CHAPTER

20

Gonadotropin Signaling in the Ovary

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INTRODUCTION

The gonadotropic hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) initiate signaling events in ovarian target cells that govern the reproductive cycle and thus the continuation of a species. The ovarian follicle is the key structure that houses the oocyte. Upon appropriate stimulation by FSH and LH, follicles produce hormones that promote the development of secondary sex characteristics and that regulate the hypothalamic–pituitary axis as well as uterine receptivity. Somatic cells of the follicle contribute to hormone production and provide an environment supportive of growth and maturation of the oocyte. Additionally, the follicle must extrude the oocyte at ovulation to allow for fertilization, and remaining cells within the follicle must differentiate into cells of the corpus luteum that produce hormones necessary to sustain pregnancy. In the absence of FSH, follicles do not develop beyond the preantral stage, and animals are infertile. Similarly, in the absence of LH, ovulation does not ensue, corpora lutea do not form, and animals are infertile. Although there is a great deal of species variability, LH is also uniformly required to initiate progesterone production by the corpus luteum, which is necessary for implantation and the maintenance of pregnancy. Both gonadotropic hormones initiate their activities by binding to cell surface protein receptors. Through complex signaling pathways, FSH and LH initiate distinct, highly coordinated programs of gene expression that are only now beginning to be unraveled.

The existence of the gonadotropins was postulated based on the pioneering physiological studies of Long and Evans in 1922, and this work was quickly followed by the separation of pituitary extracts into the distinct fractions that would become known as FSH and LH by Fevold, Hisaw, and Leonard in 1931.[1] As approaches for the purification of the gonadotropins from various species were perfected in the 1970s, it became possible to begin detailed studies of their actions, leading to our fundamental understanding of the critical roles that they play in both male and female reproduction. A classic study by Greep et al. in 1942 showed that while purified FSH was sufficient to stimulate maturation of ovarian follicles to a preovulatory stage in hypophysectomized rats, LH was required to promote follicle ovulation.[2] These results form the basis for all of the subsequent studies reviewed in this chapter that now define the mechanisms by which FSH promotes follicular maturation and LH stimulates ovulation and oocyte maturation.

Early advances in our understanding of gonadotropin receptor localization and actions were made possible by the development in the 1960s by Hunter and Greenwood of a technique to iodinate hormones.[3] The development of conditional gene knockout technology in mice using Cre-Lox-mediated recombination in the late 1980s allowed investigators to establish the requirement for, and in some cases the function of, hormones, growth factors, receptors, and signaling molecules.[4] Mapping of intracellular signaling pathways regulated by the gonadotropic hormones and growth factors occurred with the development of commercially available antibodies specific to phosphorylated proteins in the 1990s.

In this chapter we review current knowledge about the signaling pathways by which FSH promotes follicle maturation and LH promotes ovulation and corpus luteum formation. We refer the reader to excellent reviews of the FSH[5–7] and LH receptors.[8–10] We focus on FSH and LH

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signaling in granulosa cells of immature preantral follicles and mature preovulatory follicles and in theca cells primarily of the rodent, although examples from other species are also discussed. We do not consider LH signaling pathways and the regulation of LH target gene expression in the corpus luteum; readers are referred to Chapter 23.

FSH SIGNALS PROMOTE FOLLICULAR MATURATION

Follicle Maturation

Initial formation and early growth of ovarian follicles occur in a gonadotropin-independent fashion and rely on a number of characterized locally acting regulatory proteins that signal between the oocyte and the somatic cells of the follicle.[11–14] Once the follicle acquires functional FSH receptors, it can respond to this pituitary gonadotropin to undergo further maturation in preparation for ovulation. Only a small subset of ovarian follicles survives to full maturity, with most being lost along the way to a programmed cell death process termed atresia.[15,16] FSH-stimulated maturation of ovarian follicles to a preovulatory phenotype encompasses not only an explosive increase in the proliferation of granulosa cells contained within the follicle, resulting in rapid follicle growth, but also granulosa cell differentiation. As a consequence, the follicle develops a fluid-filled antrum, although markers for antrum formation are not well established.

Recent microarray results and the Ovarian Kaleidoscope Database reveal that FSH regulates the expression of ~500 target genes[17,18] in granulosa cells that support follicle maturation. Well-known responses that are often used as markers for FSH-dependent granulosa cell differentiation include increased estrogen production resulting largely from expression of the rate-limiting enzyme in estrogen biosynthesis that converts testosterone to estrogen, P-450 aromatase (aromatase); increased progesterone production as a result of increased expression of the rate-limiting enzyme in progesterone production that converts cholesterol to pregnenolone, P-450 cholesterol side chain cleavage (SCC); increased expression of 3β-hydroxysteroid dehydrogenase (3β-HSD), which converts pregnenolone to progesterone; increased expression of membrane receptors for LH and epidermal growth factor (EGF), both of which are required for the ovulatory response; and expression of the α subunit of the hormone inhibin, which feeds back to the anterior pituitary to repress production of FSH.[19–23]

FSH also stimulates the transcription of genes that encode for intracellular signaling molecules, such as the regulatory (R) IIβ subunit of protein kinase A (PKA),[24] the A-kinase anchoring protein (AKAP) microtubule-associated protein 2D (MAP2D),[25] and serum glucocorticoid kinase (SGK).[26] FSH has also been shown to induce expression of the EGF receptor agonist epiregulin[27,28]; the transcription factors early growth response factor-1 (EGR-1),[29] gonadotropin inducible ovarian transcription factor-1 (GIOT-1),[30] and liver receptor homolog (LRH)-1[31]; the follicular-fluid associated protein pregnancy-associated plasma protein-A,[32] which is recognized to be the insulin-like growth factor (IGF) binding protein-4 protease[33]; phosphodiesterases (PDE) 4D1 and 4D2[34] to degrade cAMP; and vascular endothelial growth factor (VEGF), which is reported to be critical for antrum formation, granulosa cell proliferation, and estrogen production, and for increasing the vascularity of the theca cell layer.[35,36] FSH also inhibits the expression of calmodulin kinase IV (CaM kinase IV)[37] and 3,5,3′-triiodothyronine binding protein mRNA.[38]

FSH enhances expression at the protein level of immediate early genes such as JUNB, c-JUN, c-FOS, FRA,[39] and c-MYC,[40,41] although these increases could result in part from posttranslational modifications to enhance protein stability[42] rather than strictly transcriptional regulation. FSH also promotes increased expression of a number of genes involved in reorganization of the microtubule and actin cytoskeleton, such as β-tubulin, the heavy chain of kinesin, and tropomyosin-4.[36,43]

The predominant marker for granulosa cell proliferation is increased expression of cyclin D2.[44,45] Cyclin D2-deficient mice are infertile as a result of the inability of granulosa cells to proliferate in response to FSH.[44] It is interesting, however, that granulosa cells of cyclin D2–null mice differentiate to a preovulatory phenotype, and upon stimulation with an ovulatory concentration of LH, follicles do not ovulate but granulosa cells luteinize in follicles with entrapped ova.[44] While FSH is sufficient to induce certainly the majority of granulosa cell differentiation markers in serum-free primary granulosa cell cultures, expression of cyclin D2 requires not only FSH but also activin.[46–49] These results suggest that in the intact animal, granulosa cell proliferation requires both FSH and paracrine actions of activin.

Taken together these results indicate that the response of granulosa cells to FSH involves a complex and coordinated program of gene expression that comprises the regulation of hundreds of genes.[17,18] The stimulus to promote granulosa cell differentiation requires relatively low but constant levels of FSH; withdrawal of FSH or its mediator cAMP during the 48-h time course of granulosa cell differentiation (of the 4-day rodent estrous cycle) results in incomplete induction of differentiation markers.[50,51]

FSH and the FSH Receptor

FSH is a glycoprotein hormone consisting of two distinct subunits, an α subunit shared with the related

glycoprotein hormones LH, human chorionic gonadotropin (hCG), and thyroid-stimulating hormone, and a unique β subunit (Figure 20.1). FSH is produced in gonadotrope cells of the anterior pituitary, and its synthesis and secretion are highly regulated by neural, pituitary, and gonadal factors.[53,54] The FSH receptor is a seven membrane-spanning G protein-coupled receptor (GPCR) that belongs to the rhodopsin/β-adrenergic family of GPCRs.[55] GPCRs are characterized by the presence of intracellular and extracellular loops linked by transmembrane (TM) α-helices and by their ability to couple to one or more guanine (G) nucleotide binding proteins. The FSH receptor belongs to the subgroup of class A GPCRs that contains Leu-rich-repeat motifs in their extracellular domain as well as the AsnSerxxAsnProxx-Tyr motif in TM 7 and the DRY motif at the border of TM 3 and inner loop 2.[56] The extracellular domain contains at least eight Leu-rich-repeat motifs of approximately 24 residues[6] that are believed to provide structural integrity to the receptor.[5] FSH binds to the large extracellular N-terminal domain of the receptor,[57] although one report stated that the interior of the receptor is also important for both hormone binding and signal generation.[58] Upon binding of FSH to this receptor, the receptor becomes activated. Activation of GPCRs is believed to represent a conformational change that alters the orientation of the TM domains.[59] Circular dichroism studies detected a difference in the secondary structure of the receptor when FSH was bound compared with the unbound state, consistent with the generation of an altered conformation upon hormone binding.[60] Binding of a partially deglycosylated human FSH to the extracellular domain of the FSH receptor showed that specificity is mediated by both the FSH α and β subunits and that upon hormone binding, FSH undergoes a conformational change to a more rigid structure.[61] While these investigators provided structural evidence for the existence of FSH receptor dimerization,[61] a recent report observed a trimeric association between FSH and the extracellular domain of the receptor.[62] These investigators additionally suggest that FSH first binds to a high-affinity subdomain on the receptor, which results in the formation of a sulfotyrosine binding pocket on FSH, with sulfated Tyr335 of the receptor then being inserted into the FSH pocket, leading to receptor activation.[62]

The predominant intracellular signal generated upon FSH receptor activation is cAMP, consistent with receptor coupling primarily to the stimulatory G protein (Gs).[6] Although there are reports that the FSH receptor can also couple to a pertussis toxin-sensitive Gi protein to modulate cAMP production in a Chinese hamster ovarian cell line,[63] and to activate phospholipase C (PLC) upon receptor overexpression in human embryonic kidney (HEK) cells,[64,65] the physiological relevance of these results has not been established.

The FSH GPCR is encoded by a single, rather large gene that consists of 10 exons and 9 introns.[5,6,66] The extracellular domain is encoded by the first 9 exons; the C-terminal part of the extracellular domain, the transmembrane domains, and the intracellular C-terminal domains are encoded by exon 10.[66] This receptor is expressed in females only on follicular granulosa cells[6]; expression is absent from ovaries of postmenopausal women.[6] FSH receptor-null mice are infertile, and follicles do not develop beyond the preantral stage,[67,68] consistent with the view that this receptor is necessary

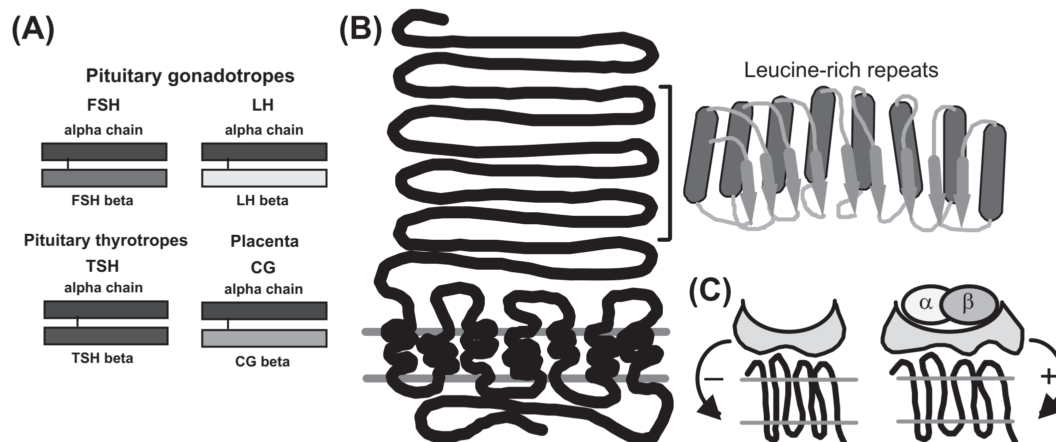

FIGURE 20.1 **The gonadotropins and gonadotropin receptors.** (A) A schematic of the family of dimeric glycoproteins hormones, which includes the pituitary gonadotropins FSH and LH, pituitary thyroid-stimulating hormone, and, in some species, placental chorionic gonadotropin. The α subunit is shared by all four hormones. (B) Generic schematic of the glycoprotein hormone receptors, indicating the large extracellular domain and the seven membrane-spanning domains characteristic of GPCRs. A significant portion of the extracellular domain is composed of leucine-rich repeats, each composed of a β-strand followed by an α-helix, and these are proposed to form a horseshoe-shaped domain as shown that likely plays an important role in ligand binding. (C) A model showing the view that the extracellular domain of the receptor plays a repressive role in the absence of ligand binding and that ligand interaction and ensuing conformational changes relieve this repression, allowing G protein activation. *Source: Modeled after Ref. 52.*

and sufficient to mediate the effects of FSH. FSH receptor expression is not detected in primordial follicles but has been detected on granulosa cells of preantral follicles from postnatal day 3 in rats; therefore, it is expressed at the very early stages of follicular development.[69–71] FSH receptor expression is increased by FSH or cAMP,[72,73] activin,[72–74] transforming-growth factorβ (TGFβ),[70] nerve growth factor,[75] and IGF-1,[72,76] and is abolished by the preovulatory LH surge that promotes ovulation.[69] Freshly harvested granulosa cells from preantral follicles of rats contain approximately 4500 receptors per cell.[77] Although the promoter of the FSH receptor gene contains an E-box, which is conserved among species and binds basic helix-loop-helix transcription factors such as upstream stimulatory factors 1 and 2, c-Myc and its dimerization partners Max or Mad, and hypoxia-inducible factor 1 (HIF-1),[78,79] and activation of the FSH receptor promoter requires steroidogenic factor (SF)-1[78,80] as well as binding of upstream stimulatory factors to the E-box, the mechanism for selective expression of the FSH receptor only in granulosa cells of female mammals has eluded identification. Exhaustive studies by Heckert and colleagues[7,79] revealed that elements required for cell-selective expression of the receptor are not present within the −5000 to +123 gene region surrounding the transcription start site.

Several naturally occurring inactivating mutations of the FSH receptor gene in humans have been described that result in arrested follicular development and hypergonadotropism. These include the following amino acid changes: Ile143Thr, Ala172Val, and Asp207Val in the extracellular domain, Lys584Val in the extracellular loop 3, and Arg556Cys in cytoplasmic loop 35. These mutations reduce or block FSH-stimulated cAMP generation, either as a result of poor hormone binding to the receptor and/or poor coupling to Gs. Directed point mutations in cytoplasmic loop 2 (Arg450His, Thr453Ala) either block or enhance receptor activation (Lys260Asp), consistent with the notion that this loop acts as a conformational switch.[81] A potential binding motif in cytoplasmic loop 3 for coupling to Gs (BXXBB, where B and X represent basic and nonbasic amino acids, respectively) has been identified.[81] A small molecule “negative allosteric modulator” that binds to the FSH-bound receptor in HEK cells overexpressing the FSH receptor and inhibits cAMP formation ($IC_{50} = 0.7 \pm 0.2\,\mu M$) by apparently stabilizing an inactive conformation of the receptor was recently reported, although the binding site for this compound was not identified.[82]

The only naturally occurring activating mutation identified for the human FSH receptor is Asp550Gly, located in cytoplasmic loop 3.[83] The FSH receptor with this mutation exhibits a modest increase in basal cAMP levels upon transfection into heterologous cells.[83] While the patient with this mutation exhibited normal testis function despite being hypophysectomized (because of a pituitary tumor), he received testosterone replacement therapy that might have contributed to the maintenance of spermatogenesis.[6,83] Directed mutation of Asp580His, a hot spot for activating mutations in other glycoprotein hormone receptors, results in enhanced granulosa cell proliferation, estrogen production, loss of small follicles, and development of hemorrhagic cysts in a mouse model.[84] It is surprising that more activating mutations of this receptor have not been identified.

Like most other GPCRs, the FSH receptor appears to exhibit desensitization or reduced FSH-stimulated cAMP production[65,85] upon exposure to saturating agonist, although granulosa cells likely never see saturating concentrations of FSH under physiological conditions. The mechanism of GPCR desensitization has been particularly well studied for the β-adrenergic receptor. Desensitization of the β-adrenergic receptor occurs rapidly after receptor activation and is characterized by both a rightward shift in the agonist dose–response curve and a decrease in maximal stimulation of adenylyl cyclase.[86] β-adrenergic receptor desensitization requires the phosphorylation of the activated receptor by a G protein-regulated kinase[87] and the consequent high affinity binding of arrestin2 (β-arrestin1) or arrestin3 (β-arrestin2) to the phosphorylated receptor.[88,89] Receptor phosphorylation functions to increase the affinity of the receptor for the arrestin.[88,90,91] Arrestins also play an integral role in receptor internalization based on their ability to bind both clathrin and β_2-adaptin of the AP-2 protein complex.[92–94] Thus, GPCR internalization often is linked to and follows receptor desensitization. Arrestins have also been shown in some cellular contexts to function as adaptors to bind signaling intermediates, such as upstream components in the mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (ERK) and c-Jun NH2-terminal kinase (JNK) pathways, to redirect receptor signals to other pathways.[95]

The FSH receptor, upon overexpression (by approximately 10-fold) in a heterologous cell model, becomes phosphorylated in response to FSH on Ser/Thr residues in intracellular loops 1 and 3[96] in a G-protein-regulated kinase 2-dependent manner.[97,98] Mutation of the phosphorylatable residues in intracellular loop 1 leads to reduced receptor internalization.[96] Most of the internalized ligand-bound receptor recycles back to the cell surface, and intact hormone dissociates.[99] FSH also promotes the phosphorylation of a cluster of five Ser/Thr residues in the C-terminal tail of the receptor expressed in heterologous cells that stabilizes the binding of β-arrestin and is required for receptor desensitization but not for receptor internalization.[80] However, neither the rate nor the extent of FSH receptor internalization or FSH receptor desensitization has been studied under physiological conditions in granulosa cells.

There is also evidence that upon overexpression in a heterologous cell model, the FSH receptor not only binds β-arrestin[80] but also binds APPL1 (adaptor protein containing PH domain, PTB domain, and leucine zipper motif).[100] While these authors identified the binding site of APPL1 within intracellular loop 1 of the receptor (Lys376),[64] the physiological significance of this interaction is difficult to establish since the APPL1-null mouse is fertile.[101]

There is a report of an alternatively spliced FSH receptor that contains the first eight exons of the classic FSH GPCR plus a unique C-terminal extension that is hypothesized to traverse the plasma membrane a single time.[102] This receptor, upon expression in HEK cells, localizes to the plasma membrane and binds FSH in a specific and high-affinity manner but does not appear to couple to Gs to activate adenylyl cyclse.[102,103] This alternative FSH receptor is reported to be expressed in mouse ovaries[104] and to activate the ERK pathway in immortalized granulosa cells.[105] The physiological significance of this alternative FSH receptor to granulosa cell function remains to be elucidated.

FSH SIGNALING PATHWAYS THAT STIMULATE GRANULOSA CELL DIFFERENTIATION AND PROLIFERATION: cAMP AND PKA-DEPENDENT SIGNALING

The predominant intracellular signal generated by FSH-dependent signaling through its GPCR is cAMP (Figure 20.2), based on the ability of forskolin to mimic FSH's ability to induce such differentiation markers as progesterone synthesis and LH receptors[50,51,106,107] (reviewed in Ref. 19). cAMP signals predominately by activating the cAMP-dependent protein kinase (PKA).[108] PKA is a tetrameric holoenzyme that consists of two regulatory (R) subunits and two catalytic (C) subunits.

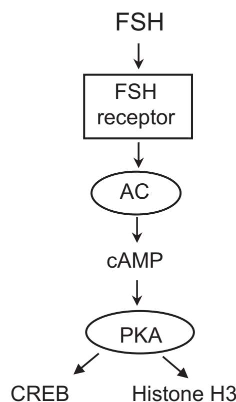

FIGURE 20.2 FSH signals via cAMP to activate PKA. Two PKA substrates in immature granulosa cells are CREB and histone H3.

PKA is activated upon binding of cAMP to the R subunits, resulting in the dissociation of the C subunits that function as active kinases to phosphorylate substrates.[109] Although representatives of an alternative family of cAMP effectors, consisting of cAMP-activated guanine nucleotide exchange factors also known as exchange proteins activated by cAMP (EPACs),[110,111] have been identified in rodent and human granulosa cells,[112] FSH either does not[113] activate or marginally (40%)[114] activates the EPAC target RAP1 in granulosa cells. Moreover, an EPAC-selective cAMP analog does not promote induction of aromatase,[115] a well-known marker of granulosa cell differentiation, or activation of the phosphatidylinositol-3 kinase (PI-3K) pathway.[116] Rather, in granulosa cells FSH activates PKA downstream of cAMP.[117] The cell-permeable selective PKA C subunit inhibitor peptide myristoylated-(Myr-) PKI, the active portion of the ubiquitous PKA inhibitor protein, inhibits the induction of a number of proteins induced by FSH, including MAP2D, inhibin-α, and progesterone producing enzymes.[113,118] PKI functions by binding to the substrate binding site of the C subunits of PKA, thus preventing other PKA substrates from binding, and preventing PKA from phosphorylating substrates.[119] While competitive ATP antagonists that are established inhibitors of PKA, such as H89 and KT5720,[120,121] also inhibit many of these responses initiated by FSH, including the induction of progesterone synthesis, aromatase, LH receptor, RIIβ, EGR-1, SGK, and MAP2D,[29,113,122] these inhibitors also inhibit other kinases, including p70 ribosomal S6 kinase (p70 S6 kinase) and RHO kinase (H89)[123] and phosphoinositide-dependent kinase 1 (PDK-1; KT5720).[124] Taken together, however, these results suggest that PKA is necessary for FSH-stimulated granulosa cell differentiation.

Two classes of PKA holoenzymes, PKA I and PKA II, exist based on the association of two possible RI subunits (RIα and RIβ) or two possible RII subunits (RIIα and RIIβ) with four possible C subunits (α, β1, β2, and γ).[109] PKA phosphorylates a large number of substrates in various cells.[125] Well-documented PKA substrates include CREB (cAMP response element binding protein),[126] PDE4D3,[127] SRC tyrosine kinase,[128] histone H3,[129] glycogen synthase kinase (GSK) α and β,[130,131] and L-type Ca^{2+} channels.[132]

But is PKA sufficient to promote granulosa cell differentiation? This remains a controversial topic that will be addressed throughout this review. The question of whether PKA is sufficient to drive granulosa cell proliferation has been addressed directly over the past 10 years by the Zeleznik group. Increasing intracellular levels of cAMP in preantral granulosa cells to levels equal to those stimulated by FSH either by transducing cells with adenovirally expressed constitutively active Gs[115] or constitutively active LH receptor[133] mimicked the ability of FSH to activate some (*Hsd3b2*, *Inha*) but not other

(*Lhcgr*, *Cyp19a1*) target genes to levels achieved by FSH 48h posttreatment. However, cotransduction with constitutively active Gs and AKT (protein kinase B) rescued gene expression to levels equal to or greater than FSH,[115] suggesting that signaling via cAMP and AKT pathways is sufficient to activate FSH target genes. Correspondingly, while reducing FSH-stimulated cAMP levels ~ three-fold by transducing granulosa cells with adenovirally expressed constitutively active Gq greatly reduced the ability of FSH to activate key FSH target genes (*Lhcgr*, *Cyp19a1*, *Hsd3b2*, *Cyp11a1*), responses were rescued by the cell permeable cAMP analogue 8-bromo-cAMP.[134] Most compelling are results from a study in which granulosa cells were transduced with lentivirally expressed constitutively active PKA C subunit and compared to cells stimulated with FSH.[17] Microarray analysis of genes regulated 48h posttreatment showed that the majority of genes were activated to the same extent by FSH and constitutively active PKA, although there were subsets that differed. The two caveats of this approach, as pointed out by the author, are: (1) constitutively active PKA cannot bind to AKAPs, thereby obscuring intracellular compartmentation, and (2) by 48h, some FSH targets (*Lhcgr*, *Cyp19a1*) may begin to undergo downregulation in response to the potential of the constitutively active PKA C subunit to mimic the surge of LH. We conclude with our present state of knowledge that PKA appears to be sufficient to mediate granulosa cell differentiation, at least qualitatively. However, we expect that additional pathways activated by FSH contribute to optimal target gene regulation to yield a preovulatory follicle.

While most PKA in ovaries with preantral follicles consists of a PKA IIβ holoenzyme,[135] based on DEAE-cellulose chromatography, RIIβ knockout mice are fertile.[136] PKA Iα holoenzyme comprises less than 5% of the PKA activity detected in preantral follicle-dominated ovaries, although there is a large amount of C subunit–free RI.[135] The presence of substantial C subunit–free RI is surprising because RIα not bound to C is reported to be rapidly degraded[137] and may suggest the presence of activated PKA Iα, since free C subunit does not bind to DEAE-cellulose. Rodent ovaries do not express RIβ.[138] Taken together, these results suggest that the actions of FSH could be mediated by PKA Iα rather than by the seemingly more abundant PKA IIβ holoenzyme. Because RIα-null mice died at embryonic day 10.5,[139] proof that RIα is crucial to granulosa cell differentiation requires the generation of mice with an RIα deletion targeted to granulosa cells.

AKAPs Target PKA to Specific Subcellular Locations

The specificity of PKA action is accomplished by the targeting of PKA as well as its substrates to specific cellular locales by virtue of the binding of PKA regulatory subunits to a large family of AKAPs. It has been estimated that more that 75% of PKA holoenzymes are targeted to specific intracellular sites via association of PKA regulatory subunits with AKAPs.[109] RII subunits bind with nM affinity to AKAPs.[109,140] The domain on the AKAP responsible for RII binding comprises an amphipathic helix that binds the N-termini of the PKA-RII dimer.[141] AKAPs direct PKA to such locations as the mitochondria, Golgi apparatus, centrosome, nuclear envelope, and actin and microtubule cytoskeletons by the presence of specific subcellular targeting domains on each AKAP,[142–144] as depicted in Figure 20.3. Localization of PKA and one or more substrates to distinct regions within the cell is thought to promote both specific and efficient substrate phosphorylation in response to a stimulus.[144] AKAPs not only bind PKA but also function as platforms to coordinate signaling cascades by binding additional signaling proteins such as CaM, PDEs, protein phosphatases such as PP1 and PP2A, and protein kinases such as the tyrosine kinase ABL and the Ser/Thr protein kinase C (PKC) enzymes.[143,144] By confining PKA and specific substrates to isolated cAMP gradients at discrete cellular locations, not only are signaling cascades optimized but also their fidelity is maintained, thereby preventing inappropriate cross talk among pathways.[143]

Most known AKAPs anchor PKA II holoenzymes and exhibit at least a 100-fold lower affinity for PKA I holoenzymes.[143] Although there are a growing number of "dual specificity" AKAPs that readily bind PKA I holoenzymes, these AKAPs still exhibit a 10- to 25-fold preference for RII.[145] However, fibrous sheath protein 1,[146] the *Caenorhabditis elegans* $AKAP_{CE}$,[147] the peripheral benzodiazepine receptor-associated protein PAP7,[148] the neurofibromatosis 2 tumor-suppresser protein merlin,[149] sphingosine kinase interacting protein,[150] and GRB2

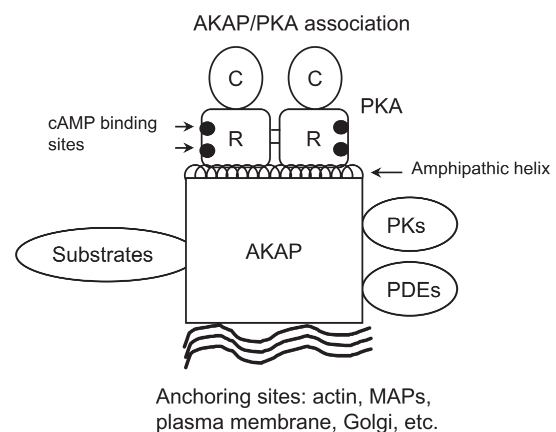

FIGURE 20.3 Schematic model of the association of AKAPs with PKA, other protein kinases (PKs), PDEs, substrates, and anchoring sites in cells.

(growth factor receptor binding protein 2) associated binding protein 2 (GAB2)[116] appear to preferentially bind RI and thus are PKA I AKAPs.

Preantral follicle-dominated ovaries express a large number of AKAPs, including AKAP-KL, AKAP79, Ezrin, AKAP-149, AKAP95, AKAP220, and AKAP100, based on RII overlay and western blotting results.[135] However, neither the selective link of each AKAP to individual PKA substrates and signaling pathways nor the cellular location of these AKAPs has been investigated. Thus, the cellular mechanism(s) by which each of the AKAPs present in granulosa cells modulates cellular functions is not known. We recently identified GAB2 as an RI AKAP and a direct PKA substrate in preantral granulosa cells that appears to orchestrate signaling of PKA into the PI-3K pathway, as discussed below, although formal proof of this link is lacking.[116]

PKA Targets in Undifferentiated Granulosa Cells: CREB and Histones H3 and H1

Defining the signaling pathways that are activated by FSH in granulosa cells and determining how PKA coordinates signaling into these pathways remains a central challenge in ovarian biology. Although details of the signaling pathways activated by FSH are just beginning to be elucidated, it is clear that FSH stimulates the PKA-dependent phosphorylation of the established PKA substrates CREB on Ser133[151,152] and histone H3 on Ser10.[117,118] Both FSH-stimulated CREB and histone H3 phosphorylations in granulosa cells are abrogated by the PKA inhibitor Myr-PKI, suggesting that PKA is the predominant kinase that phosphorylates these proteins (Figure 20.2). CREB and histone H3 are direct PKA substrates.[118,153]

It is interesting that in other cellular models, both CREB and histone H3 are phosphorylated on the same sites by protein kinases other than PKA. For example, CREB kinases in other cells include the PI-3K substrate AKT,[154] CaM kinases, the ERK substrates p90 ribosomal S6 protein kinases (RSK) and the mitogen- and stress-activated protein kinases 1 and 2 (MSK1 and -2), and the p38 MAPK substrates MAPK-activated protein kinases 2 and 3 (MK-2 and -3)[155,156]; however, inhibitors of these kinases do not reduce FSH-stimulated CREB phosphorylation in granulosa cells.[113,118] Similarly, in other cells histone H3 is commonly phosphorylated by the ERK substrate RSK2 and by the ERK and p38 MAPK substrate MSK-1.[157–159] However, inhibitors of ERK activation and of activated p38 MAPK do not reduce histone H3 phosphorylation in granulosa cells.[118] These data support the notion that PKA plays a unique role in granulosa cells to regulate signaling pathways.

FSH enhances the phosphorylation of histone H3 on Ser10 as well as the acetylation of Lys14, although it is not clear whether these modifications occur simultaneously or in a sequential manner.[118] The histone code hypothesis put forth by Strahl and Allis[160] stated that covalent modifications of core histone tails result in the remodeling of chromatin to affect downstream events. The best-known core histone modifications consist of the covalent addition of an acetyl group to Lys, the addition of one or more methyl groups to Lys or Arg, and addition of a phospho group to Ser or Thr. These additions occur primarily on the N-terminal tails of histones H3 and H4. Acetylation neutralizes the positive charge of the histone and phosphorylation adds a negative charge, thereby decreasing the affinity of histone for DNA.[161] The predicted result of histone acetylation and/or phosphorylation is the loosening of chromatin structure, resulting in increased access of select promoter regions to transcription factors and co-activators.[160,162] Methylation can correlate either with gene activation or gene inhibition and can occur on the same Lys residues that are acetylated, and Lys residues can be mono-, di-, or trimethylated, whereas Arg residues can be mono- or dimethylated. Arg methylation of histone H3 generally correlates with gene activation.[163] For histone H3, methylation of Lys4 correlates with gene activation, whereas methylation of Lys9 and Lys27 correlates with gene repression.[164,165] Histone acetylation is catalyzed by histone acetyltransferases, such as CREB binding protein (CBP), histone deacetylation is catalyzed by histone deacetylases, and histone methylation is catalyzed by methyltransferases.

Consistent with the histone code hypothesis, increased *c-Fos*, *Sgk*, and *Inha* promoter DNA is detected in the dual-phosphorylated and -acetylated histone H3-chromatin pools in response to FSH treatment of granulosa cells in chromatin immunoprecipitation (CHIP) assays.[118] Because the *c-Fos* promoter contains a functional cAMP response element,[166] signaling to activate *c-Fos* in granulosa cells probably reflects contributions from CREB. In this instance, the C subunit of PKA would function not only to phosphorylate CREB to promote binding of CBP[167,168] but also to phosphorylate histone H3, whereas CBP perhaps promotes the acetylation of histone H3 as well as recruitment of other co-activators and the basal transcription machinery.

Activation of the *Sgk* gene in response to FSH/PKA requires an Sp1 (specificity protein 1) binding site.[26] Because the phosphorylation and acetylation of histone H3 are linked to the rapid activation of the *Sgk* gene, perhaps co-activators with histone acetyltransferases activity as well as the C subunit of PKA complex with Sp1 at the *Sgk* promoter. It is possible that the stimulatory effect of PKA on *Sgk* transcription reflects H3 phosphorylation and chromatin reorganization in addition to direct phosphorylation of transcription factors. The mechanism by which PKA enhances the transcriptional activity of Sp1 is not clear, but as discussed later in "Cross Talk among FSH-Regulated Signaling Pathways and Transcriptional

Activators to Regulate Gene Expression," both ERK and the AKT target PKCζ are candidate kinases.[169] Histone H3 phosphorylation and acetylation is also linked to activation of *Inha* subunit gene expression. Activation of the *Inha* promoter requires synergism among PKA-phosphorylated CREB, SF-1, or LRH-1, and the co-activators CBP and the *Ncoa1* product steroid receptor co-activator-1 (SRC-1).[170] SF-1 and CREB constitutively bind to and interact (directly or indirectly) on the *Inha* promoter,[170] and CBP is recruited by and binds to phosphorylated CREB[167,168] and to SF-1.[171] The dependence on PKA for activation of the *Inha* gene has historically been assumed to be a consequence of the phosphorylation of CREB,[153] leading to CBP recruitment.[168,170] However, it is likely that a member of the cAMP-regulated transcriptional co-activator (CRTC) family also contributes to the ability of CREB to activate the *Inha* gene.[172] In response to elevated intracellular levels of cAMP, CRTC becomes dephosphorylated and translocates from the cytoplasm to the nucleus, where it binds to phosphorylated CREB and enhances its association with DNA.[172,173] It is also well established that PKA potentiates SF-1 transactivation activity. Although SF-1 can be phosphorylated by PKA in vitro, mutation of its consensus PKA phosphorylation site (Ser430Ala) does not affect the stimulatory effects of PKA on SF-1 transactivational activity.[174] It is now recognized that the transcriptional activity of SF-1 requires the co-activator β-catenin.[175,176] We have recently shown that FSH promotes the PKA-dependent phosphorylation of β-catenin on Ser552 and Ser675,[177] consistent with reports in other tissues,[178,179] and consequent activation of the β-catenin/T cell factor (TCF) artificial promoter-luciferase construct, TOPFlash,[177] as discussed below.

It is likely that the rapid phosphorylation of histone H3 on Ser10 by PKA and acetylation on Lys14 by CBP or other histone acetyltransferases constitute a necessary step in the transcriptional activation of many FSH responsive genes, leading to granulosa cell differentiation. FSH is also predicted to promote alterations in the methylation patterns of histone H3, although this H3 modification in granulosa cells preantral granulosa cells has not yet been reported. It is possible that histone H3, in its phosphorylated, acetylated, and possibly methylated conformation, functions as a scaffold to mediate the assembly of the multiprotein complex of transcription factors, co-activators, and basal transcription factors, which leads to transcription.[160] Based on results in a HEK cell line,[170] it is also expected that histone H4 is covalently modified in granulosa cells in response to FSH. Using the CHIP assay with an antibody to acetylated histone H4, it was shown that PKA increased histone H4 acetylation associated with the *Inha* promoter.[170] The increased histone acetyltransferase activity could result either from the recruitment of CBP[180,181] or the recruitment by CBP of a p300/CBP-associated factor PCAF.[182,183]

Histone H1 is also phosphorylated in granulosa cells in response to FSH, although with a slower time course than that of histone H3.[117] Histone H1 binds to the outer surface of the DNA that surrounds the core histones and to the stretches of linker DNA that connect nucleosomes.[184] Histone H1 is known to be an in vitro PKA substrate, with phosphorylation on Ser37,[129,185] and is phosphorylated on Ser37 in vivo in liver in response to glucagon treatment[129] and in response to forskolin addition to N18 neuroblastoma cells.[185] It is predicted that histone H1 phosphorylation contributes to chromatin remodeling, although less is known about the role of this histone in regulating transcriptional activation.

Because most of the known differentiation responses to FSH are mediated by PKA, it was initially assumed that the genes activated by FSH would all contain CREB-binding sites. However, cAMP response elements have been identified only in the promoters of a subset of FSH-regulated genes, including *Inha*,[186] *Cyp19a1*,[152] *Giot-1*,[30] *EGR-1*,[29] and *c-Fos*.[166] Therefore, FSH via PKA must phosphorylate additional targets to activate signaling pathways. Indeed, as discussed later, FSH also stimulates activation of the ERKs and PI-3K pathways in a PKA-dependent manner.

PKA Signals to Activate the ERKs in Undifferentiated Granulosa Cells

The ubiquitous ERKs, which belong to the MAPK family, are activated by a variety of receptor agonists. These kinases are classically activated by receptor tyrosine kinases such as the insulin, EGF, or IGF-1 receptors. Upon activation of these receptors, their consequent autophosphorylation creates specific binding sites for Src homology 2 (SH2) containing proteins such as GRB2.[187] GRB2, complexed with SOS, binds to the receptor tyrosine kinase, and the guanine nucleotide exchange factor SOS promotes activation of RAS by stimulating its GDP release. Active RAS then promotes activation of the Ser/Thr kinase RAF-1, which, in turn, phosphorylates/activates the ERK kinase MEK. MEK then phosphorylates ERK on Thr and Tyr residues, resulting in ERK activation. GPCRs activate ERKs generally by promoting the transactivation of a receptor tyrosine kinase,[188] resulting in its phosphorylation and consequent stimulation of the pathway leading to ERK activation, although the mechanisms of receptor tyrosine kinase transactivation vary among GPCRs and are incompletely understood.[189–191] While GPCR-generated cAMP is most commonly reported to inhibit ERK activation, especially in nonendocrine cells,[192] cAMP has been reported to activate ERK either via PKA by stimulating the phosphorylation of the small G protein RAP1, leading to the activation of B-RAF, MEK, and ERK,[193,194] or by directly binding to and activating the RAP1 guanine nucleotide exchange factor EPAC, leading to activation of B-RAF, MEK, and ERK.[110,111]

While FSH promotes the rapid albeit transient activation of ERK via a PKA-dependent pathway,[85,113,195] the mechanism by which PKA regulates ERK activity in preantral granulosa cells appears to depend in part on the extracellular matrix to which cells attach in vitro. When cells are attached to fibronectin, FSH does not promote the phosphorylation/activation of MEK; rather FSH regulates the association of a 100-kDa protein tyrosine phosphatase (PTP) with ERK in a PKA-dependent manner.[113] In the absence of FSH, a tonic stimulatory pathway promotes activation of the components of the ERK pathway upstream of ERK, including MEK; however, ERK activity is blunted by its association with a PTP that inactivates ERK (Figure 20.4). Consistent with this scheme, the MEK inhibitor PD98059 blocks FSH-stimulated ERK phosphorylation.[113] In the presence of FSH, PKA catalyzes the phosphorylation of the 100-kDa PTP that is complexed with ERK, resulting in dissociation of the phosphatase from ERK.[113] In the absence of the associated PTP, ERK is relieved from inhibition and is activated by the tonic stimulatory pathway.[113] This granulosa cell PTP is recognized by an antibody that recognizes PTP-SL, but based on its larger size and lack of recognition by other PTP-SL antibodies, the ERK-associated PTP in granulosa cells appears to be distinct from PTP-SL and has not been identified.[113]

Regulation of the tonic pathway that promotes activation of MEK in granulosa cells is poorly understood. Both extracellular Ca^{2+}, the EGF receptor, and the tyrosine kinase SRC (Rous sarcoma oncogene) appear to be necessary for FSH-stimulated ERK activation, based on the abilities of Ca^{2+} chelation with ethyleneglycotetraacetic acid, the EGF receptor inhibitor AG1478,[196] and the SRC inhibitor PP1 to abrogate this response.[113] Moreover, Ca^{2+} entry appears to be mediated by L-type Ca^{2+} channels, based on the ability of the L-type Ca^{2+} channel inhibitor nifedipine[113] to block FSH-stimulated ERK activation. Ca^{2+} entry also appears to be upstream of the EGF receptor and Src, because the EGF receptor inhibitor AG1478 and the SRC inhibitor PP1 block the ability of the Ca^{2+} ionophore A23187 to stimulate ERK phosphorylation[113] (Figure 20.4).

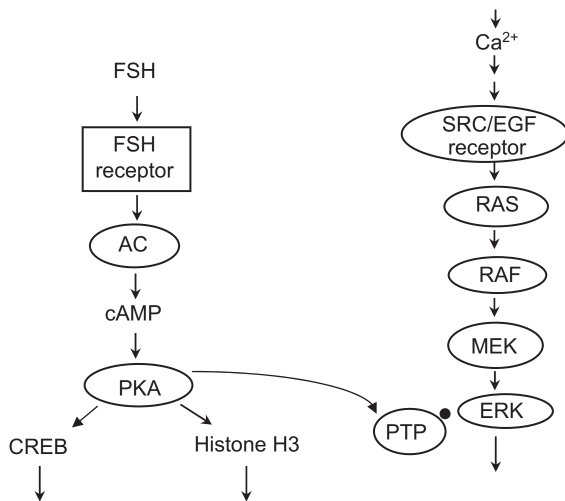

FIGURE 20.4 **FSH via cAMP/PKA activates ERK by stimulating the phosphorylation and consequent dissociation of an inhibitory protein tyrosine phosphatase (PTP).** A tonic pathway that includes Ca^{2+}, SRC, EGF receptor, RAS, and RAF promotes MEK phosphorylation.

Under similar experimental conditions, FSH-stimulated ERK activation requires PKA, SRC kinase activity, and the EGF receptor, although MEK phosphorylation in the absence of FSH (i.e., the tonic pathway) was not investigated.[185] In contrast, when cells are attached to serum-coated plates, FSH appears to signal via an EGF receptor and SRC-dependent route to activate RAS, leading to ERK activation.[107] There is also evidence that AMP-kinase (AMPK), which is active (phosphorylated on Thr172[197]) in the absence of FSH,[198] complexes with and inhibits ERK activity.[199] While FSH promotes the inactivation of AMPK,[199] the mechanism by which AMPK regulates ERK activity is not known.

Despite an incomplete understanding of the ERK activation pathway in granulosa cells, FSH-stimulated ERK appears to be necessary, but not sufficient, for induction of the MAP2D[113] and cyclin D2, based on protein/mRNA expression in the absence and presence of the MEK inhibitor, PD98059.[198,200] Similarly, this MEK inhibitor attenuates the FSH-dependent induction of the immediate early gene EGR-1 in granulosa cells,[29] suggesting that induction of EGR-1 by FSH requires MEK-dependent ERK activation.

FSH has also been shown to increase protein expression for a number of immediate early genes that comprise the AP-1 family, including JUNB, c-JUN, c-FOS, and FRA2,[39] as well as c-MYC.[40,201] JUNB has been shown in granulosa cells to regulate the expression of the *Inhb* promoter.[202] A recent report suggested ERK-dependent phosphorylation of these proteins stabilizes them by inhibiting their degradation; however, this response requires persistent ERK activation over a time course inconsistent with the expression of these immediate early genes.[42] Based on the transient activation of ERK in granulosa cells (which is generally undetectable by 1–2 h post FSH[113,195]), it is unlikely that the mechanism by which FSH increases the expression of these proteins rests with their ERK-dependent phosphorylation.

ERK can also phosphorylate or direct the phosphorylation of a number of transcription factors and co-activators that have been identified as participants in the induction of FSH target genes. Figure 20.5 reviews ERK targets that have been established in various cellular models.[156] For example, ERK is established to phosphorylate SF-1 on Ser203, resulting in recruitment of co-activators as well as driving the formation of a compact

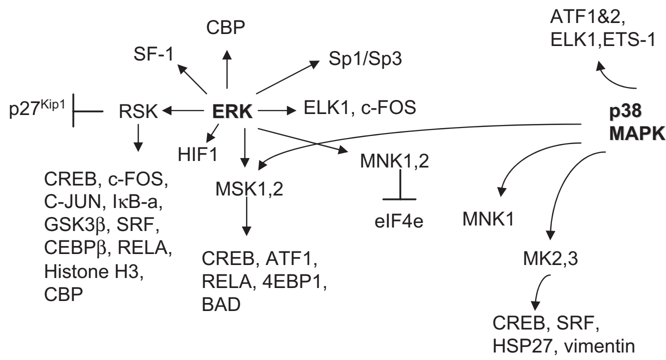

FIGURE 20.5 **Summary of identified ERK and p38 MAPK substrates.** Arrows indicate stimulation; perpendicular lines indicate inhibition of activity.

structure that results in formation of an active conformation.[203,204] ERK also phosphorylates Sp1/Sp3 on Thr266, thereby enhancing its DNA binding activity.[205] ERK is believed to phosphorylate CBP on Ser436,[206] and this phosphorylation has been suggested to be necessary for its recruitment of the AP-1 complex.[207] It is interesting that p300 lacks this phosphorylation site.[207] However, phosphorylation of CBP by ERK in vivo has not yet been reported, to our knowledge. ERK also phosphorylates a number of kinases, including RSKs 1–4, MSK1 and -2, and MAPK-interacting kinases (MNK1 and -2).[156] These kinases, in turn, phosphorylate a number of transcription factors, in a cell-specific manner, as reviewed in Figure 20.5. Only ERK-catalyzed RSK phosphorylation has been positively identified in granulosa cells treated with FSH,[118] although a number of additional ERK substrates are expected to be phosphorylated in granulosa cells in response to FSH. Although RSK can phosphorylate CREB and histone H3 in some cells, FSH-stimulated phosphorylation of these proteins is not inhibited by the MEK inhibitor PD98059,[118] indicating that neither CREB nor histone H3 is a RSK substrate in granulosa cells. Unphosphorylated RSK has been reported to sequester CBP and inhibit its histone acetyltransferase activity,[208,209] although this might be cell specific because RSK has also been reported to enhance co-activator activity of CBP.[210] An association between RSK and CBP has not been investigated in granulosa cells, but based on the number of transcription factors that require CBP in FSH-regulated signaling pathways in granulosa cells, including CREB,[167,211] HIF-1,[212] and FOXO1,[213] as described later, or that bind CBP, including SF-1[171] and SMAD,[214,215] regulation of the availability of CBP could be an important regulatory mechanism by which FSH enhances target gene expression. Thus, ERK signaling likely participates at least indirectly in the regulation of the expression of a number of FSH target genes by regulating the phosphorylation of a number of transcription factors and/or co-activators. The importance of the ERK pathway to follicle development is also evidenced by the expression of a constitutively active RAS (KRAS-Gly12Asp) in preantral granulosa cells that resulted in subfertile mice, reduced induction of *Lhcgr*, and development of abnormal follicles devoid of mitotic and apoptotic cells.[216]

Mammalian cells contain two additional MAPKs, the p38 MAPKs and the JNKs. Although activation of the JNKs by FSH to our knowledge has not been reported, the p38 MAPKs are activated by FSH in rat granulosa cells.[217–219] The effect of FSH is mimicked by forskolin[219] and appears to be PKA dependent, based on the ability of H89 to inhibit p38 MAPK phosphorylation,[219] although this result is controversial.[114,218] A downstream p38 MAPK target whose phosphorylation is increased by FSH is the small heat shock protein HSP27[219] (Figure 20.5). Although the significance of HSP27 phosphorylation in granulosa cells has not been delineated, upon phosphorylation HSP27 loses its ability to form oligomers and to block actin polymerization.[220] HSP27 is also recognized to inhibit apoptosis in various cellular models.[220] Rounding of granulosa cells, a characteristic response to FSH stimulation,[221] is inhibited by the p38 MAPK inhibitor SB203580, suggesting that the p38 MAPK pathway contributes to granulosa cell cytoskeletal changes.[222] Although FSH activates the upstream p38 MAPK kinases MKK3 and MKK6,[217] the cellular mechanism by which FSH, presumably via PKA, signals into this pathway is not known. Often, this pathway is activated by the small GTPases of the RHO family, leading to the activation of a number of possible kinases that direct activation of MKK3/6.[156] The potential ability of FSH to signal to a RHO family member has not been investigated to our knowledge.

The phosphorylation of a number of transcription factors in various cells is also regulated by p38 MAPK, as reviewed in Figure 20.5, and it promotes the phosphorylation of MNK1 (but not MNK2) and MK2 and -3.[156] HSP27 is actually phosphorylated by MK2 and -3 and not directly by p38 MAPK. CREB can also be a target of MK2 and -3 but is not likely to be phosphorylated by these kinases in granulosa cells in response to FSH, based on the inability of the p38 MAPK inhibitor SB203580 to affect FSH-stimulated CREB phosphorylation.[118] Other than HSP27, p38 MAPK targets in immature granulosa cells have not been identified.

FSH Increases Intracellular Ca^{2+} in Undifferentiated Granulosa Cells

FSH is reported to increase intracellular Ca^{2+} in a cAMP-dependent manner in porcine granulosa cells, and the Ca^{2+} appears to be derived largely from extracellular sources.[223–225] Ca^{2+} entry is inhibited with verapamil, an L-type Ca^{2+} channel blocker, suggesting that plasma membrane Ca^{2+} channels are opened by cAMP

or PKA.[225] Moreover, Ca^{2+} is required for FSH to activate the *Cyp11a1* promoter-reporter in porcine granulosa cells.[223] Although cAMP analogs that selectively activate PKA mimic the actions of FSH to raise intracellular Ca^{2+} levels in Sertoli cells,[226] equivalent studies have not been performed in granulosa cells. In contrast to reports on porcine granulosa cells, there are two reports that FSH does not mobilize intracellular Ca^{2+}, one in HEK cells heterologously expressing the human FSH receptor[227] and another in a rat ovarian granulosa cell line.[228] There is also a report that FSH increases intracellular Ca^{2+} in the human KGN cell line overexpressing FSH receptors.[64] In rat granulosa cells in culture, a Ca^{2+} signal is required for FSH-stimulated ERK activation, as discussed earlier.[113] Consistent with these data, Carnegie and Tsang[229] reported that ethyleneglycotetraacetic acid and the Ca^{2+} channel blocker verapamil inhibit FSH-stimulated progesterone production and the Ca^{2+} ionophore A23187 stimulates progesterone production in rat granulosa cells. Taken together, these results suggest that Ca^{2+}contributes to the actions of FSH. Whereas FSH appears to promote the entry of Ca^{2+} into porcine granulosa cells in a cAMP-dependent manner, the regulation of intracellular Ca^{2+} in rat granulosa cells is less clear.

Ca^{2+}-dependent downstream signaling pathways have also been studied in granulosa cells. CaM kinases II and IV are present in immature rat and porcine granulosa cells,[37,230] and FSH promotes a decline in CaM kinase IV expression to undetectable levels by 24 h in rat granulosa cells.[37] Mice deficient in CaM kinase IV exhibit impaired follicular development and ovulation.[37] Cotransfection of active CaM kinase IV with a *Cyp11a1* promoter-luciferase construct into porcine granulosa/luteal cells resulted in a significant increase in basal transcription of this gene that was blocked by a dominant negative CREB mutant.[230] Cotransfection with CaM kinase II was ineffective.[230] This result suggests that CREB is a possible CaM kinase IV target in porcine granulosa/luteal cells. Ser133 on CREB is a recognized CaM kinase IV target in neuronal cells.[231] Although these results suggest that CaM kinase IV plays a role in the basal transcription of *Cyp11a1* in mature granulosa cells, the role of CaM kinases in immature cells is less clear. An early report by Conti and collaborators[232] showed that FSH treatment of rat granulosa cells activated a cAMP-PDE that required Ca^{2+}/CaM. Additionally, FSH-stimulated progesterone production in rat granulosa cells is reported to be inhibited by the CaM inhibitor R24571,[229] although FSH-stimulated ERK activation (Hunzicker-Dunn, unpublished data) is not inhibited by the CaM kinase II inhibitor Kn62.[233] Thus, additional studies are required to clarify the role of Ca^{2+} and its regulated kinases in FSH actions.

PKA Signals to Activate the PI-3K Pathway in Immature Granulosa Cells

There is abundant evidence that FSH promotes the activation of PI-3K, leading to activation of AKT and downstream targets in granulosa cells that promote cell survival, translation, proliferation, and differentiation[113,115,218,234–236] (Figure 20.6). The PI-3K inhibitors wortmannin and/or LY294002 block FSH-stimulated induction of target gene products that characterize differentiated granulosa cells, including the LH receptor, MAP2D, VEGF, inhibin-α, and RIIβ.[235] In addition, constitutively active AKT enhances and dominant negative AKT blocks FSH-stimulated induction of the LH receptor, aromatase, inhibin-α, and/or 3β-HSD.[115,237] These data suggest that FSH-dependent activation of the PI-3K pathway is obligatory for the induction of critical genes that characterize the differentiated granulosa cell phenotype.

PI-3K. PI-3K consists of a 110-kDa catalytic subunit and an inhibitory 85-kDa regulatory subunit.[238] PI-3K is most commonly activated downstream of receptor tyrosine kinases such as the insulin receptor, IGF-1 receptor, and EGF receptor, which, upon ligand binding and

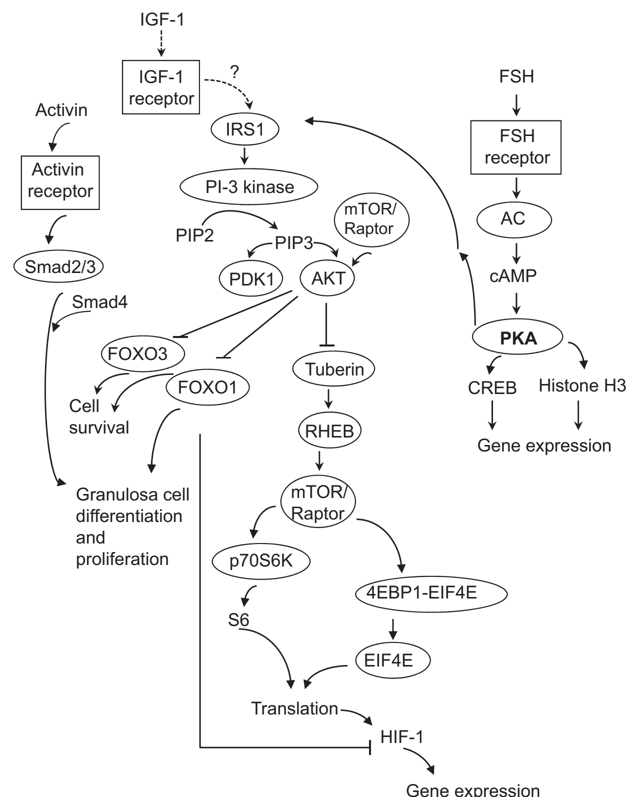

FIGURE 20.6 **FSH via cAMP/PKA signals to activate PI-3K, leading to mTOR1-stimulated translation and inhibition of FOXO1 and FOXO3 targets.** FSH and activin synergize to promote gene expression. Multiple arrows indicate more than one step in pathway. Potential contribution of IGF-1 and IGF-1 receptor is indicated by dashed lines.

dimerization, phosphorylate adaptor proteins (insulin receptor substrates 1/2 [IRS 1/2] or GAB 1/2) on specific Tyr residues. Activation of PI-3K catalytic subunit occurs on binding of both SH2 domains of the regulatory subunit to dual phosphorylated TyrXXMet (where X represents any amino acid, p-YXXM) motifs either on the adaptor protein IRS 1/2 or GAB 1/2.[238] While the catalytic subunit of PI-3K also contains a RAS binding domain, the contribution of active RAS to PI-3K activation under physiological conditions is not clear.[238] Activated PI-3K phosphorylates the 3′-OH position of the inositol ring of phosphatidylinositol 4,5-bis phosphate (PIP2), generating phosphatidylinositol 3,4,5-trisphosphate (PIP3). Increased concentrations of PIP3 at the plasma membrane create high affinity binding sites for PDK-1 and AKT through their pleckstrin homology domains, localizing both kinases in juxtaposition. PDK-1 then phosphorylates AKT on Thr308; AKT is subsequently phosphorylated on Ser473 by mTORC2 that consists of the mammalian target of rapamycin (mTOR)/Rictor complex, resulting in the full activation of AKT and its translocation to the nucleus and other cellular locations.[239] The mechanism by which GPCRs activate PI-3K is less-well understood and often involves direct activation of the 110-kDa catalytic subunit by Gβγ subunits.[238]

Consistent with the classic manner in which the PI-3K pathway is activated, in granulosa cells exogenous IGF-1 activates the PI-3K pathway via the IGF-1 receptor tyrosine kinase.[218,235,237,240] That IGF-1 is important for follicular function is evidenced by the fact that IGF-1-null mice are infertile; follicles of IGF-1-null mice do not progress beyond the preantral stage of development.[241] Although granulosa cells of preantral and antral follicles are the primary site of IGF-1 synthesis,[242] FSH does not promote IGF-1 expression by rat granulosa cells.[76,243] A recent report showed that granulosa cells express a basal level of IGF-1.[237] It is well established that FSH and IGF-1 strongly synergize to activate a number of FSH target genes, including those for *Cyp11a1*, *Cyp19a1*, and *Lhcgr*.[47,243–246] However, IGF-1 alone promotes only a minimal activation of these target genes,[47,243,247] indicating that additional FSH targets/responses are required to induce the full differentiation response.

The cellular mechanism by which FSH activates the PI-3K pathway in granulosa cells is beginning to be unraveled (Figure 20.6). FSH-stimulated AKT phosphorylation is detected within 5–10 min of FSH addition to rat granulosa cells.[116,235] Signaling by FSH to activate the PI-3K leading to the phosphorylation of AKT in granulosa cells is mimicked by cell-permeable cAMP analogs or the adenylyl cyclase activator forskolin[115,218,235] and is mediated by PKA.[116] PKA promotes the phosphorylation of IRS-1 on Tyr989, a canonical YXXM binding site for the regulatory subunit of PI-3K, resulting in phosphorylation of AKT on both Thr308 and Ser473.[116] These phosphorylations of IRS-1 and AKT are all abrogated by the specific PKA inhibitor PKI. The adaptor GAB2 appears to coordinate signaling of PKA to PI-3K, as depicted in Figure 20.7. GAB2 is present in granulosa cells in a preformed complex with IRS-1 and the PI-3K heterodimer. GAB2 is an RI-AKAP and a direct PKA target, and is rapidly phosphorylated on Ser159 in response to FSH. Overexpression of GAB2 enhances FSH-stimulated AKT phosphorylation.[116] However, there remain many unanswered questions, including the identity of the tyrosine kinase that phosphorylates IRS-1 on Tyr989, the mechanism by which PKA apparently activates this tyrosine kinase and/or inhibits a tyrosine phosphatase, and the mechanism by which GAB2 appears to coordinate this signaling cascade.

A recent report has shed new light on the contribution of IGF-1 to the FSH signaling pathway that leads to AKT phosphorylation. Granulosa cells, cultured in the presence of transferrin, selenium, insulin, amino acids, and a cholesterol source, express ~1 ng/ml IGF-1 under basal conditions.[237] FSH or dibutryl cAMP coupled with autocrine-generated IGF-1 signal via the IGF-1 receptor tyrosine kinase to activate AKT to induce *Cyp19a1*.[237] Addition of selective IGF-1 receptor antagonists or transduction of cells with lentivirus expressing short hairpin RNAs for IGF-1 or the IGF-1 receptor markedly reduced the ability of FSH to induce *Cyp19a1*. Also, AKT phosphorylation (Ser473) detected 1 h post FSH was abrogated by a selective IGF-1 receptor antagonist (NVP-AEW541[248]).[237] Together, these results suggest that in some manner FSH in combination with IGF-1

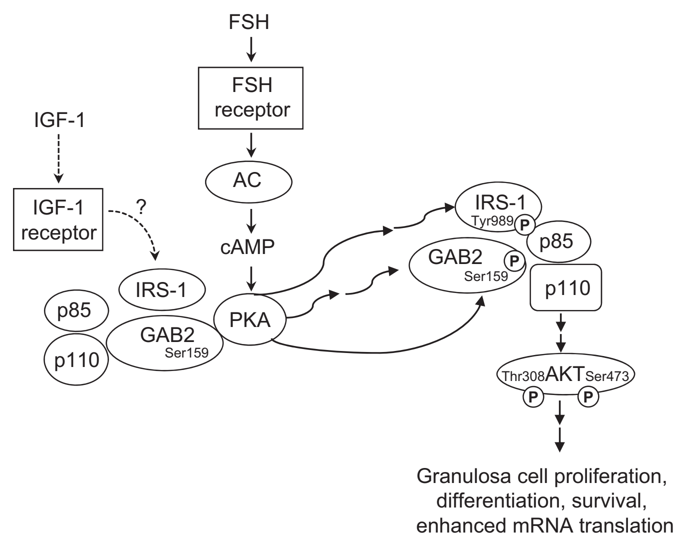

FIGURE 20.7 Mechanism by which FSH via PKA activates PI-3K leading to AKT activation. Multiple arrows indicate more than one step in the pathway. Potential contribution of IGF-1 and IGF-1 receptor is indicated by dashed lines.

activates the IGF-1 receptor to signal to AKT.[237] Additional studies are necessary to clarify the contributions of IGF-1 and the IGF-1 receptor to FSH signaling into the PI-3K pathway.

While the overexpression of constitutively active RAS (Gly12Asp) in immature granulosa cells elevates basal AKT phosphorylation,[216] transduction of granulosa cells with an adenovirus expressing a dominant negative RAS (Ser17Asn) does not prevent AKT phosphorylation on Ser473 or Thr308.[116] Taken together these results support the current view that RAS is not a major regulator of PI-3K activity under physiological conditions.[238]

AKT Substrates. General downstream targets of AKT in a variety of tissues can include the transcription factor CREB, the proapoptotic protein BAD, the phosphodiesterase PDE3β, nitric oxide synthase (NOS), the translation antagonist tuberin, GSK3β, the p53 E3 ubituitin ligase MDM2, the RAB-GTPase activating protein AS160 that regulates the cellular location of glucose transporter 4, select members of the FOXO transcription factor family, D-type cyclins, the cell cycle inhibitor $p27^{Kip1}$, the translation elongation factor E2F, and others[154,239] (Figure 20.8). Thus, AKT can signal to regulate transcription, translation, metabolism, cell survival, and differentiation, in a cell-specific and agonist-dependent manner.

CREB. In rat granulosa cells, FSH-stimulates CREB phosphorylation on Ser133. However, CREB phosphorylation is mediated by PKA in granulosa cells, as reviewed earlier, and not by AKT. This conclusion is based on the inability of the PI-3K inhibitor wortmannin[249] to reduce CREB phosphorylation[113] and the inability of CREB to be further phosphorylated in granulosa cells transduced with constitutively active AKT.[115]

Tuberin and Translational Regulation. FSH also stimulates the phosphorylation of tuberin, a product of the tuberous sclerosis (TSC) tumor suppressor gene[235] (Figure 20.6). In its unphosphorylated state, tuberin (TSC2) in a complex with TSC1 (Hamartin) is active and inhibits translation.[250] The tuberin–TSC1 complex functions as a GTPase-activating protein for the protein RAS homolog enriched in brain (RHEB).[251] This TSC complex stimulates the hydrolysis of the GTP bound to RHEB, converting RHEBGTP to RHEBGDP, resulting in the inactivation of RHEB. Phosphorylation of tuberin on Thr1462 by AKT results in an inhibition of the GTPase-activating protein activity of the TSC1–TSC2 complex.[251] As a result, RHEB is maintained in its active conformation. By a poorly understood mechanism, RHEBGTP promotes activation of mTORC1 (mTOR/Raptor complex). mTORC1 signals to at least two substrate proteins to stimulate translation, p70 S6 kinase, and a binding protein that sequesters the eukaryotic translation initiation factor eIF4E (eIF4E-BP1).[252–254] Phosphorylation of p70 S6 kinase on Thr389 leads to the phosphorylation of the ribosomal protein S6. Phosphorylation of S6, one of 30 ribosomal proteins that along with the 18S rRNA comprises the 40S ribosomal subunit complex, stimulates the translation of mRNAs containing a 5′-oligopyrimidine tract.[255] Phosphorylation of eIF4E-BP1 on Ser65 and other sites frees eIF4E[256,257] to bind to the 5′-methyl cap of mRNAs.[255,258] This stimulates cap-dependent translation and is the rate-limiting step in translation initiation.[258]

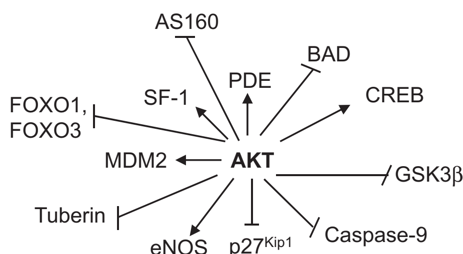

FIGURE 20.8 Summary of identified AKT substrates. Arrows indicate stimulation; perpendicular lines indicate inhibition.

FSH, via PI-3K/AKT, stimulates the phosphorylation of tuberin on Thr1462 within 10 min, leading to the activation of mTORC1 and phosphorylation of its substrates p70 S6 kinase and downstream S6 protein as well as 4E-BP1 in rat granulosa cells.[235] That mTORC1 activation is crucial for activation of a subset of FSH target genes is evidenced by the ability of the mTORC1 inhibitor rapamycin[252] to inhibit the induction of MAP2D and RIIβ protein expression as well as promoter-reporter activities for *Lhcgr*, *Vegf*, and *Inha*.[235] These results suggest that a very rapid effect of FSH in granulosa cells is to stimulate translation. Indeed, it has been suggested that activation of the PI-3K and ERK pathways in general preferentially promotes translation rather than transcription.[259] mTORC1-dependent targets of translation whose expression is increased in various cellular models include the cyclins, resulting in increased cell proliferation.[260,261] Certainly a future area of research should be the identification of FSH-targeted translation products via some of the newer proteomics approaches or via ribosome profiling.

There is also a report that FSH promotes the phosphorylation of tuberin via an ERK-dependent, AKT-independent pathway that leads to phosphorylation of p70 S6 kinase and increased cyclin D2 mRNA that is partially reduced by the mTORC1 inhibitor rapamycin, using granulosa cells seeded on serum-coated plates.[262] While the ERK target RSK has been reported to phosphorylate tuberin,[251] it is not clear how the mTORC1 pathway leads to enhanced cyclin D2 transcription.

It is quite interesting that IGF-1 also stimulates the phosphorylation of AKT, tuberin, p70 S6 kinase, and S6 protein in rat granulosa cells,[235] but as already indicated, IGF-1 does not stimulate expression of FSH target genes,[47,243] at least to levels observed with FSH. This

result suggests that additional pathways activated by FSH but not by IGF-1 are required for FSH target gene expression.

HIF-1α. One of the mRNAs whose translation is increased by FSH downstream of AKT and tuberin in granulosa cells is the transcription factor HIF-1α.[235] HIF-1 is a heterodimer, consisting of HIF-1α and HIF-1β, the latter also known as the aryl hydrocarbon receptor nuclear transporter. HIF-1 is a member of the basic helix-loop-helix/Per/aryl hydrocarbon receptor nuclear transporter/Sim family of transcription factors that bind to a modified E-box on DNA promoters.[263] HIF-1β is constitutively expressed; HIF-1α protein levels are regulated. HIF-1 is best known for its regulation of cellular responses to hypoxia.

Under normoxic conditions, HIF-1α is hydroxylated on two critical proline residues by oxygen-dependent prolyl hydroxylases, triggering HIF-1α degradation.[263,264] Hydroxylation of the Pro residues creates a binding site for the protein product of the von Hippel–Lindau tumor suppressor gene, VHL. VHL is an E3-ubiquitin ligase and promotes the polyubiquitination of proline hydroxylated forms of HIF-1α, leading to their degradation by the proteosomal system. Cobalt has been shown to inhibit the interaction of HIF-1α and VHL[265] and is often used experimentally to stabilize HIF-1α protein levels. HIF-1 activity is also regulated by a second oxygen-dependent mechanism. Under normoxic conditions, a protein called "factor inhibiting HIF-1" catalyzes the hydroxylation of an Asn residue in the C-terminal transactivation domain of HIF-1α, preventing the binding of its co-activator p300/CBP. Under hypoxic conditions, the activity of the oxygen-dependent prolyl hydroxylases is reduced, and HIF-1α protein is stabilized. The activity of the asparaginyl hydroxylase is also reduced under hypoxic conditions, thereby allowing the binding of p300/CBP to stimulate HIF-1 activity. The prolyl hydroxylases require iron as a cofactor, so agents that chelate iron such as desferrioxamine inhibit the propyl hydroxylases, thereby stabilizing HIF-1α protein levels.

HIF-1α protein can also be regulated at the level of translation upon activation of the PI-3K pathway by insulin or IGF-1.[266,267] In these studies, expression of HIF-1α protein correlated with activation of HIF-1 DNA binding activity and/or HIF-1-dependent reporter gene activities. Inhibitors that abrogate the ability of these growth factors to stimulate the phosphorylation of p70 S6 kinase and 4E-BP1 block the increase in HIF-1α protein.[266,267]

In rat granulosa cells, FSH promotes an increase in HIF-1α protein accumulation under normoxic conditions (Figure 20.6), which is detected in the presence either of a proteosomal inhibitor or $CoCl_2$.[235] FSH also promotes an increase in HIF-1 activity, detected using a minimal hypoxia response element linked to the luciferase reporter in rat granulosa cells. Moreover, co-expression of a HIF-1 dominant negative construct, lacking its DNA binding and transactivation domains,[268] reduces the ability of FSH to activate promoters for *Lhcgr*, *Vegf*, and *Inha*, as detected using promoter-reporter assays.[235] These results suggest that HIF-1 activity is required for FSH to activate *Lhcgr*, *Vegf*, and *Inha* genes. However, although each of these genes contains apparent HIF response elements and VEGF is an established HIF-1 target, only *Vegf* in granulosa cells has been shown to be a direct HIF-1 target.[247]

While there is some evidence that the granulosa cell environment may become increasingly hypoxic as the follicle grows,[269] hypoxia does not appear to be a prominent contributor to the ability of FSH to induce target genes such as *Lhcgr* (E. Donaubauer and M. Hunzicker-Dunn, unpublished).

IGF-1 also promotes an increase in the accumulation of HIF-1α protein in rat granulosa cells to levels equivalent to those achieved with FSH; however, interestingly IGF-1 does not enhance HIF-1 activity, based on a promoter-reporter assays.[247] This result shows that the mere presence of HIF-1α protein is not sufficient to generate an active HIF-1 transcriptional factor. Thus, additional unidentified signaling events independent of the PI-3K pathway that are activated by FSH are required to "activate" HIF-1.

Transcriptional activity of HIF-1α requires binding of CBP/p300 to the C-terminal transactivation domain.[264] The binding of CBP/p300 is negatively regulated in an oxygen-dependent manner by the hydroxylation of Asn803 by "factor inhibiting HIF-1".[264] HIF-1 transcriptional activity in a number of cellular models is also positively regulated by ERK, although whether ERK directly phosphorylates HIF-1α is controversial.[247] Moreover, activation of VEGF transcription in various cell models requires cooperation between HIF-1 and an AP-1 complex.[212] Thus, ERK could contribute to HIF-1 activity by phosphorylating HIF-1α protein, by phosphorylating CBP, or by phosphorylating members of the AP-1 family, as summed in Figure 20.5. In rat granulosa cells, *HIF-1* promoter-reporter activities and the interaction of HIF-1α with the *Vegf* promoter in a CHIP assay are inhibited by the MEK inhibitor PD98058, consistent with the potential contribution of ERK phosphorylation to HIF-1 transcriptional activity.[247] Moreover, IGF-1 in rat granulosa cells does not activate ERK phosphorylation, suggesting that the inability of IGF-1 to promote HIF-1 transcriptional activity is the result of the lack of ERK activation by IGF-1.[247] However, transient expression of a constitutively active MEK was not sufficient to rescue IGF-1-stimulated HIF-1 activity.[247] Based on our evidence that ERK activity in immature granulosa cells is restrained by a PTP whose activity is inhibited by FSH,[113] the potential contribution of ERK needs to be reevaluated in cells transduced with adenovirally expressed constitutively active ERK.

FOXO1 and FOXO3. A second AKT target in rat and porcine granulosa cells is FOXO1.[48,112,234,270,271] FOXO1 is a member of the forkhead O class of transcription factors that was originally identified by its disruption in the pediatric tumor rhabdomyosarcoma.[272] Three of the members of this family are AKT targets: FOXO1, FOXO3, and FOXO4.[273] FOXO-regulated genes control the cell cycle, cellular metabolism, and cell death. These transcription factors bind to DNA as monomers at the insulin response element consensus sequence TT(A/G)TT(T/G)(A/G)(T/C) on target genes. In the absence of signaling through the PI-3K pathway, these FOXO transcription factors are bound to DNA and are active. They can function as transcriptional activators to stimulate such target genes as the cell cycle inhibitor p27^{Kip1}, glucose-6-phosphatase activity,[274] and IGF binding protein-1, as well as transcriptional repressors to suppress such target genes as cyclin D1 and D2.[273] Upon phosphorylation by AKT, FOXO1, -04, and -03 exit the nucleus and are targeted for degradation. Three Ser/Thr residues on FOXO proteins are phosphorylated by AKT. For FOXO1, phosphorylation of Ser256 diminishes DNA binding and is necessary for the phosphorylation of Thr24 and Ser319.[275] Phosphorylation of Thr24 disrupts association of FOXO1 with CBP and stimulates binding to 14-3-3 proteins, and phosphorylation of Ser319 stimulates nuclear export.[273]

FOXO factors also bind nuclear receptors, including those for estrogen (preferentially ER-α over ER-β), progesterone, androgen, thyroid hormone, glucocorticoid, and retinoid acid, presumably via the conserved LeuXXLeuLeu domain located in the C-terminal region of the FOXO protein.[273] Although the actions of the FOXO factors can require binding to DNA, there is also evidence that they can affect transcription of target genes independent of their DNA binding activity, possibly via their association with co-activators such as CBP or with steroid receptors.[276] FOXO factors can also be acetylated, possibly by CBP, a modification that is thought to activate FOXO transcriptional activity, although the effect of acetylation is controversial (reviewed in Ref. 273).

There is accumulating evidence from a number of cellular models that FOXO factors promote cell cycle arrest.[273,277] Cell cycle arrest can occur as a result of increased expression of the cyclin-dependent kinase (CDK) inhibitors p27^{Kip1}, p21^{Cip1}, and p130, and/or by decreased expression of the CDK or their binding partners cyclin D or cyclin E. In general, progression from G_1 to the S phase of the cell cycle is mediated by increased expression of cyclin D, which partners with CDK 4/6, followed by increased expression of the CDK 2 partner cyclin E. Hyperphosphorylation of the retinoblastoma protein catalyzed by CDK 4/6 and CDK 2 relieves inhibition of the transcription factor E2F by retinoblastoma, allowing for the expression of genes necessary for DNA replication and S phase entry.

FOXO transcription factors have been reported to inhibit cell cycle progression by enhancing transcription of p27^{Kip1} and other CDK inhibitors, by repressing cyclin D transcription, and/or by increasing transcription of the unconventional cyclin G2, which inhibits the cell cycle.[278] Porcine granulosa cells undergo proliferation upon stimulation with FSH and insulin.[270] Transduction of porcine granulosa cells with a dominant negative FOXO1 truncation mutant, which lacks its transactivation domain, increases entry of these cells into the S phase of the cell cycle by redirecting the compartmentalization of p27^{Kip1} from the nucleus to the cytosol and decreasing p27^{Kip1} content.[270] Although p27^{Kip1} is known to be phosphorylated on Thr157 by AKT, resulting in its redistribution from the nucleus to the cytosol,[279–283] the mechanism by which FOXO1 directs relocation of p27^{Kip1} to the cytosol followed by its apparent degradation is not known.

It is not known if FSH promotes a redistribution of p27^{Kip1} to the cytosolic fraction in rat granulosa cells upon FOXO1 phosphorylation as occurs in porcine granulosa cells.[270] However, p27^{Kip1} does not show any evidence of downregulation, and interestingly, the phosphorylatable Thr157 residue believed to be responsible for exit of p27^{Kip1} from the nucleus is not conserved in rodents.[279,280]

In contrast to porcine granulosa cells, proliferation of rat granulosa cells and the obligatory induction of cyclin D2[45] and the S-phase marker proliferating cell nuclear antigen, under primary serum-free culture conditions, does not occur in the presence of FSH alone or FSH plus IGF-1.[44,47,49] However, as shown in Figure 20.6, in the presence of activin, cyclin D2 mRNA expression is strongly enhanced by FSH under serum-free culture conditions.[46,227,276] These results suggest that FSH, directly or indirectly, stimulates the expression of activin or a similar TGFβ-family ligand and that both of these ligands converge to stimulate cyclin D2 expression and consequent granulosa cell proliferation. Rat granulosa cells are reported to produce activin subunits throughout early follicular development.[284] However, activin levels produced under culture conditions are apparently too low to synergize with FSH, possibly due to contributions from the activin inhibitor follistatin and/or to utilization of activin subunits by inhibin-α, as these cells also produce follistatin and inhibin.[285]

FOXO1 functions to repress activation not only of the *Ccnd2* (cyclin D2) gene but also to repress activation of a subset of FSH targets that characterize the preovulatory phenotype, including *Nr5A1* (SF-1), *Nr5A2* (LRH-1), *Cyp19a1* and *Cyp11a1*, *Inha,* and *Ereg* (epiregulin).[48] The list of genes repressed by FOXO1 in immature granulosa cells has been expanded to include genes required for cholesterol biosynthesis and steroidogenesis as well as *Lhcgr*.[271] Phosphorylation of FOXO1 by AKT is necessary but likely not sufficient to achieve the induction of

these FSH target genes, as evidenced by the established regulation of *Inha*[170,186] and *Cyp19a1*[152,286] promoters, for example, by a number of transcriptional activators. The ability of FOXO1 to repress activation of these FSH target genes is based on studies in which granulosa cells were transduced with an adenoviral vector encoding a constitutively active FOXO1 mutant in which the three AKT phosphorylation sites were mutated to Ala (A3-FOXO1). Direct binding of FOXO1 to the cyclin D2 promoter was evidenced by CHIP assays, this association was abrogated upon stimulation of cells with FSH, and the association was enhanced and not regulated in cells transduced with the constitutively active A3-FOXO1 mutant.[48] However, it is not known if other FSH target genes regulated by inactivation of FOXO1 are repressed directly or indirectly by FOXO1.

FOXO1 also represses the ability of FSH to promote the accumulation of HIF-1α, as evidenced in cells transduced with A3-FOXO1 compared to empty adenovirus, and thus activation of HIF-1 transcriptional activity[247] (Figure 20.6). While the FOXO1 target is likely a ubiquitin ligase, this ligase has not been identified. The ability of FOXO1 to repress the accumulation of HIF-1α in the absence of FSH may be a mechanism to prevent inappropriate actions of HIF-1 in granulosa cells.

The induction of *Ccnd2*, *Nr5a1*, *Inha*, *Cyp11a1*, *Cyp19a1*, and *Ereg* was also abrogated in granulosa cells transduced with a dominant negative Smad2/3 mutant.[48] These results suggest that induction of these proliferation and differentiation responses requires not only relief from repression by FOXO1 but also the contribution of the Smad2/3 transcription factors (Figure 20.6). However, the mechanism by which Smad2/3 cooperates with the AKT pathway leading to FOXO1 phosphorylation to promote expression of at least a subset of FSH target genes has not been elucidated. It is interesting that active (unphosphorylated) FOXO1 can complex with phospho-Smad to promote expression of the cell cycle inhibitor $p21^{Cip1}$ in neuroepithelial and glioblastoma cell lines.[287] In granulosa cells, however, phospho-Smad2/3 functions upon inactivation of FOXO1 to drive proliferation. Thus, the cross talk between these two pathways is cell specific.

A similar repressive function in follicular maturation has been attributed to FOXO3, but at a much earlier stage of follicular development. Unlike FOXO1-null mice, which die at embryonic day 10.5,[288] FOXO3-null mice show global activation of primordial follicles, resulting in oocyte death and depletion of follicles as a result of increased granulosa cell mitotic activity.[289] These results suggest that FOXO3, most likely in oocytes, functions to suppress maturation of primordial follicles.

FOXO3 has also recently been shown to promote expression of the pro-apoptotic protein Bcl-2-interacting mediator of cell death/Bcl-2-related ovarian death agonist (BIM/BOD) in porcine granulosa cells.[290] CHIP assays place FOXO3 on the *Bim(Bcl2l11)* promoter in the absence of FSH.[290] FSH via the PI-3K/AKT pathway stimulated the phosphorylation of FOXO3 on Ser253, resulting in its inactivation and exit from the nucleus. Consistent with the positive effect of FOXO3 on BIM/BOD expression in the absence of FSH, FSH reduced expression of BIM/BOD.[290] These results suggest that FOXO3 is a transcriptional activator of *Bim*, and that FSH reduces the expression of the pro-apoptotic protein product to promote granulosa cell survival.

Owing to the embryonic lethality and premature ovarian failure of global FOXO1[288] and FOXO3[289] knockouts, respectively, FOXO1 and FOXO3 were recently selectively deleted from granulosa cells.[291] While targeted deletion of FOXO1 resulted in a subfertile mouse (some mural granulosa cells retained FOXO1 expression), targeted deletion of FOXO3 did not reduce fertility. However, dual targeted deletion of FOXO1 and FOXO3 generated an infertile mouse in which follicle growth was arrested at the preantral/early antral stage of follicle development.[291] Deletion of FOXO1/FOXO3 would be expected to reduce expression of those genes that are positively regulated by FOXO1/FOXO3. However, enhanced expression of genes that are repressed by FOXO1/FOXO3 might not occur due to the absence of positive transcriptional regulators. These double mutant mice exhibit severely reduced serum FSH levels[291] and thus would not be expected to express genes repressed by FOXO1/FOXO3. Granulosa cells of the double mutant mouse exhibited reduced apoptosis, associated with reduced expression of the FOXO3 target[290] *Bcl2l11* (that encodes for BIM/BOD) and *Bmp2* that encodes bone morphogenic protein 2 (BMP2), a protein selectively expressed in follicles undergoing apoptosis.[291] Granulosa cells of these double mutant mice also exhibited reduced expression of a number of apparent FOXO1/FOXO3 targets, including *Igf1* (insulin-like growth factor 1), *Ctgf* (conective tissue growth factor), *Amh* (anti-Mullerian hormone), and *Nr0b1* (nuclear receptor subfamily 0, group B, member 1; Dax1),[291] the expression of which likely contributes to the immature granulosa cell phenotype (in the absence of FSH). Studies with isolated granulosa cells showed that the positive effects of (active) FOXO1/FOXO3 on the expression of these genes was enhanced by activin, consistent with the interaction of FOXO1/FOXO3 with pathways downstream of activin.[291] It is interesting that activin signaling pathways appear to converge both with the positive effects of FOXO1/FOXO3 on target genes that maintain granulosa cells in an immature state (in the absence of FSH)[291] and with FSH signaling pathways that promote inactivation of FOXO1/FOXO3 to drive granulosa cell maturation.[48]

GSK3β and the WNT signaling pathway. An additional AKT target in many cells is the Ser/Thr protein kinase GSK3β (Figure 20.8). GSK3β is active when the PI-3K pathway is silent. Active AKT phosphorylates GSK3β on Ser9, resulting in the inactivation of GSK3β.[292] One of the GSK3β targets in a number of cellular models is the D-type cyclins. In the absence of signaling through the PI-3 kinase pathway, GSK3β phosphorylates the D-type cyclins, leading to their ubiquitination and degradation.[293] Activation of the PI-3K pathway promotes AKT-catalyzed phosphorylation of GSK3β, thereby rescuing D-type cyclins from degradation. Although there is evidence that FSH promotes the phosphorylation of GSK3β in rat granulosa cells,[218] it is not known if the stability of cyclin D2 in granulosa cells is also regulated by GSK3β. However, based on evidence that FSH stimulates only a modest two- to three-fold increase in cyclin D2 promoter-reporter activity in transiently transfected granulosa cells compared with the robust increase in cyclin D2 protein expression,[48] it is likely that FSH also stabilizes cyclin D2, possibly via inactivation of GSK3β.

GSK3β can also be phosphorylated on Ser9 by a number of other Ser/Thr kinases, including PKA, PKC, and the ERK substrate RSK, to reduce its activity.[294,295] Although FSH activates PKA, AKT, and RSK in granulosa cells,[113,115,117,118,218,234,235] FSH-stimulated GSK3β phosphorylation is strongly inhibited by PI-3 kinase inhibitors,[218] suggesting that GSK3β is predominately an AKT substrate in granulosa cells.

In addition to regulating the stability of the D-type cyclins, GSK3β is recognized to phosphorylate a number of transcriptional activators, including β-catenin, CREB, HIF-1, NF-κB, and AP-1, as well as structural proteins and metabolic and signaling proteins such as MAP2, kinesin light chains, glycogen synthase, eIF2B, PKA RIIβ subunit, and protein phosphatase 1.[294,295] Thus, GSK3β potentially regulates glucose production, translation, transcription, cell survival, and cell motility. Generally, GSK3β substrates are initially phosphorylated by another protein kinase, priming the substrate for phosphorylation by GSK3β.

The phosphorylation activity of GSK3β is also regulated by its association with a specific protein complex that includes its substrate, β-catenin, the obligatory priming kinase, and other proteins. This pathway is regulated by the paracrine/autocrine factor WNT.[296] In the absence of the WNT ligand, GSK3β is complexed with a scaffold protein (AXIN), casein kinase 1α, adenomatous polyposis coli (APC), and β-catenin, as depicted in Figure 20.9. In this complex, β-catenin is targeted for ubiquitination and proteosomal degradation as a result of a priming phosphorylation by casein kinase 1α on Ser45 followed by phosphorylation by GSK3β on Thr41, Ser37, and Ser33.[296] WNT signaling through the plasma membrane Frizzled receptor/lipoprotein receptor-related proteins 5 and 6

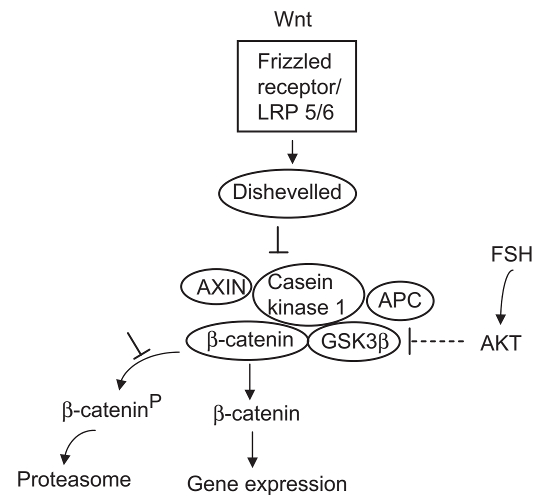

FIGURE 20.9 Schematic diagram of the rescue of hypophosphorylated β-catenin from proteosomal degradation by WNT signaling. A potential route for FSH to inhibit phosphorylation of β-catenin via AKT is depicted.

together with the scaffolding protein Dishevelled inhibits its GSK3β activity by a poorly understood mechanism. As a result, β-catenin is not phosphorylated and escapes from degradation. Nonphosphorylated β-catenin translocates to the nucleus where it functions as a transcriptional co-activator. While β-catenin is best known for its ability to bind TCF/lymphoid enhancer factor (LEF) and to activate TCF/LEF–regulated genes,[297] β-catenin is also required for the transcriptional activation of SF-1.[175,176]

Immature granulosa cells express Wnt2, Wnt4, Frizzled1, Frizzled4, and Dishevelled2 mRNAs.[298,299] Conditional deletion of Wnt4 from granulosa cells using *Amhr2-Cre* reduced the number of antral follicles ~ 50%,[300] suggesting that Wnt4 contributes to follicular maturation. However, to our knowledge, there is no evidence that FSH enhances expression of Wnt4 to activate the canonical β-catenin pathway in granulosa cells. The Richards' laboratory used an alternative approach to evaluate the contribution of β-catenin to FSH-signaling pathways. Conditional expression of a dominant stable β-catenin in granulosa cells of small antral follicles (driven by *Amhr2-Cre*), in which exon 3 that contains the N-terminal phosphorylation sites required for degradation was excised, generated the formation of either abnormal follicles that lacked oocytes and were highly vascularized or cystic structures as well as some normal follicles, resulting in subfertile mice.[301] A microarray analysis of ovaries expressing this dominant stable β-catenin compared to control ovaries revealed abnormal overexpression of Wnt/β-catenin signaling antagonists such as AXIN as well as bone and neuronal cell markers.[302] Conditional expression of the dominant stable β-catenin in granulosa cells of follicles at a slightly later stage of maturation, driven by the *Cyp19-Cre*,

showed that β-catenin inhibits apoptosis and facilitates FSH-stimulated follicular growth by enhancing expression of *Fshr*, *Cyp19a1*, *Ccnd2*, and *Nr5a1* without affecting *Lhcgr* expression.[303] The ability of β-catenin to regulate expression of *Cyp19a1* agrees with previous reports in granulosa cells that β-catenin is required for transcriptional activation of SF-1 on the *Cyp19a1* promoter.[286,304] Fan et al.[303] showed that FSH activated the artificial TCF/β-catenin promoter-reporter TOPFlash, and that FSH enhanced the nuclear accumulation of nonphosphorylated β-catenin. The authors postulate that the mechanism by which FSH enhances nuclear accumulation of nonphosphorylated β-catenin may be via AKT-dependent phosphorylation/inactivation of GSK3β,[303] as shown in Figure 20.9. An alternate pathway by which FSH regulates β-catenin activity is discussed later in "Cross Talk among FSH-Regulated Signaling Pathways and Transcriptional Activators to Regulate Gene Expression." In contrast to enhancing FSH responses, persistent expression of the dominant stable β-catenin repressed LH-induced oocyte maturation, ovulation, and luteinization by inhibiting expression of LH target genes and thus compromised fertility.[303] The negative effect of the dominant stable β-catenin was at least in part due to reduced phosphorylation of CREB, MEK, and ERK in response to hCG, although it is not clear how dominant stable β-catenin interferes with CREB and MEK/ERK phosphorylations.

SGK. FSH and forskolin also induce the immediate early gene SGK[118,122,218] and stimulate its apparent phosphorylation, based on an upward shift in mobility on sodium dodecyl phosphate polyacrylamide gel electrophoresis.[199] SGK bears similarities to AKT, as it is activated in a PI-3K-dependent manner by PDK-1[305] and phosphorylates many of the same substrates phosphorylated by AKT, including members of the FOXO family.[306] However, recent evidence suggests that the primary function of this kinase is to regulate the abundance of Na^+, K^+, and Cl^- ion channels by preventing their proteosomal degradation[307,308] by phosphorylating and inactivating a ubiquitin ligase.[309] SGK is induced in granulosa cells in a PKA-dependent manner, and its induction is prevented by the PKA inhibitor H89.[106] SGK is not induced in granulosa cells by IGF-1.[199] In contrast to the signaling pathway that regulates the FSH-stimulated induction of SGK in granulosa cells, FSH-stimulated phosphorylation of SGK in granulosa cells is abolished by PI-3K inhibitors wortmannin and LY294002, consistent with FSH signaling to stimulate phosphorylation/activation of SGK via the PI-3K pathway.[199] However, neither the substrates nor the function of SGK in granulosa cells is known.

Apoptosis-related PI-3K Substrates. The PI-3K pathway is also known to regulate cell survival. Indeed, addition of the PI-3K inhibitor LY294002 to rat granulosa cells induces apoptosis.[310] Up to 99% of the ovarian follicles undergo apoptosis primarily at the early antral stage of development (in rats).[311] Follicles survive and proceed to final stages of maturation only if they are rescued from apoptosis, predominately by FSH. It is not surprising that apoptosis is a highly regulated event in the ovary and that a number of proteins in the apoptotic pathway have been identified as being highly expressed in ovarian cells.[290,311]

There are two major pathways that lead to apoptosis: one directed by the death receptors of the tumor necrosis family (TNF) and one directed by the release of cytochrome c (and other proteins) from mitochondria as a result of various cellular stresses such as heat shock, oxidative stress, and DNA damage.[312] In both of these pathways, cysteine aspartic acid-specific proteases (caspases) become activated and cleave proteins that are crucial to cellular function, resulting in programmed cell death. Initiator caspases are activated in response to a proapoptotic signal, and they in turn activate effector caspases that catalyze substrate proteolysis, leading to cell death.

FSH appears to inhibit apoptosis primarily by controlling the release of cytochrome c from mitochondria, as depicted in Figure 20.10. The proteins that regulate this pathway are members of the BCL-2 (B-cell leukemia/lymphoma 2) family and share one or more BCL-2 homology (BH) domains.[313,314] Group I, the anti-apoptotic proteins that contain four BH (BH 1–4) domains, includes BCL-2, BCL-XL (extra large), MCL-1(myeloid cell lymphoma-1), and DIVA. They prevent apoptosis by binding to and blocking the activity of the Group II pro-apoptotic BH123 domain proteins BAX, BAK318 and possibly BOX (BCL-2–related ovarian killer).[315] BAX and BAK, upon activation, oligomerize at the outer mitochondrial membrane and create pores for the exit of cytochrome c. Group III BH3 domain-only proteins stimulate apoptosis upon receipt of an apoptotic stimulus most likely by inhibiting the interaction of Group I anti-apoptotic proteins with Group II pro-apoptotic proteins[314] and include BAD (BCL-XL/BCL-2–associated death promoter), BID, PUMA, BIM (aka BOD, BCL-2-related ovarian death agonist), and NOXA. These proteins are restrained in the absence of an apoptotic signal at the transcriptional (BIM, PUMA, NOXA), posttranslational (BAD), or precursor (BID) levels.[313] They bind with selectivity and high affinity to Group I anti-apoptotic proteins, effectively freeing Group II pro-apoptotic (BH123) proteins to oligomerize and stimulate cytochrome c release.[314]

Upon release of cytochrome c from the mitochondria, cytochrome c complexes with APAF-1 (apoptotic protease activating factor-1), resulting in the oligomerization of APAF-1 and recruitment of procaspase-9.[312] Caspase-9 becomes activated as a result of an autocatalytic event, forming the active apoptosome, leading to the activation of effector caspases-3 and -7, which promote the

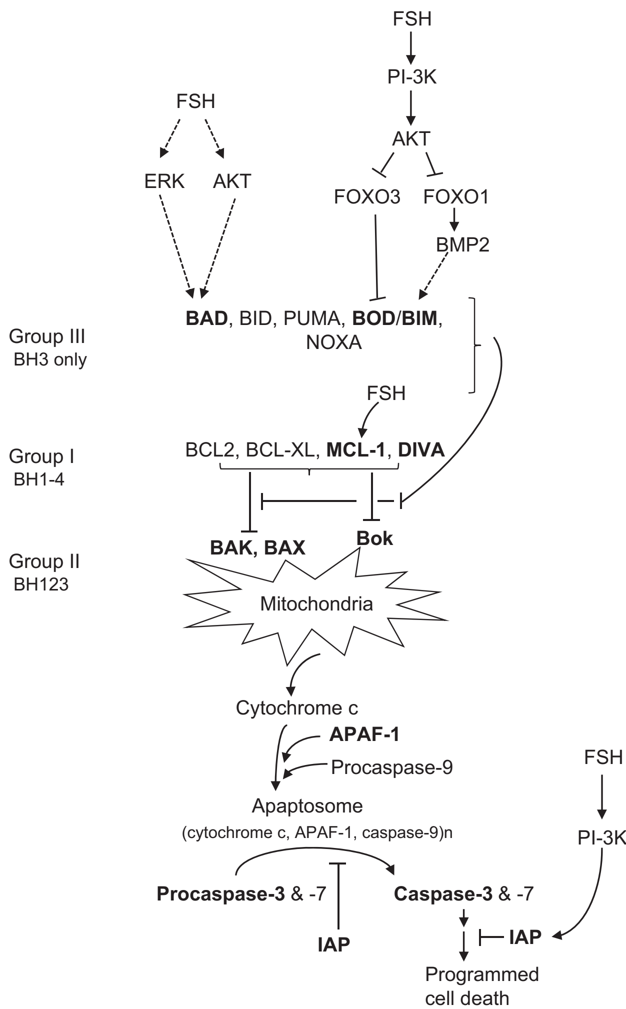

FIGURE 20.10 Schematic diagram of the mitochondrial-dependent apoptotic pathway in ovarian cells and sites at which FSH has been reported to inhibit (perpendicular lines) or stimulate (arrows). Bold names represent proteins identified in granulosa cells. Dashed lines represent expected but not demonstrated regulation.

proteolysis of a large number of proteins crucial to cell survival and consequent cell death.[312] PI-3K–stimulated AKT has been reported to phosphorylate and inactivate caspase-9[316]; however, the AKT phosphorylation site found in human caspase-9 is not conserved in rat, mouse, or monkey caspase-9.[317] Thus, regulation of caspase-9 phosphorylation/activity at least by AKT does not appear to be a widespread mechanism by which PI-3K signaling promotes cell survival. Although IGF-1 has been shown in porcine granulosa cells to inhibit apoptosis,[240] a definitive role for IGF-1 in rat granulosa cell apoptosis, to our knowledge, has not been reported.

The fate of a cell is determined by the balance between survival and apoptotic signals. Transgenic mice overexpressing the Group I anti-apoptotic protein BCL-2 in the ovary show decreased follicular apoptosis and increased folliculogenesis; however, BCL-2 does not appear to be expressed in the ovary.[318] Rather, ovaries appear to express predominately MCL-1, and expression of MCL-1 is increased by treatment of rats with the FSH receptor agonist pregnant mare's serum gonadotropin (PMSG).[319] While increased expression of MCL-1 likely contributes to the ability of FSH to promote cell survival, the mechanism by which FSH increases MCL-1 expression was not explored. Yeast two-hybrid analysis showed that MCL-1 interacted with BAK, BAX, and BOK (Group II BH123 pro-apoptotic proteins) to inhibit apoptosis, and with BAD and BIM (Group III BH3-only pro-apoptotic proteins) to promote apoptosis, consistent with the current view of the mechanism by which Group III proteins promote apoptosis. Ovaries also express high levels of the Group I anti-apoptotic protein DIVA.[298]

Yeast two-hybrid studies with an ovarian cDNA library showed that a mutant, nonphosphorylated BAD (Group III BH3-only protein) interacts with MCL-1, thereby diminishing the anti-apoptotic effect of MCL-1.[319] The phosphorylation of BAD prevents its interaction with MCL-1, thereby allowing MCL-1 to inhibit apoptosis, and promotes its binding to 14-3-3 proteins. BAD is phosphorylated on Ser136 by AKT, and on Ser112 and Ser155 by PKA or RSK in a cell-specific manner.[320] Phosphorylation of Ser155 is thought to be rate limiting and to require priming phosphorylations at Ser112 and Ser136.[320] Based on the ability of FSH to activate PKA, AKT, and RSK, it is likely that BAD phosphorylation contributes to the ability of FSH to promote granulosa cell survival. However, FSH-stimulated BAD phosphorylation has not been formally demonstrated in granulosa cells.

FSH has recently been shown to promote granulosa cell survival by inhibiting the transcription of *Bcl2l11* that encodes BIM/BOD, a BH3-only Group III pro-apoptotic protein.[290] In the absence of FSH, FOXO3 is a transcriptional activator *Bcl2l11*.[290] The effect of FOXO3 appears to be facilitated by FOXO1 activation of *Bmp2* expression; BMP2 also enhances expression of *Bcl2l11* to promote apoptosis.[291] FSH via the PI-3K/AKT pathway stimulates the phosphorylation of FOXO3 on Ser253, resulting in its inactivation and exit from the nucleus.[290] AKT-stimulated FOXO1 phosphorylation in response to FSH is also expected to reduce expression of BMP2 and consequently of *Bcl2l11*, although this has not been demonstrated. Together, these results show that in the absence of FSH, FOXO3 and FOXO1 via BMP2 are activators of *Bcl2l11* expression. FSH reduces the expression of BIM/BOD in a PI-3K/AKT–dependent manner by regulating the activity of FOXO3 and likely of FOXO1 (Figure 20.10). Thus, in the absence of FSH, FOXO1 and FOXO3 contribute to granulosa cell apoptosis.

Granulosa cells express APAF-1 and, in the absence of gonadotropin support, exhibit increased activation of caspase-3.[321] There is also evidence that gonadotropins decrease APAF-1 expression[294] (Figure 20.10), a response expected to be anti-apoptotic. Although granulosa cells

of caspase-3-null mice show attenuated apoptosis, these cells do undergo apoptosis, suggesting that other effector caspases can compensate for caspase-3.[322]

Additional regulation of the apoptotic pathway occurs via a family of inhibitors of apoptosis (IAPs) that act as substrate inhibitors of caspases and block the activation of both effector caspases and, at higher concentrations, initiator caspases.[297] Of these IAPs, the X-linked IAP (XIAP) is reported to be the most potent.[297] FSH has been shown to increase expression of XIAP in rat granulosa cells[236,310] (Figure 20.10). Based on evidence that the addition of XIAP antisense oligonucleotides to granulosa cells enhances apoptosis,[310] it is likely that the FSH-dependent regulation of XIAP expression contributes to the anti-apoptotic effect of FSH.[323] There is also a report that AKT interacts with and phosphorylates XIAP, protecting it from ubiquitin-directed degradation.[324] These results suggest that the regulation of XIAP is crucial to the ability of FSH to rescue granulosa cells from programmed cell death and that FSH potentially regulates XIAP at multiple levels. However, FSH-stimulated XIAP phosphorylation via AKT has not been demonstrated in granulosa cells. A second group of regulators of the apoptotic pathway function as inhibitors primarily of the initiator caspases. One of the members of this group of proteins is FLICE inhibitory protein, or FLIP.[297] Although FSH has not been shown to regulate the expression of FLIP in granulosa cells, the prosurvival effects of TNF-α in rat granulosa cells are accompanied by increased expression of both XIAP and FLIP.[325,326]

NF-κB. The PI-3K pathway can also signal to regulate cell survival by promoting the nuclear translocation of the ubiquitous transcription factor NF-κB. Seven proteins comprise the NF-κB family: p105 and its proteolytic product p50, p100 and its proteolytic product p52, RELA (p65), RELB, and c-REL.[327] These transcription factors function as heterodimers and bind to κB response elements on target genes. More than 200 genes have been reported to be regulated by NF-κB.[328] The prototypical NF-κB transcription factor complex consists of p50 and RELA.[327] p50/RELA exists in the cytosol as a latent transcription factor complexed to an IκB inhibitor, as shown schematically in Figure 20.11. The family of IκB inhibitors include IκB-α, the prototypical IκB inhibitor, as well as IκB-β, γ, and ε.[327] In response to growth factor or cytokine stimulation, the IκB kinase (IKK2; which exists in the cytosol in a complex with IKK1 and the scaffold protein NEMO) is activated and phosphorylates the IκB inhibitor, targeting IκB for ubiquinitation and proteosome-mediated degradation.[327] With the dissociation of IκB, the nuclear localization signal of RELA becomes exposed and p50/RELA translocates into the nucleus. Transcriptional activation of this complex, however, additionally requires the phosphorylation of RELA in a cell-specific manner.[327] RelA kinases include

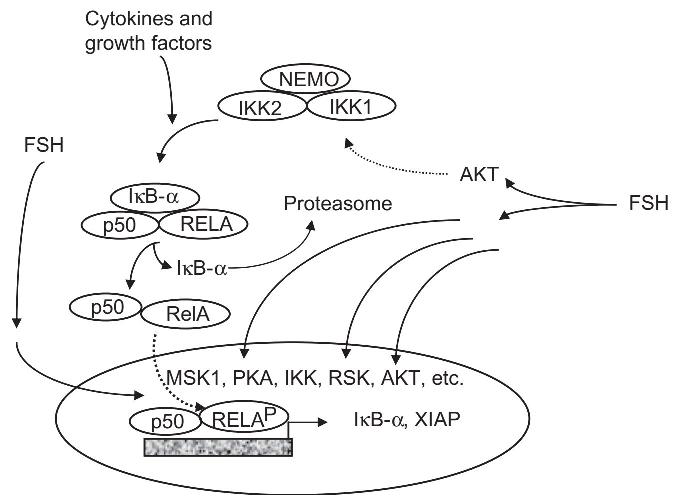

FIGURE 20.11 Schematic diagram of the pathway leading to NF-κB-dependent activation of target genes. Potential sites of FSH stimulation of this pathway are indicated. Dashed lines represent expected but not demonstrated regulation.

PKA, MSK1, RSK, AKT, PKCζ, casein kinase 2, IKK, and NF-κB-activating kinase.[327,329] Phosphorylation of RELA generally increases the binding of co-activators, like CBP, and/or DNA binding activity.[313]

FSH has been shown to promote the translocation of RELA from the cytoplasm to the nucleus of rat granulosa cells (as shown in Figure 20.11) and to increase NF-κB–DNA binding activity, detected by formation of a protein–DNA complex using the consensus κB binding site and granulosa cell nuclear extracts in mobility shift assays.[236] Additionally, the DNA–protein complex could be supershifted by antibodies to p50 and RELA, consistent with the presence of these proteins in the protein–DNA complex.[236] Inhibition of the nuclear translocation of p50/RELA with SN50, a drug that binds to and blocks the nuclear localization signal of NF-κB, inhibited the ability of FSH to induce the anti-apoptotic protein XIAP and suppressed NF-κB–DNA binding activity.[236] These results suggest that the caspase inhibitor XIAP may be an NF-κB target in granulosa cells (Figure 20.11). Pretreatment of granulosa cells with the PI-3K inhibitors LY292004 or wortmannin inhibited FSH-stimulated activation of p50/RELA DNA-binding activity as well as XIAP expression, suggesting a role for PI-3K in this pathway.[236] However, these authors did not determine whether the PI-3K inhibitors blocked translocation of NF-κB or affected its transactivation activity. Because AKT has been reported to enhance IKK activity[292] to phosphorylate IκB and to promote transactivation of RELA,[315] either/or both sites of action of PI-3K is possible. However, these authors did not detect the phosphorylation of IκB-α or its degradation during the first 30 min after treatment with FSH, in contrast to results with TNF-α-treated granulosa cells.[236] Moreover, modest (2.5-fold) overexpression of a dominant

negative IκB-α (which had its IKK phosphorylation sites mutated from Ser to Ala) did not inhibit the ability of FSH to increase p50/RELA DNA-binding activity or XIAP expression but did inhibit the ability of TNF-α to increase p50/RELA DNA-binding activity.[236] One interpretation of these results is that FSH regulates the nuclear translocation of p50/RELA by an alternative pathway independent of IκB phosphorylation. Because the transactivation of RELA can also be enhanced by PKA,[313] it is tempting to speculate that FSH-stimulated PKA also participates in RELA transcriptional activation (see Figure 20.11). Additional studies are thus required to clarify the mechanism by which FSH enhances NF-κB activity and to identify additional NF-κB target genes in granulosa cells.

NOS. An additional AKT substrate is endothelial nitric oxide synthase (eNOS; *Nos3*). NOS catalyzes the oxidation of L-arginine to nitric oxide (NO) and L-citrulline. NO regulates a number of biological functions including apoptosis, cell migration, and angiogenesis.[330] Phosphorylation of eNOS on Ser1177 by AKT results in its activation.[331] A major target of NO is the soluble guanylyl cyclase, the activation of which results in the production of cGMP.

Ovarian cells have been shown to express both eNOS (*Nos3*) and inducible NOS (iNOS; *Nos2*) in both a gonadotropin-dependent and -independent manner. Both eNOS and iNOS appear to be expressed in theca and stroma of immature rat follicles in the absence of gonadotropin stimulation[332]; eNOS, but not iNOS, is also expressed on the surface of oocytes.[332] The injection of PMSG to immature rats or addition of FSH to porcine granulosa cells induces the expression of eNOS, but not iNOS, in mural granulosa cells (~2.5-fold).[332] hCG injection to immature rats further increases eNOS expression 5–7 fold.[332] While iNOS-null mice do not exhibit a striking reproductive phenotype,[333] eNOS-null mice exhibit reduced rates of ovulation and oocyte maturation (>50%), and a five-fold increase in estrogen at a time when estrogen is 50% lower than proestrus peak levels in wild-type mice.[333,334] eNOS expression in response to both PMSG and hCG is regulated, in part, by nuclear orphan receptor LRH-1 (*Nr5a2*).[335] Conditional deletion of *Nr5a2* from granulosa cells, driven by *Amhr2-Cre*, results in reduced *Nos3* expression at 40 h post PMSG and 4 h post hCG, and CHIP assays place *Nr5a2* on the *Nos3* promoter 40 h post PMSG.[335] Conditional deletion of *Nr5a2* from granulosa cells also prevents the fall in estrogen that normally precedes ovulation, as discussed below in "Luteinization," consistent with the notion that LHR-1–regulated *Nos3* expression contributes to the LH/hCG-induced down-regulation of *Cyp19a1*. These results suggest that eNOS is most likely activated in response to the preovulatory surge of LH.

The signaling pathways that regulate eNOS activation to generate NO have not been studied in granulosa cells, although regulation by the PI-3K pathway downstream, at least of LH/hCG, is likely, based on evidence that the phosphorylation of eNOS on Ser1177 by AKT is activating.[331] FSH has been shown to produce only a minimal increase in cGMP levels,[336] and direct activation of soluble guanylyl cyclase in rat granulosa cells results in reduced FSH-stimulated cAMP production, estrogen, and inhibin-α synthesis.[336,337] These latter results suggest that it is unlikely that FSH signals to activate guanylyl cyclase activity in mural granulosa cells. However, FSH-dependent NO signaling to regulate pathways other than guanylyl cyclase cannot be excluded.

Testosterone Synergizes with FSH

It is common for investigators to include either testosterone or estrogen in primary granulosa cell cultures, based on evidence that they enhance FSH actions.[19] However, the mechanisms by which either estrogen or testosterone contributes to FSH-stimulated responses are undefined. A recent study showed that conditional deletion of the androgen receptor from preantral follicles, using *Amhr2-Cre*, resulted in subfertile mice that ovulated ~75% fewer oocytes, with ovaries that contained fewer preovulatory follicles and corpora lutea, and increased preantral and atretic follicles.[338] Follicles grew more slowly and appeared to be halted at the preantral stage of follicle development, consistent with the notion that the androgen receptor in conjunction with FSH is required for follicular maturation to the preovulatory state. In agreement with these results, addition of 1 μM testosterone to primary granulosa cells for 96 h promotes a striking (>15-fold) increase in *Cyp19a1* mRNA expression that is mediated, in part, by increased expression of *Nr5a2* (LRH-1), and increased association of *Nr5a2*, and not *Nr5a1*, with the *Cyp19a1* promoter, as evidenced by CHIP assay.[339] Testosterone plus FSH yields a synergistic increase in *Cyp19a1* mRNA expression.[339] Estrogen elicits a more modest ~two-fold induction of *Cyp19a1* mRNA while the testosterone metabolite 5α-dihydrotestosterone (DHT) is ~50% as effective as testosterone.[339] In contrast to these studies, there are reports that DHT inhibits follicular maturation.[19] Indeed, Menon's group showed that DHT (~ 25 nM, 24 h) inhibits FSH-stimulated ERK, Tuberin, and p70 S6 kinase phosphorylations resulting in reduced induction of cyclin D2 expression in granulosa cells.[262] The actions of DHT were mediated, in part, by promoting the phosphorylation of AMPK on Thr172, thereby activating this kinase.[199] While AMPK co-immunoprecipitates with ERK, the mechanism by which active AMPK inhibits FSH-stimulated ERK activation was not investigated.[199]

CROSS TALK AMONG FSH-REGULATED SIGNALING PATHWAYS AND TRANSCRIPTIONAL ACTIVATORS TO REGULATE GENE EXPRESSION DURING GRANULOSA CELL DIFFERENTIATION

As reviewed in the preceding sections, FSH regulates gene expression to inhibit apoptosis, drive proliferation, stimulate steroidogenesis, and promote a complex differentiation response that enables granulosa cells to respond appropriately to the LH surge. FSH accomplishes this array of functions by regulating posttranslational modifications of proteins that function as transcriptional activators, co-activators, repressors, or co-repressors, and of proteins that remodel chromatin to modulate gene activation. The majority of the responses to FSH appear to be mediated by PKA, as reviewed earlier. PKA can directly phosphorylate transcriptional activators, like CREB, and co-activators, like β-catenin, as discussed below, or can initiate signaling cascades that promote posttranslational modifications of transcriptional activators, such as GATA-4/6, co-activators, repressors, and co-repressors. PKA also enhances the translation of preexisting mRNA, such as that for HIF-1α, and newly transcribed mRNA. Figure 20.12 reviews some of the FSH target genes that are regulated directly or indirectly downstream of PKA-regulated transcriptional activators, co-activators, and repressors, as described in preceding and subsequent sections. As we will review below, it is apparent that no single transcriptional activator is sufficient to activate a target gene, and that a number of pathways converge on the promoters of these genes to activate multiple transcriptional activators and co-activators, to remodel chromatin, and to remove repressors and co-repressors. FSH also represses expression of target genes, such as those that stimulate apoptosis (*BIM*),[290] as previously discussed; however, this topic is less well defined. FSH also stabilizes mRNA, at least for the *Lhcgr*; this FSH response is also poorly understood.[340] In this section, we will summarize our current understanding of the convergence of signaling pathways on various transcriptional regulators to stimulate or inhibit expression of select FSH target genes.

LH Receptor

The rate of *Lhcgr* transcription and *Lhcgr* mRNA stability are increased by FSH.[340] While cAMP is required to stabilize *Lhcgr* mRNA in granulosa cells, the mechanisms of its actions are complex and poorly understood.[340] Transcriptional regulation of *Lhcgr* has been more extensively investigated. cAMP responsiveness of the 5′-*Lhcgr* promoter was attributed, in part, to three Sp1 binding sites.[341] Sp1 is a common PKA-dependent transcriptional activator for a number of FSH target genes, including *Sgk*,[26] *Egr-1*,[29] and *Ereg*.[27] Sp1 binds to "GC"-rich motifs, and its transcriptional activity is enhanced upon phosphorylation at a number of Ser/Thr residues by various kinases including ERK or PKCζ downstream of PI-3K.[169] As both the ERK and PI-3K pathways are activated in a PKA-dependent manner in granulosa cells, as previously reviewed, Sp1 could be regulated by either or both kinases. Consistent with a role for ERK in FSH-stimulated expression of EGR-1, FSH-stimulated Sp1 binding to the *Egr-1* promoter is reduced by pretreatment of granulosa cells with the MEK inhibitor PD98059.[29] However, the ability of either ERK or PKCζ to phosphorylate Sp1 and regulate its binding to FSH target genes has not been demonstrated in granulosa cells.

We recently showed that the co-activator β-catenin contributes to the ability of PKA to activate *Lhcgr*

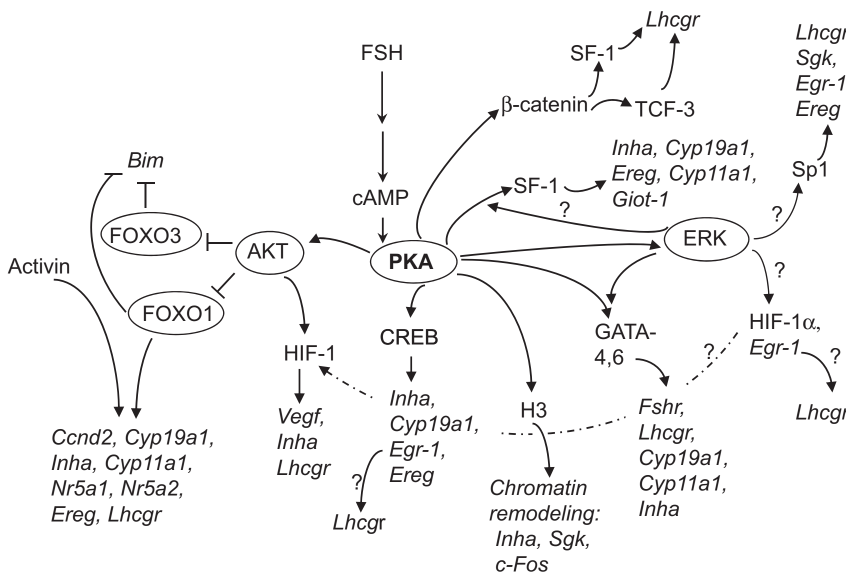

FIGURE 20.12 Composite regulation of FSH-responsive gene targets. Question marks (?) for ERK signaling reflect inhibition by a MEK inhibitor but not proof of target phosphorylation. Regulation of Lhcgr by Egr-1 is suggested by literature.

transcription[177]. Transduction of granulosa cells with an adenovirus that expresses a mutant SF-1 that cannot bind β-catenin[342] blocks the ability of FSH to promote *Lhcgr* mRNA expression. This result suggests that SF-1 is necessary for *Lhcgr* mRNA expression and that FSH activates β-catenin. FSH stimulates the PKA-dependent phosphorylation of β-catenin on Ser552 and Ser665, sites that have been linked with the ability of β-catenin to act as a co-activator for transcriptional factors,[178,179] and activation of the TCF artificial promoter-reporter TOPFlash, consistent with a previous report.[303] In support of these results, transduction of granulosa cells with an adenoviral β-catenin mutant that mimics phosphorylation of these sites (Ser552Asp, Ser665Asp) enhanced *Lhcgr* mRNA expression in FSH-treated cells.[177] The ability of β-catenin to function as a co-activator for SF-1 is now recognized in a number of cell models.[175,176,286] SF-1 is also necessary for expression of other FSH target genes, such as *Cyp19a1*, *Ereg*, *Cyp11a1*, *Inha*, and *Giot-1*. We hypothesize that the PKA-dependent phosphorylation of β-catenin contributes to the PKA-dependent regulation of these genes and is the predominant mechanism by which β-catenin activity is regulated in granulosa cells. However, this hypothesis has not been formally tested for genes other than the *Lhcgr*.

Results also suggest that β-catenin functions in granulosa cells to promote expression of *Lhcgr* mRNA by displacing co-repressors from TCF3 bound to the *Lhcgr* promoter.[177] This conclusion is based on data showing that an adenoviral dominant negative TCF,[343] in which the β-catenin binding site was deleted, abrogated the ability of FSH to induce *Lhcgr* mRNA. This suggests that β-catenin functions not only as a co-activator for SF-1 but also competitively displaces a member of the co-repressor Groucho-related genes family to activate TCF3.[344] Consistent with these findings, CHIP results showed that TCF3 is constitutively associated with *Lhcgr* promoter, and that FSH stimulates association of β-catenin phosphorylated on Ser552 and Ser675 as well as SF-1 with *Lhcgr* promoter.[177] Microarray results suggest that TCF3 is a common activator of a number of FSH target genes, such as *Inha*, *Cyp19a1*, and others, but additional experiments are required to confirm this hypothesis.

Lhcgr mRNA expression also appears to require expression of the immediate early gene EGR-1, based on evidence that follicles of EGR-1-null mice do not acquire LH receptors and thus cannot ovulate.[345,346] EGR-1 is a zinc-finger transcription factor that binds GC-rich enhancer elements.[347] FSH transiently increases EGR-1 expression in granulosa cells.[29] Activation of the *Egr-1* promoter is PKA dependent and involves ERK-dependent binding of Sp1 and Sp3 and phosphorylated CREB, as evidenced by electromobility shift assays and promoter mutational analyses.[29] Evidence that EGR-1 regulates *Lhcgr* expression has only been provided indirectly by electrophoretic mobility shift assays and by the ability of overexpressed EGR-1 to modestly enhance *Lhcgr*-promoter luciferase activity in a Leydig cell line.[346] Additional studies are required to define more completely the regulation of *Lhcgr* by EGR-1.

Lhcgr mRNA expression also appears to require HIF-1 and relief from repression by FOXO1, both of which are regulated downstream of PKA and PI-3K signaling. Although formal evidence for the regulation of *Lhcgr* mRNA expression by HIF-1 is not available, indirect data showed that dominant-negative HIF-1α missing its DNA binding and transactivation domains abolished FSH-stimulated *Lhcgr* promoter-luciferase reporter activity.[235] *Lhcgr* has also been identified as a FOXO1 target whose FSH-stimulated mRNA expression is reduced in cells transduced with adenoviral A3-FOXO1.[177,271]

In summary, pathways that contribute to the transcriptional activation of this gene include: PKA via either ERK or PKCζ to phosphorylate/activate Sp1; PKA to phosphorylate/activate β-catenin to activate both SF-1 and TCF3; PKA via PI-3K to phosphorylate/inactivate FOXO1; PKA via PI-3K and another pathway, possibly ERK, to promote accumulation of HIF-1α and activate HIF-1; and PKA to phosphorylate/activate CREB plus ERK or PKCζ to phosphorylate/activate Sp1 to promote expression of the immediate early gene EGR-1. Finally, we hypothesize that PKA also promotes remodeling of chromatin associated with *Lhcgr* gene by stimulating the phosphorylation of Ser10 and acetylation on Lys14 of histone H3, as previously discussed for the *Inha* promoter.[118] However, there is no direct evidence that either of these histone H3 modifications is linked to the *Lhcgr* promoter.

Inhibin-α and Aromatase

The expression of both inhibin-α and aromatase have been extensively studied regarding the combinations of factors required to promote their transcription. Unlike the *Lhcgr* promoter, the *Inha* promoter contains a functional nonconcensus CREB binding site as well as an SF-1 binding site. SF-1/LRH-1, both of which bind to the same DNA element,[348] and cAMP/PKA synergize to activate the *Inha* promoter.[170,349] The SF-1 and CREB sites are adjacent to each other on the promoter, and although SF-1 and CREB can interact in extracts, it is not known if this interaction is direct.[170] The co-activators CBP and SRC-1 further increase *Inha* transcriptional activity in a mouse granulosa cell line, and the association of both with the *Inha* promoter is enhanced by forskolin, as evidenced by CHIP assays.[349] Recent studies suggest that the LIM domain protein four and a half LIM domain 2 (FHL2) also functions as a co-activator of SF-1/LRH-1 and CREB in granulosa cells.[350] FHL2 is recruited to the *Inha* promoter in a granulosa cell line in response to

elevated cAMP levels, although the mechanism of this recruitment was not investigated.[350] Unlike CBP and the co-activator SRC-1, FHL2 lacks enzymatic activity and may function as a scaffold to integrate the association of other co-activators.

CHIP assays also revealed that while SF-1 is associated with the *Inha* promoter in the basal state, forskolin stimulates the release of SF-1 and binding of LRH-1 (which is induced by forskolin) in a granulosa cell line.[349] These cAMP-dependent associations of SF-1/LRH-1 with the *Inha* promoter, as well as cAMP-stimulated *Inha* promoter-luciferase activity, were inhibited by the MEK inhibitor PD98059,[349] possibly reflecting ERK phosphorylation of SF-1[203] and LRH-1.[351] The mechanistic basis for the dynamic associations of SF-1 and LRH-1 with the *Inha* promoter is not known, but might reflect distinct posttranslational modifications or distinct associations of the co-activator β-catenin[175,176] with SF-1 versus LRH-1. While SF-1 and LRH-1 tend to have overlapping actions,[352] selective regulation of the *Cyp19a1* expression by LRH-1 in testosterone-treated granulosa cells has been reported and seems to be due to preferential expression of LRH-1 by testosterone.[339]

The ability of cAMP to activate the *Inha* promoter thus likely reflects actions at a number of sites. cAMP-activated PKA phosphorylates CREB on Ser133, promoting recruitment of CBP.[167,211] PKA also promotes the phosphorylation on Ser10 and acetylation on Lys14 of histone H3, as previously discussed, thus remodeling chromatin in the region of the *Inha* promoter.[118] PKA is also recognized to potentiate SF-1 transcriptional activity both via the phosphorylation/activation of β-catenin and via ERK-mediated phosphorylation of SF-1, as previously described. *Inha* expression is also regulated by HIF-1, based on the ability of a dominant-negative HIF-1α to reduce significantly the ability of FSH to activate *Inha* promoter-reporter activity.[235] Additionally, expression of constitutively active FOXO1 in granulosa cells reduces the ability of FSH plus activin to activate *Inha* expression,[48] although there is no formal evidence that FOXO1 represses *Inha* expression.

There is also accumulating evidence that GATA transcription factors regulate a number of FSH targets, including *Inha* in granulosa cells. GATA factors are zinc-finger DNA binding proteins. GATA-4 and -6 are highly expressed in granulosa cells,[353] and promoters of several FSH target genes contain functional (*Inha*, *Nr5a1*, *Hsd17b*, *Cyp11a1*, *Cyp19a1*, *Star*) or putative (*Lhcgr*, *Hsd3b1*) GATA regulatory elements.[354,355] GATA-4 is phosphorylated on Ser261 directly by PKA[356] and on Ser105 by ERK.[357] *Inha* gene expression is activated by GATA-1 in testicular cell lines,[358,359] and PKA-phosphorylated GATA-4 enhances an *Inha* promoter-reporter construct in CV-1 cells.[356] GATA-4 also regulates expression of *Cyp19a1* in granulosa cells.[360] FSH stimulates the association of GATA-4 with the *Cyp19a1* promoter, as shown by CHIP assays, and GATA-4 siRNA reduces the ability of FSH to promote *Cyp19a1* mRNA expression. A nonphosphorylatable Ser105 GATA-4 mutant prevents the induction of *Cyp19a1* mRNA by FSH, and the MEK inhibitor U0126 abolishes FSH-stimulated phosphorylation of GATA-4 that was transfected into granulosa cells.[360] That GATA-4 is required for follicular maturation was recently demonstrated with conditional deletion of both GATA-4 and GATA-6 in granulosa cells with the *Cyp19-Cre*.[353] Mice are infertile, follicular maturation is arrested prior to antrum formation, and granulosa cells exhibit reduced *Fshr*, *Lhcgr*, *Cyp19a1*, and *Cyp11a1* mRNA. Together these results show that GATA-4 is necessary for follicular maturation and that its transcriptional activity is regulated by PKA.

FSH also regulates the expression of a protein, DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on the X-chromosome, gene 1, *Nrob1*), that functions as a transcriptional repressor of Nr5a1 (SF-1) and Nr5a2 (LRH-1).[342] FSH reduces DAX-1 mRNA and protein levels, and overexpression of DAX-1 has been shown to prevent activation of *Giot-1* promoter activity by SF-1 in granulosa cells.[30] Expression of the *Cyp19a1* gene is also inhibited by DAX-1,[361] suggesting that for those genes that require binding of SF-1 or LRH-1, decreased expression of DAX-1 may be a conserved response to FSH. However, the mechanism by which FSH downregulates *Dax-1* mRNA has not, to our knowledge, been investigated.

Taken together, FSH-stimulated target gene activation requires input from a number of pathways, as summarized in Figure 20.12. Inhibitors of PKA, PI-3K, and MEK abrogate the ability of FSH to activate select target genes. The requirements for both PKA-phosphorylated CREB as well as for SF-1 and/or Sp1 and/or GATA-4/6 to promote transcriptional activation of a number of FSH target genes coupled with their dependence on the PI-3K pathway explains, in part, why activation of the PI-3K pathway by IGF-1 is not sufficient to induce FSH target genes. Although some of the transcription factors and co-activators required to activate a small subset of FSH target genes have been identified, much needs to be learned regarding the combinatorial requirements for transcription factors and co-activators and how the activity of these factors is regulated. Moreover, it is likely that additional signaling pathways are regulated by FSH. Finally, we know very little about how FSH-dependent pathways converge with the many signaling systems involved in initial formation and early growth of the follicle, as discussed in Chapter 21. Thus, there is a great deal remaining to be elucidated regarding how FSH signals to promote follicular maturation.

LH SIGNALS THAT REGULATE THECA CELL FUNCTIONS

Follicles are exposed to relatively low levels of LH as follicle maturation proceeds. The resulting increased production of androgens provides the substrate for aromatase in granulosa cells to synthesize estrogen. In addition to facilitating the actions of FSH, these increasing levels of estrogen act in a positive feedback fashion on the brain to induce the preovulatory LH surge. In pathological conditions such as polycystic ovarian syndrome, theca cells produce elevated levels of androgens. Thus, an understanding of how LH regulates androgen production is important.

LH has been shown to enhance mRNA expression of the transcription factor required for cholesterol biosynthesis, sterol regulatory element binding transcription factor 1a (*Srebf1a*), and HMG-CoA reductase(*Hmgcr*), the rate-limiting enzyme in cholesterol biosynthesis.[362] LH also induces 17α-hydroxylase/C17-20-lyase (CYP17; *Cyp17a1*), the rate-limiting enzyme in androgen biosynthesis in theca cells,[363] and the low-[364] or high-[365] density lipoprotein receptor, depending on the species, to provide cholesterol for steroid biosynthesis. While the signaling pathways and transcriptional regulation of LH-regulated genes in theca cells have not been intensely investigated, recent studies have placed SF-1 on both the *Cyp17a1* and *Star* promoters, and GATA-6 on the *Cyp17a1* promoter, as shown by CHIP studies.[366] There is also evidence that the nuclear orphan receptor NR4A1/NUR77 contributes to the ability of LH to induce genes required for androgen biosynthesis including *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b2*.[367] Overexpression by adenoviral transduction of *Nr4a1* increases and knockdown of *Nr4a1* reduces the expression of all of these genes in response to LH. The induction of these mRNAs as well as that of *Nr4a1* is mimicked by forskolin, consistent with a cAMP-regulated pathway.[367] LH via a PKA-dependent step has been shown to signal into the AKT, Tuberin, mTORC/Raptor pathway to stimulate the phosphorylation of p70 S6 kinase, leading to the upregulation of cyclin D3, *Hsd3b1*, *Cyp11a1*, and *Cyp17a1*.[368] Although this pathway is generally reserved for regulating translation,[250] in theca cells p70 S6 kinase phosphorylates CREB on Ser133. CHIP assays place CREB on the *Cyp17a1* promoter.[368] The requirement for the mTORC/Raptor pathway to regulate expression of *Hsd3b1*, *Cyp11a1*, *Cyp17a1*, and *Ccnd3* is based on inhibition by the mTORC/raptor inhibitor rapamycin and by mTORC siRNA studies.[368]

Thus, while some of the signals leading to enhanced transcription of genes that promote androgen production have been identified, the persistent regulation of these pathways during the life of the follicle until the surge of LH remains incompletely understood. It is quite interesting, as discussed below, that the signaling pathways initiated by LH to regulate gene expression in theca cells are quite distinct from the pathways used by LH to regulate ovulation and corpus luteum formation.

THE LH SURGE PROMOTES OOCYTE MATURATION, OVULATION, AND CORPUS LUTEUM FORMATION

A Brief Overview of the Physiology of Oocyte Maturation, Ovulation, and Luteinization

Follicles are exposed to a single high level surge of LH, driven by the hypothalamic release of gonadotropin-releasing hormone that promotes ovulation and differentiation of mural granulosa and theca cells (but not cumulus granulosa cells) into luteal cells (luteinization) and triggers final maturation of the oocyte. These responses to LH require the induction of more than 500 genes.[369]

Cumulus Expansion. Preceding ovulation, the cumulus granulosa cells and oocyte become surrounded by a hyaluronan-rich extracellular matrix that facilitates the expansion/movement of cumulus granulosa cells within this matrix. Cumulus expansion and oocyte maturation require, for example, the induction of hyaluronic acid synthase-1 *Has-2*, pentraxin-related protein 3 *Ptx3*, and TNF-α-stimulated gene-6 *Tnfaip-6*.[370] Mice that are deficient in *Ptx3* and *Tnfaip-6* exhibit ovulation defects,[371,372] consistent with a link between cumulus expansion and ovulation.

Ovulation. Ovulation is a complex, incompletely understood process that results in the breakdown of the follicular wall and release of the oocyte and surrounding cumulus granulosa cells. The oocyte and surrounding cells escape through the mural granulosa cell layer, the basement membrane, the theca cell layer, and ovarian surface epithelium. LH induced genes that are required for ovulation include the cyclic nucleotide degrading phosphodiesterase *Pde4d*[34], *Ptgs2* that encodes the rate-limiting enzyme (prostaglandin-endoperoxide synthase) in prostaglandin synthesis,[373] and *Pgr* that encodes the progesterone receptor[374] and its downstream targets pituitary adenylate cyclase activating polypeptide (*Pacap*),[375,376] lysosomal protease cathepsin L (*Ctsl)*,[377] and the matrix metalloproteinase *Adamts-1* (a disintegrin and metalloproteinase with thrombospondin-like repeats).[378]

Oocyte maturation. Oocytes are restrained in prophase I of meiosis until just before ovulation. Breakdown of the nuclear membrane, also known as germinal vesicle breakdown, marks an oocyte that has reinitiated meiosis. Completion of the second meiotic division after fertilization results in the production of two polar bodies and a single haploid oocyte.

Luteinization. Luteinization is associated with a cessation of granulosa and theca cell proliferation and is characterized by hypertrophy of both granulosa and theca cells[379] as well as by increased steroid hormone synthesis and secretion. Luteinization requires the downregulation of cyclin D2 (*Ccnd2*),[45] and expression of the rate-limiting enzyme in progesterone synthesis encoded by *Cyp11a1*,[380] the cholesterol-mobilizing protein *Star*,[376] and cell cycle inhibitors p21^{Cip1} and p27^{Kip1}[45,381] encoded by *Cdkn1a* and *Cdkn1b*, respectively. The surge of LH also terminates the expression of select FSH target genes that were required for follicular maturation but are not required for luteal function. For example, the following genes are transcriptionally silenced, as discussed below, in response to LH: the FSH receptor (*Fshr*),[69] estrogen receptor β (*Esr2*),[382] *Cyp19a1* (aromatase),[383] *Inha* (inhibin-α),[384] and *Foxo1*.[112] The LH receptor[385] (*Lhcgr*) is transiently downregulated by the LH surge; however, the mechanism is not via reduced transcription, as discussed below. The expression of other FSH targets whose functions are required either during luteinization or for luteal function are not affected by the surge of LH include, for example, *Map2d*,[25] *Cyp11a1*,[380] and *Sgk*.[112] Some of these responses are also variable among species; for example, inhibin-α is repressed by the LH surge in the rodent,[384] but inhibin A is actively secreted during the luteal phase in the primate reproductive cycle.[386]

LH and the LH Receptor

The pituitary gonadotropin LH shares a common alpha subunit with FSH, but its synthesis and secretion from pituitary gonadotropes is under distinct regulation. For many of the studies discussed in this chapter, the related placental gonadotropin hCG (Figure 20.1) is used as an agonist of the LH receptor. The LH receptor, like the FSH GPCR, contains nine Leu-rich-repeat motifs in its extracellular domain and is classified in subgroup A of the rhodopsin/β-adrenergic family of GPCRs[9,387] (Figure 20.1). These Leu-rich-repeats contribute to hormone binding and structural integrity of this receptor.[388] The LH receptor is encoded by a gene with 11 exons and 10 introns.[6] Exons 1–7 encode the regions of the large extracellular domain that are essential for high affinity binding of LH receptor agonists and the placental-derived hCG.[389,390] Exons 9 and 10 also encode the extracellular domain, but this region appears to be less important for agonist binding.[386] Exon 11 encodes the seven TM domains and the C-terminal tail. The LH receptor in female mammals is expressed in the ovary in theca cells of ovarian follicles, in mural granulosa cells of mature preovulatory follicles, and in corpora lutea. LH receptor-null mice are infertile; their ovaries contain only early antral follicles and lack preovulatory follicles or corpora lutea.[391]

The absence of follicular development to a preovulatory stage in LH receptor-null mice likely reflects, at least in part, the reduced levels of theca-derived androgens and thus granulosa-derived estrogens,[391] and the apparent requirement of these steroid hormones for final follicular maturation. Granulosa cells also express androgen receptors.[392] Androgen receptor-null mice exhibit increased granulosa cell apoptosis and defective cumulus cell expansion in response to the LH surge,[393] and conditional deletion of the androgen receptor from granulosa cells results in subfertile mice that ovulate ~75% fewer oocytes.[338] Together these results suggest a role for androgens in granulosa cells distinct from their function as an aromatase substrate. Although granulosa cells express primarily ER-β and theca cells express ER-α, mice null for ER-α or ER-β ovulate in response to exogenous gonadotropins, although ovulation efficiency is reduced, suggesting that estrogen facilitates ovulation.[394] Incomplete follicular maturation in the LH receptor-null mice could also result from effects of basal LH on granulosa cells distinct from those described for the surge of LH.

In the absence of agonist, GPCRs are believed to exist in the membrane primarily in an inactive conformation that is in equilibrium with an active conformation.[59] Agonist binding is thought to stabilize the receptor in the active conformation. Consistent with this notion, and unlike the FSH receptor, a number of mutations in the LH receptor have been identified that stabilize the receptor in an active conformation in the absence of agonist.[395] The first human mutation of the LH receptor identified was Asp578 in TM 6 in patients with familial male-linked precocious puberty.[396] This mutation results in a constitutively active LH receptor, which in males leads to precocious puberty. For reasons that are not clear, females with this LH receptor mutation do not exhibit abnormal fertility.[395] This may reflect a stringent requirement for postpubertal levels of FSH to induce LH receptor expression in the female ovary. The absence of a phenotype in women with constitutively active LH receptors is especially unexpected in light of the clear phenotype in mice overexpressing a longer-lived LH mutant protein, which is characterized by precocious puberty, anovulation, and resulting infertility.[397] TM 6 and its extension into the cytoplasm as the third intracellular loop have been defined as hot spots for LH receptor mutations that promote a constitutively active receptor, and more than 16 mutations have been identified in this region.[395] Additionally, Ser431 in TM 3,[398] Arg442 in the second intracellular loop,[399] Asp397 in exoloop 1,[400] and Lys583 in exoloop 3[401] of the rat LH receptor appear to contribute to the stabilized agonist-dependent active conformation of the LH receptor to generate cAMP (without affecting agonist binding), whereas Lys583 in exoloop 3 also appears to stabilize the agonist-stimulated active state

of the receptor to generate inositol trisphosphate (IP_3).[402] A large number of inactivating mutations of the LH receptor in women have been reported.[395] These women exhibit amenorrhea and no preovulatory follicles or corpora lutea, consistent with the established functions for LH, and do not respond to exogenous hCG.

The LH receptor in granulosa cells is induced by FSH[403]; LH receptor synthesis then ceases in response to the surge of LH, as discussed below, and is reinitiated with the formation of the corpus luteum. Hormonal regulation of the induction of LH receptors in corpora lutea is species dependent,[404,405] consistent with the high degree of species specificity in the factors that regulate formation, maintenance, and regression of the corpus luteum. Theca cells appear to express LH receptors coincident with the morphological formation of this cell layer at very early stages of follicular development[69]; however, it is not known if LH receptor expression is constitutive in these cells or is regulated by a paracrine factor produced either by the oocyte or by adjacent granulosa cells.

The LH receptor couples to activate Gs,[406,407] uniformly resulting in activation of adenylyl cyclase to increase synthesis of cAMP.[408] The LH receptor has also been shown to activate Gi,[409–411] Gq/11, and G13, based on the ability of LH to stimulate binding of a GTP photoaffinity analog to these Gα proteins.[411] Although LH receptor activation can stimulate Gi to inhibit adenylyl cyclase activity upon expression in a heterologous cell model,[412] LH-stimulated adenylyl cyclase activity in porcine follicular membranes is not increased by pretreatment of membranes with pertussis toxin, which uncouples GPCRs from Gi.[409] Thus, the physiological significance of LH receptor coupling to Gi is not known. Constitutively active LH receptors expressed in heterologous cells also activate PLC, resulting in elevated levels of IP_3 and Ca^{2+}.[408,413,414] Similarly, when LH receptors are expressed at sufficiently high concentrations, receptor activation raises intracellular Ca^{2+} and activates PLC.[415–417] This response is pertussis toxin insensitive[410] and thus most likely mediated via activation of Gq/11. In rat granulosa cells, which express approximately 10,000 receptors per cell,[411] LH raises intracellular levels of IP_3 presumably via activation of PLC.[418] Thus, signaling through cAMP appears to be the predominant but not the only second messenger through which the LH receptor can modulate responses.

Although the exact residues that bind Gs on the LH receptor have not been identified, the C-terminal portion of the third intracellular loop extending into TM 6 domain is important, based on the ability of a synthetic peptide corresponding to this region to activate adenylyl cyclase in the absence of LH receptor expression.[419] Truncation of the C-terminal region of the rat LH receptor at residue 653 does not affect agonist-stimulated adenylyl cyclase activation but hampers receptor-stimulated PLC activation, suggesting that the G protein that activates PLC couples to a distinct region of the receptor from that which signals to Gs.[420]

The LH receptor also exhibits desensitization and, in heterologous cell models, relatively rapid internalization in response to saturating levels of agonist. However, the preovulatory LH surge does not promote rapid internalization of the LH receptor in granulosa cells. Rather, cell surface receptor numbers around the time of ovulation decrease with a T1/2 of approximately 8.5h.[421–423] Although this receptor can be phosphorylated on up to five serine residues located in the C-terminal tail[424,425] in a G protein-regulated kinase-dependent manner,[426,427] phosphorylation of the human and porcine receptors does not contribute to either uncoupling from Gs/adenylyl cyclase[424,428–430] or receptor internalization.[431] Similarly, for the rat LH receptor, mutation of the serine residues in the C-terminal tail retards the rate of desensitization but does not affect the extent of LH receptor desensitization.[420,424,426] These results are consistent with evidence that truncation of the C-terminal tail of the LH receptor does not prevent LH receptor desensitization.[432] A protein that interacts with the C-terminus of the human LH receptor, the type I PDZ domain protein GIPC,[433] appears to regulate levels of cell surface LH receptor during hormone internalization and to play a role in ligand recycling.

Thus, unlike the β-adrenergic and rhodopsin GPCRs, in which high affinity binding of arrestin to the receptor is facilitated by receptor phosphorylation,[89,434] binding of arrestin to the LH receptor and consequent uncoupling from Gs appears to depend on receptor activation rather than phosphorylation.[431,435–437] Indeed, arrestin2 binds with pM affinity to a synthetic peptide corresponding to the third intracellular loop of the porcine LH receptor to promote desensitization.[438,439] The accessibility of arrestin2 to the LH receptor in porcine membranes and in HEK kidney cells stably transfected with the murine LH receptor is regulated by the association of arrestin2 with the small G protein ADP-ribosylation factor (ARF)6.[439,440] LH receptor activation promotes activation of membrane-delimited ARF6[441] by ARNO (ARF-nucleotide binding site opener) or a similar ARF6 activator,[442] releasing arrestin2 from its membrane docking site.[438] The binding of arrestin to GPCRs has been shown to sterically hinder the ability of the receptor to bind Gs.[439] Consistent with this model, both Gs binding and arrestin2 binding appear to require the C-terminal portion of the third intracellular loop of the LH receptor.[419,438] Although this pathway for ARF6-dependent desensitization has not been tested in other cellular models, overexpressed ARNO is reported to stimulate arrestin-dependent β-adrenergic receptor internalization, and an inactive ARF6 that cannot bind GTP inhibits β-adrenergic receptor internalization.[443] These results

suggest that ARF6 might play a more universal role in regulating arrestin availability to GPCRs.

Coupling of the LH receptor to Gs is also regulated in a poorly understood manner by PDE4D.[34] Unexpectedly, in granulosa cells of PDE4D null mice, the immediate (detected by 1 h) and the sustained (over 6 h) cAMP synthetic response to an ovulatory concentration of hCG (both in vivo and in vitro) is significantly blunted (and not increased), whereas the response to the adenylyl cyclase activator forskolin (in vitro) is equivalent compared with that of wild-type mice.[34] This result suggests that PDE4D in some manner facilitates coupling between the LH receptor and Gs to activate adenylyl cyclase and that in the absence of PDE4D, coupling between LH receptor and Gs is impaired. LH receptor-Gs coupling is likely regulated by the sustained levels of PDE4D induced in granulosa cells by FSH rather than that induced by LH.[34] How PDE4D enhances LH receptor-Gs coupling remains to be elucidated.

MECHANISMS BY WHICH LH STIMULATES OOCYTE MATURATION, OVULATION, AND LUTEINIZATION

LH-Regulated Signaling Pathways in Preovulatory Cells

The remainder of this chapter will focus on our current understanding of the cellular mechanisms by which LH stimulates oocyte maturation, ovulation, and luteinization. The reader is also referred to Chapter 22 on ovulation. We begin by summarizing LH-stimulated signaling pathways; however, additional information on specific pathways is provided in the context of the following sections.

cAMP- and PKA-Dependent Signaling to CREB and ERK. The LH receptor is believed to signal predominately via Gs to activate adenylyl cyclase and raise intracellular levels of cAMP. Consistent with this notion, the adenylyl cyclase activator forskolin mimics LH to stimulate expression of a number of LH target genes.[29,364,444–447] The primary cAMP target is PKA. Indeed, ovulatory concentrations of LH/hCG in preovulatory follicles lead to activation of PKA,[448] and the specific PKA inhibitor PKI[119] or the selective PKA inhibitor H89[123] inhibits the induction of a number of LH targets or responses.[29,364,448]

As in preantral granulosa cells, hCG or forskolin treatment of preovulatory granulosa cells stimulates the phosphorylation of the PKA target CREB on Ser133.[151,449] Although LH-stimulated CREB phosphorylation is undetectable when granulosa cells are pretreated with the selective PKA inhibitor H89,[424] it is possible that other pathways downstream of PKA also contribute to CREB phosphorylation. LH target genes that contain cAMP response elements and have been shown to bind CREB include EGR-1,[29] ICER,[450] and StAR.[451]

As will be highlighted in the following discussion, one of the most important signaling pathways by which LH regulates gene expression is via the ERK1/2 (MAPK3/1) pathway (Figure 20.13). The initial route to ERK is via PKA, based on the ability of H89 and Myr-PKI to strongly inhibit LH-stimulated ERK activity in mural granulosa cells.[452] However, in contrast to regulating the association of a PTP with ERK as is observed in preantral granulosa cells,[113] PKA signals to regulate ERK activity in preovulatory granulosa cells at a step upstream of MEK. While LH stimulates MEK phosphorylation,[452] the site of regulation by PKA has not been elucidated in preovulatory granulosa cells. In MA-10 and primary Leydig cells, LH signals via PKA into the ERK pathway to activate RAS.[452,453] However, the relevant direct PKA substrate in either of these cell types has yet to be identified.

Sustained ERK activation is required for LH responses that lead to oocyte maturation, ovulation, and luteinization. This is achieved by the ability of LH to promote activation of an EGF receptor located in both mural and cumulus granulosa cells in a PKA-dependent manner.[451,454] The EGF receptor family consists of the EGF receptor (ErbB1, HER), ErbB2 (Neu, HER2), ErbB3 (HER3), and ErbB4 (HER4); granulosa cells express ErbB1, ErbB2, and ErbB3.[455] ErbB1 binds EGF or EGF-like ligands to promote homo- or heterodimerization and activation of its tyrosine kinase activity.[456] An elegant series of studies have shown that LH generates a group of EGF-like ligands that include amphiregulin, epiregulin, and β-cellulin that are synthesized as transmembrane precursors and then shed as mature proteins by cleavage at the membrane surface by a metalloproteinase[454] (Figure 20.13). LH is believed to promote both the de novo synthesis of these ErbB ligands, based on inhibition by puromycin, and to activate a metalloproteinase, based on inhibition by pan-metalloproteinase inhibitors, to generate these EGF-like ligands in a PKA-dependent manner in mural granulosa cells.[451] The ErbB1 ligands promote activation of ErbB1 on both mural and cumulus granulosa cells to signal through the canonical EGF receptor pathway to activate ERK.[456] The functional significance of the ERK pathway to LH actions is discussed below. The ErbB1 ligands also signal via the EGF receptor to activate p38 MAPK (MAPK14), also discussed below.

LH also stimulates expression of the EGF-like factor neuroregulin-1, via an ERK-dependent pathway, and this ligand binds ErbB3.[455] The ErbB3 receptor appears to signal predominately to the PI-3K/AKT pathway in granulosa cells, based on the ability of neuroregulin-1 to enhance AKT but not ERK phosphorylation.[455] Both amphiregulin and neuroregulin-1 contribute to luteinization by enhancing expression of genes required for progesterone production and oocyte maturation.[455]

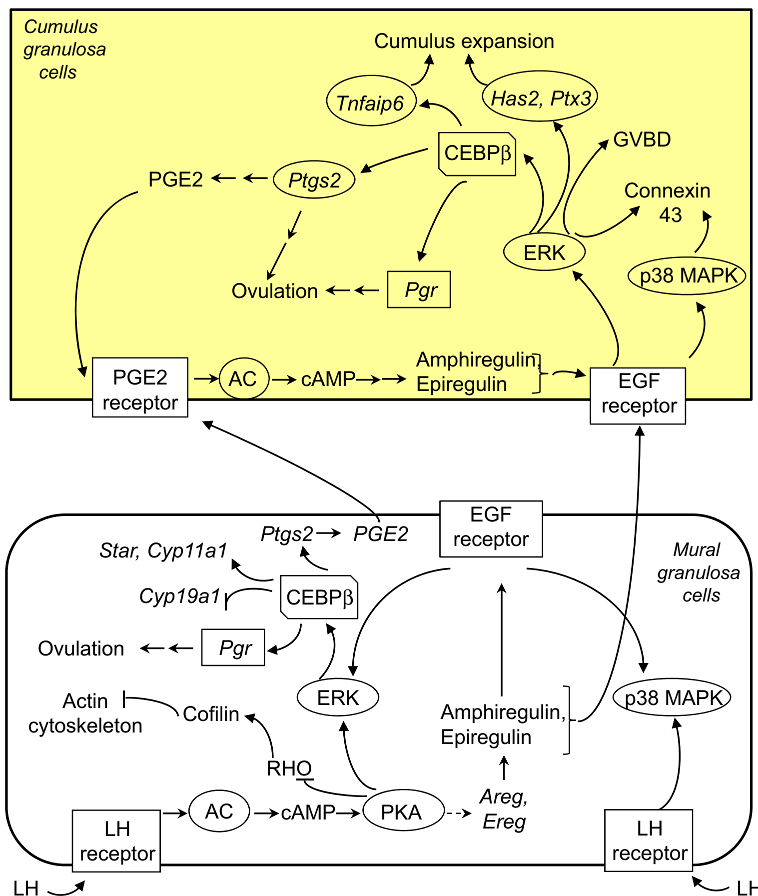

FIGURE 20.13 **LH signals that regulate ovulation, cumulus granulosa cell expansion, and luteinization.**

LH signaling to p38 MAPK. hCG signals to rapidly phosphorylate p38 MAPK(MAPK14) in preovulatory granulosa cells[452] as well as its upstream kinases MKK3 and MKK6.[217] However, at least the initial signaling into the p38 MAPK pathway appears to be independent of PKA, based on the inability of H89 to modulate hCG-stimulated p38 MAPK phosphorylation in cells in which hCG-stimulate CREB phosphorylation was blocked by H89.[452] The functional significance of the p38 MAPK pathway activated by hCG and ErbB1 ligands is discussed later.

LH Increases Intracellular Ca^{2+} but Does Not Appear to Activate PKC. It is well established that the LH receptor, upon expression in a heterologous cell, activates PLC to raise IP_3 levels and/or intracellular Ca^{2+}.[408,410,417,457] The rise in intracellular Ca^{2+} in one report was shown to be independent of the concomitant rise in cAMP and thus likely dependent on IP_3.[457] In rat granulosa cells, LH is reported to increase IP_3.[458] In porcine theca cells, LH has been shown to generate an oscillatory Ca^{2+} signal that is not inhibited by pertussis toxin (thus independent of Gi) but is inhibited by the Ca^{2+} chelator ethyleneglycotetraacetic acid and by the PLC inhibitor U73211.[459] The rise in intracellular Ca^{2+} likely activates a CaM kinase, and LH-stimulated progesterone production is reduced by 50% by the CaM kinase inhibitor Kn93.[444] Ca^{2+} has recently shown to be required for hCG/amphiregulin-stimulated increases in the activity of the protease calpain-2 in cumulus granulosa cell–oocyte complexes.[460] Active calpain-2 promotes degradation of proteins within the focal adhesion complex of cumulus cells, leading to cell detachment, and is believed to contribute to cell movement within the cumulus cell–oocyte complex.[460] It is also possible that LH activates one or more PKC isoforms.

PKC consists of a number of isoforms[461]: the conventional PKCs (α, β, and γ) require Ca^{2+}, diacylglycerol, and phosphatidylserine for activation; the novel PKCs (δ, ε, η, θ) do not require Ca^{2+}; and the atypical PKCs (ζ, ι/λ) require only phosphatidylserine for activation. Subovulatory concentrations of LH plus 200 nM PMA (phorbol myristate acetate), which binds the diacylglycerol binding site on conventional and novel forms of PKC, mimic the effect of an ovulatory concentration of LH to promote progesterone production, *Ptgs2* induction, and luteinization of granulosa cells[462]; 200 nM PMA mimics the surge of LH to stimulate expression of LH targets such as the *Pgr*[463] and *Egr-1*.[29] However, in contrast to these results, hCG treatment of granulosa cells does not appear to promote activation of the

conventional or novel isoforms of PKC.[449] The ability of LH to activate the atypical forms of PKC has not been thoroughly investigated, but LH is reported to stimulate the membrane translocation of the atypical PKC isoform PKCζ, and this pathway is proposed to mediate the induction of the immediate early transcription factor *NGF1B/EGR-2* in these cells[464] that is implicated in *Cyp19a1* gene repression,[465] as discussed later.

LH and Insulin/IGF-1 Synergize to Activate AKT and LH Target Genes. There is abundant evidence in preovulatory granulosa and theca cells that LH/hCG synergizes with insulin/IGF-1 to promote target gene expression. This synergism is observed in granulosa cells with respect to stimulation of progesterone production,[466,467] *Star* expression,[468,469] *Ldlr* (low-density lipoprotein receptor) promoter-reporter activity,[364] and, surprisingly, cAMP production.[468,469] Synergism is also evident in vivo with conditional deletion of the lipid phosphatase PTEN (phosphatase and tensin homolog) that degrades PIP3, as shown by increased numbers of ovulated oocytes and increased litter size.[470] Synergism is reported in theca cells for the *Cyp17a1* promoter-reporter.[363,468] Co-transfection with the PKA inhibitor PKI inhibited both LH stimulation and the LH/hCG synergism with insulin/IGF-1 in regulation of *Star*[364] and *Ldlr*[364] promoter-reporter activities, consistent with LH signaling to these targets via PKA. Synergism between these pathways to stimulate *Ldlr* promoter-reporter activity was also inhibited with the PI-3K inhibitors wortmannin and LY24002.[364]

Insulin/IGF-1 are expected to signal through their canonical receptor tyrosine kinase pathways to activate IRS-1/2, PI-3K, and AKT.[238] However, the cellular mechanism by which LH regulates the PI-3K/AKT pathway is poorly understood. There is one report, using an in vivo model in which LH was infused into the vena cava of diestrus rats, that investigated signaling to AKT.[469] These authors showed, through immunoprecipitation of indicated targets from ovarian extracts, that LH stimulates the tyrosine phosphorylation of the cytokine receptor-associated tyrosine kinase Janus kinase 2 (JAK2), the phosphorylation of JAK2 targets signal transducer and activator of transcription (STAT)-1 and -5b, the tyrosine phosphorylation of IRS-1 and its association with PI-3K, and AKT phosphorylation. They also demonstrated that LH stimulates the tyrosine phosphorylation of SHC,[469] an adaptor protein that binds to phosphorylated tyrosine residues and associates with GRB2/SOS to activate RAS[471] and leads to an activation of ERK.[469] Moreover, these authors reported that LH and insulin synergized to increase all these responses except for ERK activation and tyrosine phosphorylation of STAT-1.[469] Additional studies are required to confirm these observations and to determine the mechanism by which LH signals to regulate these phosphorylation events.

Cumulus Granulosa Cell Expansion and Oocyte Maturation

Oocyte Arrest. Oocytes are arrested in prophase I of meiosis prior to the surge of LH, as shown in Figure 20.14. Arrest is initially maintained by the absence of at least the maturation promoting factor (MPF) kinase CDK1,[472] and possibly other proteins in oocytes. Oocytes acquire competence to resume meiosis sometime between preantral and antral stages.[473] Arrest is then maintained by elevated levels of cAMP and cGMP within the oocyte.[474] cAMP levels sufficient to maintain meiotic arrest appear to be the product of signaling by the orphan GPCRs GPR3[475] and GPR12[476] to Gs and adenylyl cyclase. Downregulation of GPR3 and GPR12 promotes meiotic resumption.[476] The ligands that activate these receptors remain unidentified. A second key player is the oocyte-specific cAMP hydrolyzing phosphodiesterase PDE3A, whose activity is inhibited prior to the LH surge primarily by cGMP,[477] thereby maintaining elevated intra-oocyte levels of cAMP. PDE3A-null mice ovulate but are infertile because oocytes do not undergo germinal vesicle breakdown and thus remain arrested.[478]

cAMP via PKA maintains oocyte arrest by inhibiting the activation of maturation promoting factor MPF, which consists of the cyclin-dependent kinase CDK1(CDC2) and Cyclin B. PKA maintains CDK1 in

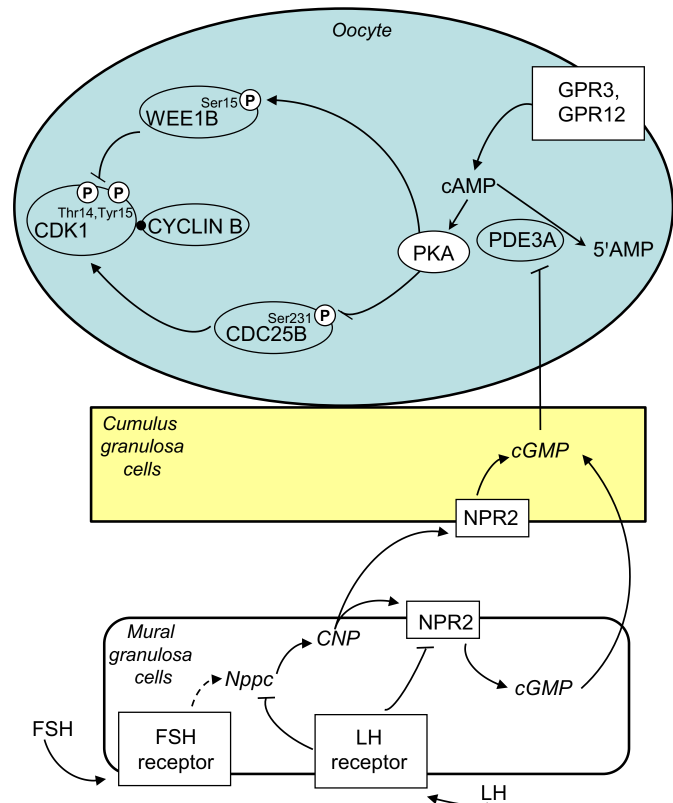

FIGURE 20.14 Mechanism by which LH promotes oocyte maturation.

an inactive conformation that resides in the nucleus by phosphorylating/activating the oocyte-specific dual-specificity kinase WEE1B on Ser15.[479] WEE1B phosphorylates CDK1 on Thr14 and Tyr15, inhibiting its activity.[480] PKA also maintains CDK1 in an inactive conformation by inhibiting the dual-specificity phosphatase CDC25B that can dephosphorylate Thr14 and Tyr15 on WEE1B. Phosphorylation of CDC25B on Ser321 promotes the association of CDC25B with 14-4-3 proteins in the cytosol, thus restricting accessibility to the substrate CDK1.[481]

Recent studies have elucidated key pathways that elevate levels of cGMP within cumulus granulosa cells to inhibit oocyte PDE3A. FSH enhances the expression of natriuretic peptide precursor C (*Nppc*) mRNA in mural granulosa cells.[482–485] The active peptide, C-type natriuretic peptide (CNP), is the ligand for the membrane guanylyl cyclase receptor NPR2 (natriuretic receptor B). NPR2 is expressed predominately in cumulus granulosa cells, to a lesser extent in mural cells, and not in oocytes.[482] The addition of CNP to cumulus–oocyte complexes elicits a rise in cGMP levels in both cumulus granulosa cells and oocytes; meiotic arrest is not maintained in oocytes from mice that are null for either *Nppc* or *Npr2*.[482] cGMP is believed to diffuse from the granulosa cell continuum into oocytes via gap junctions. Mice deficient in the major gap junction protein that connects cumulus cells and oocytes, Gja4 (Connexin 37), are infertile.[486] Expression of NPR2 in cumulus cells and the inner layer of mural granulosa cells is stimulated by oocyte-derived bone morphogenic protein 15 (BMP15), growth differentiation factor 9 (GDF9), and fibroblast growth factor 8B (FGF8B).[482]

Response to LH. The surge of LH rapidly reduces mural granulosa cell NPR2 activity[484] and more slowly reduces the expression of NPPC,[483] resulting in reduced levels of CNP in cumulus cells[484] (Figure 20.14). Additional pathways, described below, contribute to a rapid decrease in levels of cGMP in the oocyte.[477,487] The cGMP-dependent inhibition of the cAMP-hydrolyzing PDE3A is relieved, cAMP is hydrolyzed to 5′AMP, and PKA activation ceases.[474] As a result, CDC25B relocates to the nucleus[488] to dephosphorylate/activate CDK1, and meiosis resumes.

LH promotes rapid activation of ERK in mural granulosa cells via a PKA-dependent pathway[449] (Figure 20.13). LH also promotes the PKA-dependent[451] transactivation of the EGF receptor (ErbB1) in mural granulosa cells, leading to the sustained activation of ERK in mural and especially cumulus granulosa cells.[23,451,489] As described earlier, LH enhances expression of EGF-like ligands, especially epiregulin and amphiregulin, by mural granulosa cells. These EGF-like ligands act in an autocrine manner on mural granulosa cell EGF receptors and in a paracrine manner by diffusing through follicular fluid/extracellular spaces to activate EGF receptors in cumulus granulosa cells.[23] As a result, EGF receptor activation in cumulus cells is somewhat delayed compared to that in mural cells.[23,487] Expression of the EGF receptor on cumulus granulosa cells is regulated by oocyte-derived GDF9 and BMP15 via SMAD2/3 dependent pathways[490]; expression of EGF receptors in mural granulosa cells is regulated by FSH.[19]

Consistent with a major role for amphiregulin and epiregulin as mediators of LH actions, recombinant amphiregulin or epiregulin has been shown to mimic many of the responses elicited by LH, including cumulus expansion,[369,491] oocyte maturation,[487] and induction of genes required for ovulation, such as *Pgr* and *Ptgs2*.[369] Moreover, these responses are consistently inhibited by the EGF receptor antagonist AG1478 and are blunted with reduced expression paradigms for the EGF receptor.[369,487,489,492]

Amphiregulin has also been shown to promote a rapid decline in cGMP levels via mechanisms that are poorly understood but do not appear to be via activation of cGMP phosphodiesterases.[477,487] The pathway is inhibited by the EGF receptor antagonist AG1478, but cGMP levels are still greatly reduced in granulosa cells of mice with a conditional deletion of the EGF receptor, suggesting the presence of an alternative secondary pathway by which LH rapidly reduces cGMP levels.[487]

One of the major targets activated downstream of amphiregulin/EGF receptor in cumulus granulosa cells is ERK (Figure 20.13). Activated ERK closes the gap junctions that connect adjacent cumulus granulosa cells by directly phosphorylating connexin 43 on Ser255 and Ser262,[487] an event that contributes to, but apparently is not required for, oocyte maturation.[493] Activated ERK is necessary[369] but not sufficient for oocyte maturation and does not appear to contribute to the rapid fall in follicle cGMP induced by amphiregulin.[487] Activated ERK is required for expression of key cumulus expansion genes, such as *Has2* (hyaluronan synthase) and *Ptx3* (pentraxin-related protein), that generate the mucified, hyaluronan-rich matrix that contributes to the expansion of the cumulus cells surrounding the oocyte.[23,369] Activated ERK contributes to the expression of C/EBPβ (CCAAT-enhancer binding proteinα/β), and directly phosphorylates C/EBPβ.[369] ERK-induced C/EBPβ is necessary for amphiregulin to induce expression of genes required for ovulation, including *Pgr* that encodes for the progesterone receptor[369]; *Pgr* null mice do not ovulate.[374] C/EBPβ is also required for amphiregulin to promote expression of *Ptgs2* that encodes the rate-limiting enzyme for PGE2 synthesis.[23] PGE2 appears to function in a critical autocrine regulatory loop that increases the expression of amphiregulin and epiregulin by cumulus granulosa cells that in turn functions to sustain the actions of these EGF-like ligands[491] (Figure 20.13). Cumulus granulosa cells of *Ptgs2*-null mice express greatly reduced levels

of epiregulin and amphiregulin in response to an ovulatory concentration of hCG[491] and *Ptgs2*-null mice do not ovulate.[373] PGE2 via its receptor (EP2 subtype, *Ptger2*) and a cAMP/PKA pathway also promotes expression of *Tnfaip6* that contributes to cumulus expansion.[370,494] C/EBPβ is also required for expression of the EGF-like ligand type III neuroregulin I that, together with amphiregulin, apparently contributes to the fidelity of the expression of cumulus expansion genes.[455] C/EBPα/β-null mice exhibit normal cumulus expansion but fail to ovulate.[495] These results suggest that C/EBPβ targets are not required for cumulus expansion.

A potential mechanism to downregulate or modulate expression of C/EBPβ targets following ovulation was provided by evidence that theca and subsequently granulosa cells express a basic leucine zipper transcription factor, NFIL3 (nuclear factor interleukin-3), that can bind to the same response elements as C/EBPβ and CREB.[496] These proteins are expressed 8–24 h post hCG, and appear to act as repressors by competing with C/EBPβ and CREB for binding sites on both *Ptgs2* and *Areg* promoters. Overexpression of NFIL3 by adenoviral transduction of granulosa cells greatly reduces expression of *Areg*, *Ereg*, *Pgr*, and *Ptgs2* mRNA.[496]

The importance of ERK activation to oocyte maturation and ovulation was elegantly demonstrated with the conditional knockout of *Mapk1/3* (ERK 2/1) from granulosa cells using the *Cyp19a1-Cre*.[369] Mice did not exhibit cumulus expansion in response to hCG, due to a lack of expression of cumulus-expansion genes, and did not ovulate due, in part, to the absence of *Pgr* and *Ptgs2* expression (Figure 20.13). The absence of ERKs in cumulus granulosa cells prevents exogenous amphiregulin from stimulating cumulus expansion genes and genes required for ovulation. Oocyte maturation is also prevented, although how ERK contributes to this process is not clear. Follicles exhibit the normal initial increases in amphiregulin and epiregulin in response to hCG, but the induction is not sustained, likely as a result of the absence of PGE2 resulting from the absence of *Ptgs2* gene expression.

The conditional knock-in of a constitutively K-RAS, driven by *Cyp19a1-Cre*, that leads to activation of the ERK signaling pathway results in defects in cumulus expansion, oocyte maturation, and ovulation.[216] The majority of luteinized follicles exhibited entrapped oocytes. These results support a critical role for the appropriate regulation of ERK activation in response to the LH surge.

EGF receptor activation also appears to signal to activate p38 MAPK (MAPK14), based on reduced p38 MAPK phosphorylation (~50%) with conditional deletion of EGF receptor from granulosa cells.[487] p38 MAPK also appears to contribute to the phosphorylation of connexin 43, based on reduced phosphorylation of both Ser262 and Ser255 (50–60%) in follicles pretreated with the p38 inhibitor SB202190. The conditional deletion of p38 MAPK from granulosa cells had no effect on fertility.[497] However, while cumulus oocyte complexes respond normally to amphiregulin with the induction of cumulus expansion genes, *Areg* and *Ptgs2*, they fail to respond to PGE2, suggesting that the PGE2 regulatory loop in cumulus cells is mediated by p38 MAPK. Mural granulosa cells appear to compensate for the reduced expression of amphiregulin on cumulus cells by expressing elevated levels of amphiregulin and epiregulin.[497] One interpretation of these data is that while p38 MAPK contributes to EGF receptor-regulated pathways in cumulus cells, it is not essential.[474]

Ovulation

Ovulation requires both *Pgr* and *Ptgs2* induction by the LH signals that transactivate the EGF receptor leading to ERK activation and expression of C/EBPβ, as discussed earlier. PDE4D-null mice do not ovulate as a consequence of severely reduced expression of both *Pgr* and *Ptgs2*.[34] Reduced expression of these genes likely reflects the altered cAMP production that is two- to three-fold reduced and no longer transient. However, oocyte maturation and luteinization appear to be normal. Similarly, in anovulatory PGR-null mice, *Ptgs2* is induced, *Cyp19a1* is downregulated, luteal cells express *Cyp11a1*, and entrapped oocytes are functional.[377] Remodeling of the follicle wall is also believed to require one or more metalloproteinases, based on the ability of pharmacological inhibitors of this family of enzymes to block follicle rupture.[498] ADAMTS-1, a PGR target,[378] has recently been identified as a metalloproteinase that is required for follicle rupture; ADAMTS-1-null mice exhibit a 75% reduction in the rate of ovulation.[499] The proteoglycan versican is a major target of ADAMTS-1. Versican is induced by the LH surge in mural granulosa cells by a pathway that is independent of *Pgr* and *Ptgs2*, it binds hyaluronan, and relocalizes to the granulosa/theca cell boundary and to the cumulus oocyte complex.[500] ADAMTS-1-null mice exhibit a 75% reduction in versican cleavage at the granulosa/theca cell interface and within expanding cumulus cells.[500] ADAMTS-1-null mice also fail to exhibit the characteristic invagination at the theca cell border that is involved in vascularization of the follicular structure required for corpus luteum formation.[500] The remaining 25% of versican cleavage is presumably mediated by additional unidentified proteases.

The absolute requirement of *Pgr* expression for ovulation suggests that its ligand, progesterone, is required to activate PGR-regulated genes.[501] Progesterone production by mural granulosa cells requires induction of *Cyp11a1* and *Star*.[380,502] Reduced progesterone biosynthesis decreases ovulation rates.[503] Progesterone accumulation in granulosa cell medium also requires

dynamic reorganization of the actin cytoskeleton.[504] The key downstream target whose phosphorylation is regulated by hCG is the actin depolymerizing factor cofilin. Cofilin phosphorylated on Ser3 does not bind actin filaments, while dephosphorylated cofilin binds actin and enhances actin dynamics. hCG in a PKA-dependent manner, independent of ERK, inhibits the pathway from RHOGTP via RHO kinase and Lim kinase that stimulates the phosphorylation of cofilin on Ser3. hCG promotes inactivation of RHO (converting RHOGTP to RHOGDP), leading to the dephosphorylation/activation of cofilin. Transduction of granulosa cells with an adenoviral cofilin phospho-mimic, cofilin Ser3Glu, reduces hCG-stimulated progesterone production by 70%.[504] These results show that the surge of LH also regulates intracellular signaling pathways distinct from those leading to transcriptional regulation (see Figure 20.13).

Ovulation and cumulus expansion also require the orphan nuclear receptor LRH-1 (*Nr5a2*).[335] Conditional deletion of *Nr5a2* from granulosa cells, driven by *Amhr2-Cre*, abrogates LH-induced expression of *Ptgs2* and its target *Tnfaip6*, thus blocking cumulus expansion, as well as expression of *Star* and *Cyplla1*, thus preventing progesterone production and ovulation.[335] *Nr5a2*-null mice also fail to downregulate *Cyp19a1*, resulting in elevated estrogen production. The LH-induced downregulation of estrogen expression appears to be required for *Ptgs2* expression, based on evidence that *Sult1e1*-null mice, that fail to express the enzyme estrogen sulfotransferase that inactivates estrogen, exhibit elevated estrogen and reduced expression of both *Ptgs2* and *Tnfaip6*.[505] One relevant LRH-1 target in granulosa cells that appears to be required for downregulation of *Cyp19a1* is *Nos3*.[335] *Nr5a2*-null mice expressed reduced levels of *Nos3* mRNA, and CHIP assays show direct binding of LRH-1 to the *Nos3* promoter.[335] As discussed under the AKT target eNOS, *Nos3*-null mice exhibit reduced ovulation rates and oocyte maturation, and a marked increase in estrogen levels following the LH surge (rather than a decrease).[333,334] Together these results suggest that LH signals via the nuclear receptor LRH-1 not only to enhance expression of *Star* and *Cyplla1* but also to promote expression of *Nos3*. LH also potentially promotes activation of the *Nos3* product eNOS that is some manner is required for the downregulation of *Cyp19a1* and consequent upregulation of *Ptgs2*. However, the mechanism by which LH regulates LRH-1, how eNOS contributes to *Cyp19a1* downregulation, and how elevated estrogen inhibits expression of Ptgs2 are not known.

Luteinization

Luteinization defines a program of terminal differentiation that converts mural granulosa cells and theca cells to luteal cells. Proliferation ceases, the structure formed following ovulation becomes highly vascularized, and luteal cells produce high levels of progesterone. Genes specific to granulosa cells are downregulated, and genes required for luteal function are upregulated.

The LH surge suppresses granulosa cell proliferation by promoting downregulation of cyclin D2, encoded by *Ccnd2*,[45] and upregulation of cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$,[45,381] encoded by *Cdkn1a* and *Cdkn1b*. Conditional deletion of ERK1/2 from granulosa cells prevents cell cycle arrest and luteinization.[369] Although luteinization is associated with cell cycle arrest, luteinization and exit from the cell cycle are independent responses to the surge of LH. Thus, in both $p27^{Kip1}$-null[381,506] and $p27^{Kip1}/p21^{Cip1}$ double-null mice,[381] preovulatory follicles undergo ovulation and luteinization to form a corpus luteum in response to the LH surge even when granulosa cells continue to proliferate, as evidenced by retention of cyclin D2 expression and high bromodeoxyuridine incorporation in cells expressing *Cyp11a1*. In cyclin D2–null mice, follicles do not ovulate, potentially due to reduced cumulus cell numbers,[507] but granulosa and theca cells still luteinize in follicles with entrapped ova.[44] Similarly, in PDE4D and C/EBPα/β-null mice, granulosa and theca cells luteinize without release of oocytes.[34,369]

Progesterone production is the hallmark of a functional corpus luteum. The preovulatory surge of LH accordingly induces the persistent and elevated expression of the rate-limiting enzyme in progesterone synthesis *Cyp11a1*[380] and the cholesterol-mobilizing protein *Star*.[376] Expression of both genes is blocked by conditional deletion of *Mapk1/3*[369] and *Cebpa/Cebpb*[495] from granulosa cells and reduced in mice conditionally expressing partially inactive EGF receptors.[492] These results show that ERK activation downstream of LH-stimulated EGF receptor transactivation is necessary to promote expression of both *Star* and *Cyp11a1*. Expression of both genes, however, requires input from additional signaling pathways.[48,353,355,367,369,495]

Luteinization involves the silencing of a number of genes induced by FSH that define the preovulatory phenotype, such as *Inha* and *Cyp19a1*. The surge of LH induces permanent silencing of the *Inha* gene in the rodent. Repression of inhibin expression during the periovulatory time window permits the secondary surge of FSH on the morning of estrous that contributes to follicle recruitment.[508] Reduced *Inha* transcriptional activity is initially accomplished by the rapid, albeit transient, induction in mural granulosa cells of the immediate early gene *Crem* (cAMP response element modulator) that encodes the inducible cAMP early repressor ICER.[346] ICER, along with CREB, C/EBPβ, and NFIL3 are basic leucine zipper transcription factors that bind DNA and homo- or heterodimerize to activate or repress transcription. As shown by CHIP assays, ICER competes with

phospho-CREB-Ser133 homodimers for occupancy of the cAMP response element on the *Inha* promoter.[509] As a result, the multiprotein complex that promotes transcription assembled on the CREB-occupied *Inha* promoter is disassembled, and *Inha* transcription ceases. Consistent with the transient induction of ICER, a second immediate early gene C/EBPβ is induced by 1h and persists for 24h that continues to repress transcription from the *Inha* promoter.[510] C/EBPβ-null mice do not form corpora lutea[511] and do not exhibit *Inha* downregulation following injection of hCG to PMSG-primed mice.[510] Although C/EBPβ can form inactive heterodimers with CREB, the predominant repressive mechanism appears to be via the binding of C/EBPβ homodimers to a nonconcensus binding site.[510]

Recent results show that histone modifications, which alter the accessibility of DNA to transcriptional activators, temporally followed by DNA methylation that further reduces binding of transcriptional activators, additionally contribute to LH-induced *Inha* silencing.[512] CHIP assays showed that histone H3 trimethylated on Lys4 (H3K4me3), a modification linked to active genes, was enriched on the *Inha* promoter in preovulatory cells, obtained 48h post PMSG, and then declines 18–20h post hCG. Conversely, histone modifications linked to repressed genes, H3K27me3 and H3K9me3, are low in preovulatory cells and substantially increase in ovulatory cells and further increase in luteal cells obtained 6 days post hCG.[512] DNA methylation within the cAMP response element, as determined by bisulfite sequencing, is low in preovulatory and ovulatory cells and markedly enhanced in corpora lutea. It is hypothesized that the repressors like ICER and C/EBPβ recruit histone-modifying enzymes that promote methylation of Lys27 and Lys9, and that these histone modifications contribute to recruitment of DNA methylation enzymes. Together these modifications prevent the binding of CREB and other transcriptional activators to *Inha*, resulting in its silencing.

Cyp19a1 is also downregulated as a result of the LH surge, at least in part as a negative-feedback pathway to release the hypothalamic–pituitary axis from stimulatory effects of estrogen that initiated and sustained the surge of LH. Mechanisms of repression at the promoter are less well defined for *Cyp19a1* compared to that for *Inha*. The immediate-early orphan nuclear receptors NR4A2 (nuclear receptor subfamily 4 group A member2)/NURR1 and NGF1B (nerve growth factor 1B)/EGR-2 have been implicated in the negative regulation of the *Cyp19a1* gene after the LH surge, using human granulosa-like tumor cell line, KGN, based on promoter-luciferase results and siRNA(silencing RNA)-mediated downregulation of *Nurr1*.[465] *Nurr1* and *Egr-2* mRNAs are rapidly and transiently upregulated in gonadotropin-primed rat ovaries in response to hCG, the former via a PKCζ pathway.[438] A member of the *Jun* family of immediate early genes may also contribute to the downregulation of *Cyp19a1* by binding to the cAMP response element usually occupied by phospho-CREB(Ser133).[513] The redistribution of CREB from the nucleus to the cytoplasm during luteinization may also contribute to the downregulation of targets such as *Cyp19a1*.[122]

ERK is required for *Cyp19a1* downregulation. ERK1/2-null mice do not exhibit *Cyp19a1* downregulation in response to an ovulatory concentration of hCG[369] (Figure 20.13). C/EBPα/β-null mice exhibit the same phenotype,[495] consistent with evidence that expression and activation of C/EBPβ is dependent on ERK1/2 activation[369] and with evidence of the repressive actions of C/EBPβ.[510] Likewise, granulosa cells expressing EGF receptors with compromised receptor tyrosine kinase activity exhibit decreased C/EBPβ and fail to downregulate *Cyp19a1*[492] (see Figure 20.13). Activation of the EGF-receptor/ERK1/2/C/EBPβ pathway is also required for the downregulation of *Fshr*[369,492] and *Lhcgr*,[369] and for the upregulation of *Cyp11a1*,[369,492,495] *Star*,[369,495] the estrogen-inactivating enzyme *Sult1e1*,[369] and runt-related transcription factor *Runx2*.[495,514] *Cyp19a1* downregulation also appears to be a consequence of LRH-1-induced *Nos3* expression,[335] as discussed earlier in "Ovulation," although the mechanism by which eNOS contributes to *Cyp19a1* expression is not known. As with *Inha*, the downregulation of *Cyp19a1* is linked with inactivating histone modifications, while the upregulation of *Star* is linked with activating histone modifications.[515] Similarly, DNase1 protection assays suggest that hCG enhances chromatin condensation for *Cyp19a1* and reduced chromatin condensation for *Star*.[515]

The ovulatory surge of LH promotes a transient downregulation of LH receptors that recover some 48–72h later, in a species-specific manner, with the formation of the corpus luteum. LH receptor downregulation in granulosa cells is relatively slow, with a T1/2 of at least 8.5h,[421–423] compared to that of most other GPCRs.[86,516] LH receptor downregulation in response to an hCG bolus has been extensively studied, as described below, using day-5 corpora lutea from superovulated pseudopregnant rats as a model. Recent studies have used primary cultures of human granulosa cells with equivalent results. A decline in *Lhcgr* mRNA is first detected ~ 6h post hCG; *Lhcgr* mRNA is undetectable by 12h post hCG, by northern analysis.[517] However, unlike the downregulation of *Inha* and *Cyp19a1*, there is no change in the rate of *Lhcgr* transcription. Rather, hCG promotes increased *Lhcgr* mRNA degradation.[518] Enhanced *Lhcgr* mRNA degradation is mediated by a 50kDa LH receptor mRNA-binding protein (LRBP)[519] identified as mevolanate kinase.[520,521] Suppression of LRBP expression abrogated the ability of hCG to downregulate *Lhcgr* mRNA, suggesting that LRBP is not only

necessary but sufficient for this regulation.[522] LRBP binds an 18-base pair sequence within the coding region of *Lhcgr* mRNA.[519] LRBP binds *Lhcgr* mRNA at the ribosome, yielding an untranslatable complex that is targeted for degradation.[523] *Lhcgr* mRNA downregulation and the two-fold upregulation of LRBP protein detected 12 h post hCG is prevented by H89, consistent with a PKA-dependent response.[524] *Lhcgr* mRNA downregulation is also reversed by the ERK1/2 inhibitor U0126. The requirement of ERK1/2 activation for *Lhcgr* mRNA downregulation is supported by results with ERK1/2-null mice that fail to show a decline in *Lhcgr* mRNA post hCG.[369] The relevant ERK1/2 target has not been identified, but might be LRBP itself, especially since LRBP is readily detectable in granulosa cell extracts in the absence of hCG, consistent with regulation by a posttranslational mechanism.[524] While the mechanism by which surface LH receptors are downregulated in granulosa cells and corpora lutea following a bolus of LH or hCG has been clearly defined, the physiological consequence of blocking this response, especially in granulosa cells, has not to our knowledge been investigated.

MOLECULAR BASIS FOR THE DIFFERENTIAL REGULATION OF TARGET GENE EXPRESSION BY FSH IN PREANTRAL AND LH IN PREOVULATORY GRANULOSA CELLS

There have been many advances in the seven years since we last reviewed the cellular signaling pathways by which FSH and LH regulate target gene expression. Cyclic AMP/PKA play a major role in the actions of FSH and LH in granulosa cells. Despite the fact that these two gonadotropins are structurally related, act on similar receptors, and signal predominantly through cAMP-dependent mechanisms, FSH and LH elicit very different responses in preantral versus preovulatory granulosa cells. For example, FSH acts via cAMP/PKA to activate transcription of target genes such as *Cyp19a1*, *Inha*, and *Lhcgr* in preantral granulosa cells but does not stimulate expression of the *Ptgs2* or *Pgr*, whereas LH has the opposite effects in preovulatory granulosa cells and stimulates the downregulation of *Cyp19a1*, *Inha*, and *Lhcgr*, and activates transcription of the *Ptgs2* and *Pgr* genes. Thus, a central question in reproductive biology is how the same second messenger/effector exerts such different responses during the continuum of granulosa cell differentiation. Are the different responses attributable to the activation of distinct signaling pathways, such as only PKA for FSH and PKA plus PKC for LH, or to the duration of signaling? Or, do the different responses of the two cell types reflect the fact that preovulatory granulosa cells have differentiated and contain a different proteome, including distinct AKAPs, kinases, phosphatases, transcription factors, and other regulators important in generating their unique response?

To address the question of different signaling pathways for FSH versus LH, Zeleznik's group[115,133] cleverly investigated target gene expression after adenoviral transduction of preantral granulosa cells with constitutively active LH receptors (Asp578His). Results from these experiments showed that the LH receptor expressed in preantral cells partially mimics the responses of FSH and induces *Inha* and *Hsd3b2* but interestingly does not induce or minimally induces *Cyp19a1* or *Lhcgr*, even when cAMP levels are equivalent. These results suggest that these two GPCRs not only couple to overlapping signaling pathways but also appear to couple, perhaps more efficiently, to different signaling pathways to regulate distinct cellular events in preantral versus preovulatory granulosa cells.

Alternatively, rather than there being different second messengers generated by FSH versus LH receptor activation in preantral versus preovulatory granulosa cells, differences in the amount, duration, or cellular location of cAMP may distinguish FSH signaling from that of LH signaling. It is well known that sustained FSH at relatively low concentrations is necessary to stimulate granulosa cell differentiation,[106] whereas a bolus of LH is required to stimulate ovulation. Indeed, it has been proposed that the actions of LH and FSH are distinct in mature versus immature granulosa cells as a result of the ability of LH receptor activation in mature cells to generate greater levels of cAMP.[525] Although this view is supported in mature, preovulatory granulosa cells by the observation that high doses of recombinant FSH can substitute for LH/hCG in promoting ovulation and luteinization in the rat,[526] high levels of cAMP in immature cells do not seem to mimic actions of the LH surge in mature cells.[115,133] To our knowledge, it is not known if overexpression of the constitutively active LH receptor in immature cells promotes induction of genes characteristic of mature cells, such as *Pgr* and *Ptgs2*; however, the reduced expression of *Cyp19a1* and *Lhcgr* are reminiscent of downregulated genes in mature cells.

FSH and LH receptor activation also regulate the expression of the cAMP degrading PDEs.[527] PDEs certainly participate in LH receptor signaling because PDE4D-null mice exhibit an ovulation defect.[527] However, the contribution of the PDEs to the distinct signaling by FSH and LH receptors in immature and mature granulosa cells is not fully understood. There is also abundant evidence for the cellular compartmentalization of cAMP in various cellular models.[143,528] Consistent with the conceivably restricted production of cAMP in select cellular sites, PKA is recognized to be anchored to select cellular locations by the growing family of AKAPs.[142] Potentially, then, the distinct expression

of one or more AKAPs between immature and mature granulosa cells could also localize PKA and distinct PKA substrates to different cellular locals and account for distinct signaling in immature versus mature cells by FSH versus LH receptors. That PKA regulates ERK activity by distinct mechanisms in preantral versus preovulatory granulosa cells,[113,449,529] and the more prominent role of ERK signaling in preovulatory versus preantral cells[369] indicates that these two cell types are indeed quite distinct and that preovulatory cells are not simply preantral granulosa cells that express, for example, LH receptors.

CONCLUSION

As reviewed in this chapter, tremendous progress has been made in understanding mechanisms of gonadotropin action in regulating ovarian follicle maturation, oocyte maturation, and ovulation, and luteinization. The structures of FSH and hCG are now known to atomic level resolution; there is a fundamental understanding of the gonadotropin receptors and how they selectively bind FSH and LH; it is clear that cAMP serves as the key second messenger for nearly all gonadotropin actions in the ovary; substantial inroads have been made in defining the signaling pathways activated by gonadotropins; and many of the target genes activated by FSH or LH have been identified and their physiologic functions explored. In addition, genetic studies in mice and naturally occurring mutations in women have verified the fundamental importance of the gonadotropins and their receptors to ovarian function and allowed new insight into the precise stages and processes in which they act. Finally, some insight into what differentiates FSH and LH action in the ovary is now being obtained.

Despite this wealth of information, much remains to be learned about gonadotropin signaling pathways in the ovary. While the crystal structure of FSH in complex with the extracellular domain of the FSH receptor was recently reported,[62] the complete structures of the gonadotropin receptors, extending to the full-length proteins, are needed to gain further insight into hormone-binding selectivity and into the conformational changes that underlie receptor activation and G protein coupling. The recent ability to solve crystallographic structures of several model GPCRs sets the stage for these future challenges. Studies to more fully understand the tissue- and cell-specific expression and hormonal regulation of the gonadotropin receptors are also important. Although some of the major signaling pathways downstream of gonadotropin-regulated cAMP generation have been explored in depth, as discussed in this chapter, others are just beginning to be appreciated, and more detailed analyses are required. New advances in proteomics are likely to be important here, particularly methods to carefully examine the phosphorylation status of kinase target proteins in ovarian cells and to more fully characterize the kinases and phosphatases involved in particular regulatory events. As discussed, limited data also point to activation of additional signaling mechanisms via cAMP-independent pathways in ovarian cells, and this is an area worthy of further investigation. A wealth of new information is accumulating from gene expression profiling and other high throughput analyses with respect to potential target genes activated by gonadotropin signaling, and verifying these targets, establishing mechanisms of their regulation, and exploring their functions in the ovary promises to be a significant focus of future work.

Cross talk between signaling pathways is an often discussed but poorly understood aspect of signaling that is likely to be increasingly important as we begin to understand the molecular details of individual pathways. It will be particularly valuable to understand how gonadotropin signaling integrates with the many other signaling pathways important for follicle formation and maintenance. For example, TGFβ family proteins play numerous roles in cell communication in the follicle, but little is known regarding how these pathways impact gonadotropin signaling and vice versa. Similarly, understanding how other signaling pathways influence the fundamental processes of cell proliferation, cell differentiation, and cell death in the ovary, and how gonadotropin action might modify these events remains a key challenge.

We raised the issue of spatially regulated signaling as it relates to the AKAPs and their roles in determining cell-specific responses to cAMP, but the broader issue of spatially restricted signaling domains is likely to be an important one that applies to many pathways and contributes in a significant way to tissue- and cell-selective responses. Further characterization of such spatial domains in ovarian cells is warranted. This also reinforces the view that signaling principles learned in generic cell systems are not always applicable to granulosa or theca cells and must be tested in the ovary. The temporal aspects of regulation are likely to be just as important. We broadly define for the purposes of this discussion preantral and preovulatory granulosa cells as distinct stages of differentiation in the follicle that respond differentially to the gonadotropins, yet the reality is that there is a continuum of differentiation states that defines the precise nature of the response to gonadotropins or other stimuli.

Gene disruption technologies in mice have been an integral component of studies of gonadotropin signaling pathways. A significant number of gene knockouts yield reproductive phenotypes, and as discussed throughout the chapter, these mice have been invaluable in establishing roles for particular signaling proteins and pathways

in the ovary. However, many gene disruptions are not particularly informative with respect to reproduction because of early lethality or broad spectrum phenotypes that impact reproduction in an indirect fashion. Thus conditional disruption approaches, including both ovary-specific and temporally regulated knockouts, are likely to be very informative for deciphering details of gene and protein functions in gonadotropin-dependent signaling. In the same vein, recent advances in RNA interference technologies that include in vivo delivery hold much promise for downregulating selective gene products in targets like the ovary so as to establish their functional importance.

Finally, studies of gonadotropin action in the ovary continue to be informed by clinical investigation and genetic analysis of reproductive disorders, and this is an area that will surely receive much more attention in the future. Naturally occurring mutations in the gonadotropins and their receptors provide a rich context in which to learn about interaction with and activation of the gonadotropin receptors. An increasing number of mutations in signaling molecules and transcription factors exhibit some reproductive phenotype, and these are tremendously informative in inferring the functional importance of particular genes and pathways. Even complex diseases that impact the ovarian reserve, leading to premature ovarian failure, or that impact follicle maturation, leading to polycystic ovarian syndrome, are now being explored in genetic detail, and aspects of gonadotropin signaling will likely come into play in these as well. Thus, the future holds much promise for better understanding the molecular details of how FSH and LH function as master regulators in the maturation, ovulation, and luteinization of the ovarian follicle.

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