this PAM
427 exhibited nanomolar FSHR agonistic effects and selectivity over the structurally related

428 LHCGR and TSHR. When co-incubated with FSH, Org 214444-0 increased FSH binding
429 and cAMP activation. Furthermore, *in vivo*, Org 214444-0 recapitulated the action of FSH
430 in a model of fertility in rat. More recently, another series of small molecules acting as
431 agonist-PAMs were reported (160-162). These thiazolidinone derivatives have been
432 shown to activate FSHR signaling in CHO cells and to enhance estradiol production in
433 cultured rat granulosa cells (161). In addition, optimization of substituted benzamides led
434 to more FSHR-selective molecules (162). Small molecules have also been reported to
435 behave as competitive antagonists. First, suramin has been shown to inhibit testosterone
436 production and FSHR signaling (163,164). Another compound displaying similar
437 antagonistic properties as suramin but with much better specificity for FSHR was later
438 described (165). With the notable exception of the ADX series, whether these compounds
439 lead to balanced or biased agonism/antagonism compared to FSH remains unknown and
440 will need further investigation.

441
442 In the same line, it has long been suggested that naturally occurring FSH
443 glycoforms may also elicit distinct effects on target cells (166). The common α -subunit
444 and FSH β each contain two N-linked oligosaccharides (at positions Asn52 and Asn78 on
445 FSH α and at Asn7 and Asn24 in FSH β) that play a pivotal role in the functional properties
446 of the hormone (167-170). Removal of the carbohydrate residue at position 78 on the α -
447 subunit significantly increases receptor binding affinity of human FSH. Likewise,
448 carbohydrate at position 52 on the α -subunit was found to play an essential role in signal
449 transduction since its removal resulted in a significantly decreased potency. A
450 hypoglycosylated FSH (FSH21/18) was 9- to 26-fold more active than fully-glycosylated

451 FSH (tetra-glycosylated FSH24) in binding and functional assays (171,172). Another
452 human FSH deglycosylated variant, which possesses only α -subunit oligosaccharides,
453 was significantly more bioactive *in vitro* than the tetra-glycosylated form of the hormone
454 (173,174). In contrast, hyperglycosylated FSH enhanced the number of ovulated eggs
455 and subsequent *in vitro* embryo development (175). Interestingly, partially deglycosylated
456 eLH (eLHdg) behaved as a biased agonist at the FSHR, eliciting β -arrestin recruitment to
457 FSHR as well as ERK and rpS6 phosphorylation in a β -arrestin-dependent and Gs/cAMP-
458 independent manner (50).

459

460 The effects of FSH21/18 and FSH24 has further been studied *in vivo* (176).
461 Administration of these FSH glycoforms to *Fshb* null female mice induced expression of
462 several FSHR downstream signaling pathway components, including phosphorylated
463 forms of CREB, PKA, Akt, p38, and p42/44 ERK, and FSH-regulated genes (e.g. *Cy19a*,
464 *inha*, and *inhbb*) in a similar manner, albeit with some differences in the kinetics of
465 response between the two glycoforms. In contrast, when FSH21/18 or FSH24 was
466 injected to *Fshb* null males, differential up- and down-regulation in expression of the nine
467 FSH-responsive genes analyzed (e.g. in *cdo1*, *Clu*, *Tbx22*, *Zic3*, *Car13*, *Amh* and *FSHR*)
468 was observed, suggesting biased agonism of the FSH glycoforms at the Sertoli cell level
469 *in vivo* (176). Whether the functional selectivity shown by hypo- and fully-glycosylated
470 FSH is only due to differences in binding affinity or to stability of interaction with the FSHR
471 (171,177), or actually to induction of distinctly different receptor conformations leading to
472 selective activation of downstream signaling pathways, still remains to be analyzed in
473 more detail.

474
475 Another class of potential ligand bias comes from cases where structural
476 constraints are conveyed on the hormone by an antibody, leading to the modification of
477 its pharmacological properties (14). Polyclonal anti-peptide antibodies against ovine
478 FSH β subunit led to a significant enhancement of biological activity *in vivo* in mice (178).
479 Likewise, the use of a monoclonal antibody against bovine FSH in snell dwarf mice
480 showed an increase in uterine weight (179). Studies also evaluated the impact of equine
481 chorionic gonadotropin (eCG)/anti-eCG complexes on gonadotropin bioactivities. They
482 showed that anti-eCG IgGs were either inhibitory or hyper-stimulatory on LH and FSH
483 bioactivity (180). Furthermore, Wehbi and colleagues (49) discriminated the nature of
484 these complexes on FSH signaling and revealed their biased properties.

485

486 **Conclusions**

487
488 From the above discussion, it is clear that FSH-stimulated intracellular signaling is
489 mediated through an intricate network whose dynamic properties depends on the stage
490 of target cell development and cell context, which may in turn be regulated by numerous
491 factors intrinsic to the cell and by the ligand. These factors include changing ligand pulse
492 frequency and local concentrations, particular structure (166,172,176,181-183), and
493 interaction dynamics with the FSHR (177).

494

495 Since the multiple components of the FSHR-regulated signaling network (Fig. 2)
496 are activated neither with the same amplitude nor at the same time, the temporal and
497 dynamical aspects that govern the kinetics of the biochemical reactions involved has to
498 be considered. These complex dynamics can be approached with computational

499 modeling using ordinary differential equations, as proposed previously for the FSHR
500 (78,184). For example, in granulosa cells, FSH-induced cAMP pathway dynamics has
501 been modelled with careful examination of steady-states and parameter sensibility (184).
502 Dynamic interactions between cAMP and mTOR pathways, that converge onto p70S6
503 kinase activation by FSH or insulin was modelled using experimental data from primary
504 Sertoli cells (48). This model provided clues on p70S6 kinase phosphorylation rates and
505 was experimentally validated using pharmacologically perturbed conditions. Further
506 developments of FSHR network modeling will help to fill the gap between detailed
507 knowledge on molecular interactions and comprehensive understanding of hormonal
508 dynamics. Functional interactions of the FSHR with several tyrosine kinase receptors
509 other than the IGF-1R and EGFR described above, may further increase the landscape
510 complexity. These include the neurotropic factors GDNF (glial cell line-derived
511 neurotropic factor) (185) and BDNF (brain-derived neurotrophic factor) (186) receptors,
512 as well as receptors of the TGF β family members (187), known to potentiate the mitogenic
513 action of FSH in Sertoli cells and/or granulosa cells. These aspects will require further
514 investigation.

515

516 Since the first pioneering evidence that the gonads exhibit binding sites specific for the
517 FSHR and respond to FSH by increasing intracellular cAMP levels (16,188-191),
518 significant advances in our understanding of the structure and function of the FSH/FSHR
519 system has been achieved. Nonetheless, there are still important issues that remain to
520 be elucidated and that will require the application of new experimental paradigms, “omic”
521 technologies (genomic, transcriptomic, and proteomic techniques), and systems biology

522 tools to facilitate this challenging task. These issues include, for example, a more detailed
523 elucidation of the mechanisms underlying differential gene expression and protein
524 phosphorylation during granulosa cell differentiation and follicle maturation under the
525 influence of FSH variants, FSH analog compounds, and FSHR allosteric modulators,
526 which may convey important therapeutic benefits to the clinical arena. To this end, *in vitro*
527 experimental models such as intact follicle culture or three-dimensional bioprosthetic
528 ovary models that may better reproduce the physiological environment of the developing
529 follicle may be applied; these systems might allow to explore more deeply the signaling
530 pathways and gene expression profile induced by FSH both in target cells in their in-situ
531 arrangements and within different granulosa cell compartments throughout follicle
532 maturation (192-194). As noted above, further studies are also needed not only to validate
533 the existence of extragonadally-expressed FSHRs but also to dissect in more detail their
534 corresponding signaling networks and pathways as well as gene expression profiles,
535 which might eventually allow to assign them physiological roles according to their
536 particular location. Integration of large-scale information obtained from these techniques
537 will undoubtedly facilitate the building up of more integrative signaling framework models
538 that may be applied to simulate *in silico* the biological consequences of intermittent FSH
539 stimuli on its target cell in multiple-way combinations. Beyond any doubt, past and present
540 studies may just represent the prologue to what remains to be unveiled.

WORDS: 5355

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1210 **Figure legends**

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1212 **Figure 1. Schematic representation of the human FSHR with the cytoplasmic face**
1213 **of the TMD and the C-tail magnified and the extracellular domain removed at**
1214 **residue 352.** The schematics show the locations of sequences, motifs, and residues
1215 involved in internal agonist (FNPCEDIMGY sequence shaded in dark red); G protein
1216 coupling and receptor activation (ERW motif, NPXXY motif, and BXXBB motif reversed
1217 at the IL3); interaction with APPL1 (residues 393, 394, and 399 at the IL1), 14-3-3 τ (IL2),
1218 ubiquitin, and PKC2 binding (IL3); receptor trafficking [F(X₆)LL and BXXBB motif reversed
1219 at the NH₂-terminal end of the C-tail]; phosphorylation of the receptor by GRK (putative
1220 class B cluster); palmitoylation (Cys644, 646, and 672) and postendocytic fate (residues
1221 684 and 688-690). Also indicated is the location of the most common FSHR
1222 polymorphism (Asn680, light yellow circle) as well as of inactivating (red circles) and
1223 activating (green squares) FSHR mutations involving the TMD and IL3, which may impact
1224 receptor trafficking and/or activation and signaling [reviewed in refs. (4-6)]; the mutation
1225 at position M512 (magenta circle in the EL2), has been associated with biased signaling
1226 (7). Numbering of the amino acid residues includes the leader sequence. EL, extracellular
1227 loop; IL, intracellular loop; TMD, transmembrane domain, C-tail, intracellular COOH-
1228 terminus.

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1230 **Figure 2. A simplified overview of the FSHR signaling network.** The color code,
1231 depending on the arbitrarily main effector, is as follows: green for PI3K-dependent
1232 signaling, orange for cAMP-dependent signaling, red for β -arrestin-dependent signaling,

1233 regardless that some signaling effectors (e.g. ERK or p70S6K) are commonly activated
1234 by these three pathways. Mechanistic details have been omitted for the sake of clarity. Of
1235 note, the phosphorylation state of the FSHR conveyed by GIPC is not known. This
1236 network has been established by using the Cell Designer algorithm (45) and the meaning
1237 of symbols is shown in the rectangle at the bottom. AC, adenylyl cyclase; AMPK, AMP-
1238 activated protein kinase; DAG, diacylglycerol; DUSP, dual specificity phosphatase 6;
1239 eIF4, eukaryotic translation initiation factor 4; GIPC, G α i-interacting protein C terminus;
1240 PARK, E3 ubiquitin protein ligase Parkin; PINK, PTEN-induced putative kinase; PIP3,
1241 phosphatidylinositol (3,4,5) triphosphate; VEE, very early endosome.

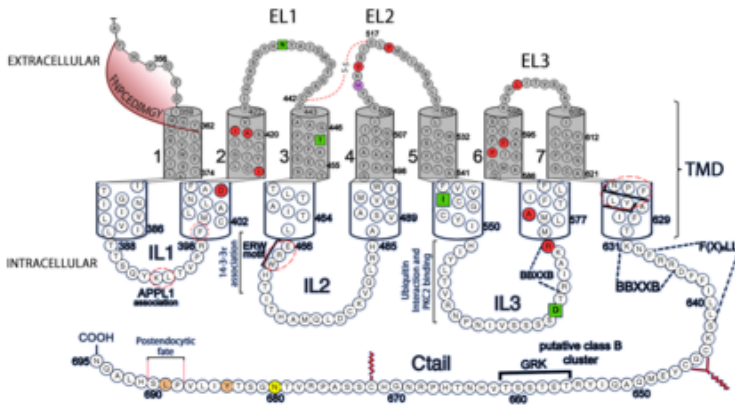
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1243 **Figure 3. Principle of biased agonism triggered at the FSHR by allosteric ligands.**

1244 **(a)** Upon agonist binding, FSHR (yellow) recruits Gs (red), leading to cAMP production
1245 and ERK phosphorylation. Agonist binding also triggers β -arrestins (light green)
1246 recruitment and cAMP-independent ERK phosphorylation. **(b)** Balanced allosteric ligand
1247 (inverted green triangle) can activate FSHR in the absence of FSH and recruits similar
1248 proportion of Gs and β -arrestin as FSH. **(c)** Gs-biased allosteric modulator (inverted
1249 orange triangle) preferentially induces Gs-dependent signaling. **(d)** β -arrestin-biased
1250 allosteric modulator (inverted blue triangle) preferentially elicits β -arrestin-dependent
1251 signaling. Other possible combinations are not represented. FSHR ECD: Follicle-
1252 stimulating hormone receptor extracellular domain.

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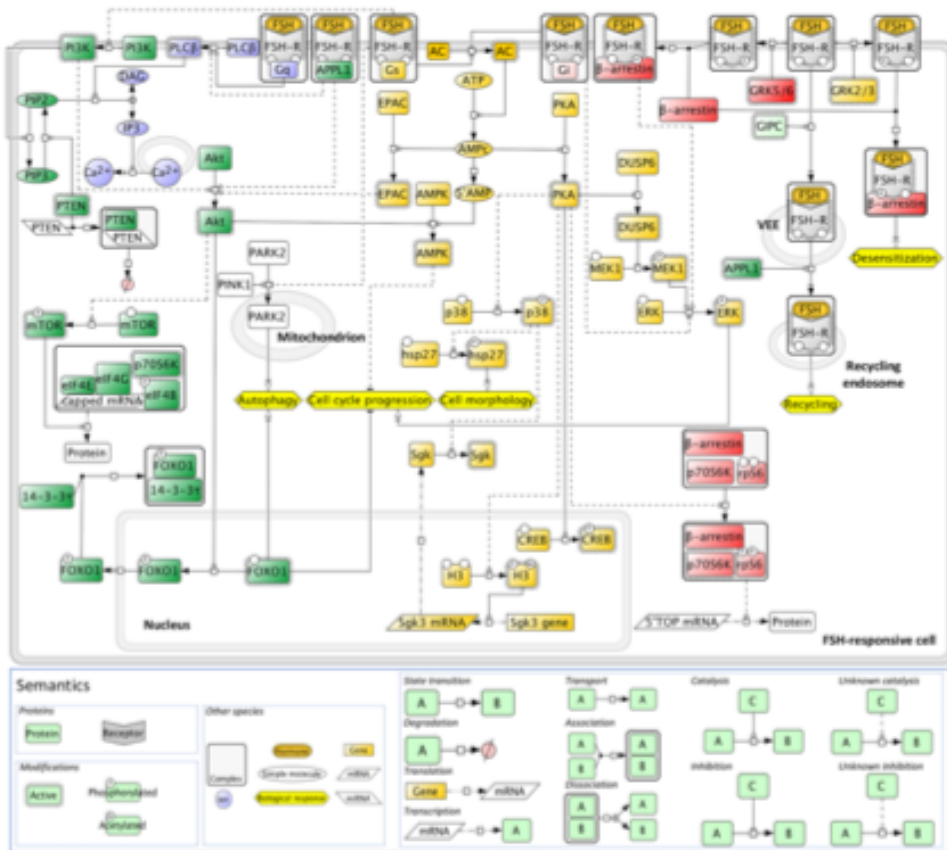


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1256 Figure 1

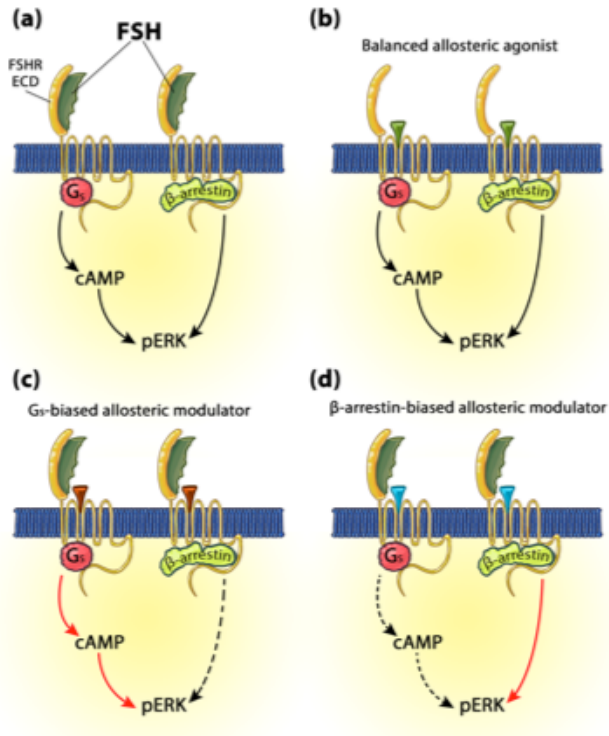
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1260 Figure 2



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1262 Figure 3