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FSH signaling pathways in immature granulosa cells that regulate target gene expression: Branching out from protein kinase A

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Abstract

Follicle-stimulating hormone (FSH) is necessary and sufficient to induce maturation of ovarian follicles to a mature, preovulatory phenotype in the intact animal, resulting in the generation of mature eggs and production of estrogen. FSH accomplishes these actions by inducing a complex pattern of gene expression in target granulosa cells that is regulated by input from many different signaling cascades, including those for the extracellular regulated kinases (ERKs), p38 mitogen-activated protein kinases (MAPKs), and phosphatidylinositol-3 kinase (PI3K). The upstream kinase that appears to be responsible for initiating all of the signaling that regulates gene expression in these epithelial cells is protein kinase A (PKA). PKA not only signals to directly phosphorylate transcription factors like cAMP response element binding protein and to promote chromatin remodeling by phosphorylating histone H3, this versatile kinase also enhances the activity of the p38 MAPK, ERK, and PI3K pathways. Additionally, accumulating evidence suggests that activation of a single signaling cascade downstream of PKA is not sufficient to activate target gene expression. Rather, cross-talk between and among signaling cascades is required. We will review the signaling cascades activated by FSH in granulosa cells and how these cascades contribute to the regulation of select target gene expression.

Keywords

Follicle-stimulating hormone; Mitogen-activated protein kinase; Female reproduction; Hypoxia-induced factor 1; Histone H3; Protein kinase A

Abbreviations

AKAP, A kinase anchoring protein; Aromatase, P450 aromatase; CBP, CREB binding protein; ChIP, chromatin immunoprecipitation assay; CREB, cAMP response element binding protein; EGF, epidermal growth factor; Egr-1, early growth response protein-1; ERK, extracellular regulated kinase; Epac, exchange proteins activated by cAMP; FSH, follicle-stimulating hormone; GIOT-1, gonadotropin-inducible ovarian transcription factor-1; GPCR, G-protein-coupled receptor; HIF-1, hypoxia-induced factor-1; IGF, insulin-like growth factor; LH, luteinizing hormone; LHR-1, liver receptor homolog-1; MAP2D, microtubule-associated protein 2D; MAPK, mitogen-activated protein kinase; MEK, mitogen- and extracellular-regulated kinase kinase; MK, MAPK-activated protein kinases; MNK, MAPK-interacting kinase; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal S6 kinase; PDE, phosphodiesterase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKI, PKA inhibitor peptide; PTP, protein tyrosine phosphatase; R, PKA regulatory subunits; RSK, p90 ribosomal S6 protein kinase; SCC, P450 cholesterol side chain cleavage; SF-1, steroidogenic factor-1; SGK, serum glucocorticoid kinase; Sp1/Sp3, specific

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protein 1/3; TGF β , transforming growth factor β ; TSC, tuberous sclerosis complex tumor suppressor gene; VEGF, vascular endothelial growth factor

1. Introduction

The ovarian follicle plays a critical role in female reproduction. The follicle contains an oocyte surrounded by epithelial-type granulosa cells, a basal lamina, and peripheral thecal cells which receive a vascular supply. Follicular maturation in adult females is cyclic owing to the cyclic recruitment of a cohort of immature follicles by follicle-stimulating hormone (FSH). Maturation of ovarian follicles to a preovulatory phenotype results in the production of estrogen by granulosa cells. Estrogen is required for development of secondary sex characteristics in females, for triggering production of the hormone that promotes follicular ovulation, and for preparation of the uterus for implantation of a fertilized egg.

FSH receptors are located exclusively on granulosa cells in females. FSH drives the proliferation, growth and differentiation of granulosa cells, characterized by: increased vascularization of the theca interna layer of cells peripheral to the basal lamina, formation of a fluid-filled antrum within the maturing follicle, and development of two “classes” of granulosa cells with distinct polarities and gene expression (the cumulus granulosa cells that surround the oocyte and mural granulosa cells that are peripheral to the antrum and line the basal lamina). These physiological responses to FSH are accomplished by the activation of more than 100 different target genes in granulosa cells [1–3], as depicted in Fig. 1. Activated target genes in mural granulosa cells encode proteins such as G-protein-coupled receptors (GPCRs) like that for luteinizing hormone (LH) [4]; intracellular signaling proteins such as the type II beta regulatory subunit (RII β) for protein kinase A (PKA) [5], phosphodiesterase (PDE) 4D [6], serum glucocorticoid kinase (SGK) [7], and the A kinase anchoring protein (AKAP) microtubule-associated protein (MAP) 2D [8]; transcription factors such as early growth response factor (Egr)-1 [9], liver receptor homolog (LRH)-1 [10,11], and gonadotropin-induced ovarian transcription factor-1 (GIOT-1) [12]; immediate early gene products such as c-Fos and c-Jun [13] and c-Myc [14]; autocrine factors such as epiregulin [15] and vascular endothelial growth factor (VEGF) [16]; the alpha and beta subunits of the heterodimeric hormone inhibin [17,18]; cell cycle proteins such as cyclin D₂ [19]; the extracellular matrix protein cartilage link protein (Crtl1) [20]; and rate-limiting enzymes that regulate steroidogenesis, such as P-450 aromatase for estrogen production [21] and P-450 cholesterol side chain cleavage (SCC) for progesterone production [22].

We have utilized two models to evaluate FSH signaling in granulosa cells. One is an in vitro model in which (primarily mural) granulosa cells are obtained from immature female rats primed for 3 days with estrogen. These cells contain FSH receptors (but not LH receptors) and when placed in serum-free medium in primary culture in the presence of 10nM estrogen, remain in the G₀ phase of the cell cycle but readily differentiate in response to FSH (reviewed in [23]). Results seen in primary cell cultures are confirmed using an in vivo model in which immature rats are injected with pregnant mare's serum gonadotropin (PMSG), a hormone that binds both FSH receptors and LH receptors [24].²

2. FSH activates protein kinase A (PKA)

FSH signals via activation of surface FSH GPCRs on granulosa cells to stimulate adenylyl cyclase activity and increase production of cAMP. Although the number of FSH receptors

²Granulosa cells in these immature rats do not express LH receptors; LH receptors at this stage of follicular maturation are expressed only on thecal cells.

(~1600–4500 receptors per cell [25,26]) and consequently cAMP product is relatively low, a predominate role for cAMP in granulosa cell differentiation is evidenced by the ability of forskolin to mimic differentiation responses of FSH (reviewed in [23]). In addition, forskolin mimics the ability of FSH both to stimulate phosphorylation of cAMP response element binding protein (CREB) [27–29] and histone H3 [28] as well as to activate signaling pathways in granulosa cells discussed below, including those for the extracellular regulated kinases (ERKs) [27], p38 mitogen-activated protein kinase (MAPK) [30], and phosphatidylinositol-3 kinase (PI3K) [16,31], as discussed below. FSH promotes rapid activation of PKA [32] and PKA-selective inhibitors such as myristoylated (Myr)-protein kinase inhibitor peptide (PKI) abrogate the effects of FSH to activate signaling pathways and target genes that lead to granulosa cell differentiation, as detailed below. These results suggest that activation of PKA is necessary for FSH to direct granulosa cell differentiation. However, it remains to be shown that PKA is sufficient to direct the entire granulosa cell differentiation program. While granulosa cells also express exchange proteins activated by cAMP (Epacs) [31], the Epac target Rap 1 is not activated by FSH [27] and an Epac-selective cAMP analogue does not promote induction of the FSH-target aromatase [33]. Taken together, these results point to PKA as an initial protein kinase activated in response to FSH and suggest that cAMP signals are mediated largely via PKA. Based on this conclusion, we sought to identify PKA targets in granulosa cells activated by FSH.

3. Identified PKA targets in granulosa cells

3.1. CREB

CREB is the best-known transcription factor regulated by PKA [34,35] and was initially predicted to regulate expression of most if not all PKA-regulated target genes in granulosa cells. FSH-stimulated CREB phosphorylation on S133 is detected within 1 min of FSH addition to granulosa cells [28], it is inhibited by Myr-PKI [27] but not affected by the p38 MAPK inhibitor SB203580, the MAPK/ERK kinase (MEK) inhibitor PD98059, the epidermal growth factor receptor (EGFR) inhibitor AG1478, the PI3K inhibitor wortmannin, or the protein kinase C (PKC)/ribosomal S6 kinase-2 (RSK-2) inhibitor GF109203X [28]. CREB phosphorylation is not stimulated by EGF, ionomycin, phorbol esters [27], or by insulin-like growth factor (IGF)-1 [unpublished]. These results suggest that in granulosa cells, CREB is directly phosphorylated by PKA, as depicted in Fig. 2, and not by alternate CREB kinases downstream of Akt, ERK, p38 MAPK, or PKC. However, cAMP response elements have been identified in only a small subset of FSH-regulated genes, namely inhibin- α [36], aromatase [37], GIOT-1 [12], Egr-1 [9], and *c-fos* [38]. Thus, CREB is not sufficient to activate the majority of FSH target genes.

3.2. Histone H3

FSH also promotes rapid phosphorylation of histone H3 on S10 which is concomitant with or rapidly followed by acetylation on K14 [28]. Phosphorylation on S10 and acetylation on K14 is transient: peak signal is detected at 1 h and signal is no longer detectable 4 h post FSH using an antibody that detects both modifications [28,32]. Histone H3 phosphorylation appears to be mediated directly by catalytic subunits of PKA (see Fig. 2), consistent with early identification of H3 as a PKA substrate [39]. FSH-stimulated H3 phosphorylation in granulosa cells is mimicked by forskolin and abrogated by Myr-PKI and the PKA/p70 ribosomal S6 protein kinase (p70S6K) inhibitor H89; it is not affected by inhibitors of p38 MAPK, MEK, RSK-2/PKC, or PI3K; and it is not stimulated by phorbol esters, EGF, or activin [28,32]. Granulosa cells appear to be unique in their use of PKA as the S10 histone H3 kinase since in other cells, S10 histone H3 kinases include the ERK substrate RSK-2 or the ERK/p38 MAPK substrates mitogen- and stress-activated protein kinases (MSK) 1 and 2 [40,41], the AMP-kinase homologue in yeast [42], p21-activated protein kinase [43], or aurora kinase B [44]. However,

it is a reasonable conjecture that in those cells in which differentiation events are regulated by PKA, such as thyroid, adrenal, and neuronal cells, the S10 histone H3 kinase will also be PKA.

Chromatin immunoprecipitation (ChIP) assays in granulosa cells show that phosphorylated/ acetylated histone H3 is selectively associated with promoters of the immediate early and early FSH target genes inhibin- α , SGK, and c-Fos [28]. These results suggest that the predicted chromatin remodeling ensuing from these covalent modifications of H3 on S10 and K14 are associated with the activation of FSH target genes that lead to differentiation and are not associated with mitosis since granulosa cells do not proliferate under serum-free conditions in the presence of FSH alone (reviewed in [23]). While it is likely that activation of additional FSH target genes is associated with H3 phosphorylation and acetylation, the transient nature of H3 phosphorylation/acetylation suggests that only those target genes activated during the first couple of hours post FSH are affected.

3.3. Protein tyrosine phosphatase (PTP) SL-like PTP

FSH stimulates the rapid yet transient phosphorylation of ERK1/2 in granulosa cells: the response is readily detected 10min post addition of FSH and waning by 1h [27]. ERK activation is mimicked by 8-chlorophenylthio-cAMP, a cell-permeable cAMP analog, and is PKA-dependent, based on inhibition by Myr-PKI [27]. While FSH-stimulated ERK activity is inhibited by the MEK inhibitor PD98059, consistent with activation of ERK by MEK, surprisingly MEK is already phosphorylated in vehicle-treated cells, and FSH does not further increase phosphorylation of MEK. Similarly, upon evaluation of the activities of the upstream components Raf-1 and Ras in the ERK cascade either by immune complex kinase assay for Raf-1 or by a Ras activation assay (using GST-tagged Raf-1 Ras binding domain which only binds active Ras), both Raf-1 and Ras exhibit activity in vehicle-treated cells that is not further increased by FSH [27]. Participation of the EGFR, Src, and Ca^{2+} in ERK activation in granulosa cells is evidenced by the abilities of the EGFR inhibitor AG1478, the Src inhibitor PP1, and the Ca^{2+} chelator EGTA to abrogate FSH-stimulated ERK activity [27]. Moreover, FSH-stimulated ERK activation is mimicked by the Ca^{2+} ionophore A23187 [45]. As shown in Fig. 2, we concluded that a tonic pathway consisting of Ca^{2+} , Src, and the EGFR led to Ras activation in vehicle-treated cells, based in part (a) on the ability of the EGFR inhibitor AG1478 to block ERK activation by the calcium ionophore A23187 but not to reduce Src activity, detected by an active Src antibody, and (b) on the ability of the Src inhibitor PP1 to block ERK activation by A23187. However, we have not yet identified the signal that initiates this tonic pathway.

The ability of FSH to activate ERK notwithstanding the presence of active MEK suggests that ERK activity is restrained in vehicle-treated cells and that this restraint is lifted in response to FSH. We showed that this restraint is mediated by a 100kDa phosphoprotein tyrosine phosphatase (PTP), based on an in-gel tyrosine phosphatase assay, that cross-reacts with an antibody directed to the "step-like" PTP-SL but is a distinct PTP [46,47], based on its size and lack of cross-reactivity with other PTP-SL antibodies [27]. FSH increases the phosphorylation of the 100kDa PTP, as detected upon immunoprecipitation of the PTP, and leads to dissociation of the PTP from ERK, as detected in anti-ERK pull-down [27]. The 100kDa PTP in granulosa cells remains to be identified.

A consequence of ERK activation in granulosa cells is phosphorylation of RSK-2 [28]; however, neither additional ERK nor RSK-2 targets in granulosa cells have been identified. A relevant potential target for ERK is the orphan nuclear receptor steroidogenic factor (SF)-1, which is recognized to be phosphorylated likely by ERK on S203 in human kidney COS cells, resulting in recruitment of coactivators and enhanced transcriptional activity [48,49]. SF-1 is necessary for the activation of a number of FSH target genes, including inhibin- α [52], epiregulin [51], Giot-1 [12], aromatase [37], and SCC [52]. Therefore, ERK activation in

granulosa cells potentially impacts expression of these target genes via regulation of SF-1. There is also evidence that ERK- and RSK-2-catalyzed phosphorylation of a number of immediate early genes, such as c-Jun and c-Fos of the AP-1 family, c-Myc, and Egr-1, results in their stabilization and thus prolonged activity [53]. While FSH increases expression of these immediate early genes [9,13,14], the only reported AP-1 family protein target in granulosa cells, to our knowledge, is the inhibin β A subunit [54]; c-Myc target genes in granulosa cells have not been identified. ERK is also reported to phosphorylate CREB binding protein (CBP) on S436 [55] resulting in enhanced coactivator activity and enhanced recruitment to the AP-1 complex [56]. The ubiquitous transcription factor specific protein (Sp)-1, which has been shown to regulate expression of a number of FSH target genes such as Egr-1 [9] and LH receptor [57], is also reported to be phosphorylated by ERK resulting in enhanced DNA binding activity, although phosphorylation of this transcription factor is complex and variable among cell types (as reviewed in [58]).

Evidence that FSH-stimulated ERK activity is necessary for activation of at least a subset of FSH target genes is based on the effects of the MEK inhibitor PD98059. This inhibitor blocks the induction of MAP2D by FSH [27] as well as the activation of FSH-stimulated hypoxia-induced factor-1 (HIF-1) activity and thus HIF-regulated genes, as discussed below. Consistent with this result, PD98059 strongly inhibits forskolin-stimulated activation of an inhibin- α promoter reporter [10]. PD98059 is also reported to reduce binding of Sp1/3 to a GC-region of the upstream regulatory sequence of Egr-1, as detected by EMSA assays [9], suggesting that Egr-1-regulated genes such as the LH receptor [59] would also be modulated by ERK.

4. Additional PKA-regulated pathways

4.1. p38 MAPK

FSH also stimulates the rapid but transient phosphorylation of p38 MAPK in granulosa cells, with signal readily detected by 10min post FSH and reduced by 1h [28]. Phosphorylation of upstream MAPK kinase MKK3/6 and p38 MAPK is also detected in ovaries 1h post PMSG injection (subcutaneously) [60]. While FSH-dependent activation of p38 MAPK is reported to be dependent on PKA based on inhibition by H89 [30,61], the PKA target that regulates p38 MAPK activity has not been identified. Based on inhibition by the p38 MAPK inhibitor SB203580, FSH-stimulated activation of p38 MAPK leads to phosphorylation of the actin-capping protein HSP-27 in granulosa cells and granulosa cell rounding and aggregation [30], a recognized response to FSH [62]. It is tempting to speculate that phosphorylation of HSP-27, which is reported to stimulate actin polymerization thus promoting microfilament reorganization and stabilization [63], contributes to the cytoskeletal reorganization in granulosa cells induced by FSH. Based on the selective expression of the HSP-27 kinase [64] p38 MAPK-activated protein kinase 2 (MK-2, formerly known as MAPKAPK-2) in immature ovaries [60], it is likely that FSH via p38 MAPK activates MK-2 to phosphorylate HSP-27 (see Fig. 2). The p38 MAPK inhibitor SB202190 is also reported to partially inhibit the ability of FSH to induce Crt11 [20] and to inhibit induction of aromatase [61], suggesting involvement of this pathway in regulation of these target genes. However, the impact of the p38 MAPK pathway on other kinases and transcription factors and the resulting potential regulation of FSH target gene expression remains to be more thoroughly investigated.

4.2. Phosphatidylinositol-3 kinase

FSH also promotes rapid activation of the PI3K pathway in rat granulosa cells, resulting in phosphorylation/activation of the downstream branch-point kinase Akt [16,27,31]. Phosphorylation of Akt is transient: phosphorylation signal is detected by 10min, peaks at 1h, and is undetectable by 4h post FSH addition [16]. Akt in ovarian extracts is similarly phosphorylated in response to PMSG injection into intact rats [16]. FSH-stimulated Akt phosphorylation in

granulosa cells is mimicked by forskolin or cell-permeable cAMP analogs but not inhibited by the typical PKA inhibitor H89 [16,31]. The inability of H89 to inhibit FSH-stimulated Akt phosphorylation could reflect the ability of H89 to inhibit p70S6K [16] preferentially over other kinases including PKA [16,65] and thus to inhibit an unidentified negative feedback pathway from p70S6K to Akt in granulosa cells. Consistent with this notion, a recent report suggests that FSH may signal into PI3K via a PKA-dependent pathway [66], although neither the PKA substrate nor the site of PKA's regulation to enhance Akt phosphorylation has been reported.

A critical role of the PI3K pathway in FSH-stimulated follicle maturation is evidenced by the ability of pharmacological PI3K inhibitors (wortmannin and LY294002) or dominant negative Akt to inhibit activation of aromatase, inhibin- α and Crt11 genes as well as LH receptor, inhibin- α , and VEGF promoter-reporters, and by the ability of IGF-1 or constitutively active Akt to synergize with FSH to increase expression of the LH receptor, inhibin- α , aromatase, and SCC in rat granulosa cells [16,20,33,67–69]. In view of the importance of the PI3K pathway to maturation of granulosa cells, we sought to identify downstream Akt targets and their regulation of FSH target genes.

4.2.1. FOXO1—The forkhead box-containing proteins in the O subfamily (FOXO1, FOXO3a, and FOXO4) are recognized Akt substrates [70], and FOXO1 is phosphorylated in response to FSH in granulosa cells [51,71,72]. FOXO transcription factors bind to DNA as monomers in the unphosphorylated state and function as both activators and repressors of transcription, depending on the gene, to regulate the cell cycle, metabolism, and/or survival [73]. Phosphorylation of FOXO proteins by Akt at three identified residues results in release from DNA, exit from the nucleus, and degradation (reviewed in [74]). One of the recognized functions of active, unphosphorylated FOXO is to maintain cells in the G₀ stage of the cell cycle, via direct [75] or indirect [76] repression of cyclin D and/or activation of the cell cycle inhibitor p27^{Kip1} (reviewed in [74]). Since induction of cyclin D₂ in granulosa cells of FSH-treated mice is required for granulosa cell proliferation [19], and follicular maturation is compromised in cyclin D₂ [19] but not in p27^{Kip1} [77] null mice, we determined whether FOXO1 functioned to repress cyclin D₂ expression in rat granulosa cells. Utilizing ChIP assays, we showed that cyclin D₂ promoter DNA (–680 to –285 nucleotides) is associated with FOXO1 in vehicle-treated granulosa cells, and that treatment of cells with FSH for 1h is sufficient to promote dissociation of FOXO1 from cyclin D₂ promoter [51]. This result suggests that active FOXO1 indeed functions to repress expression of cyclin D₂ in granulosa cells. Thus, based on our ChIP assay results, repression of cyclin D₂ gene by FOXO1 in granulosa cells appears to be direct and does not require induction of an additional repressor, as occurs in a human lymphoid cell line [76]. Yet, FSH does not promote expression of the cyclin D₂ gene [51].

Indeed, it is recognized that FSH is not sufficient to stimulate activation of the cyclin D₂ gene and consequent proliferation of rat granulosa cells (reviewed in [23]); rather, activin or another member of the transforming growth factor (TGF) β family plus FSH is required [78–81] (see Fig. 1). Consistent with these results, we showed that FSH plus activin activates a cyclin D₂ promoter-reporter, transiently transfected into granulosa cells, and stimulates expression of cyclin D₂ mRNA and protein by 24h post addition of FSH plus activin [51]. Activin alone is also not sufficient to stimulate cyclin D₂ gene expression [51,78]. Induction of cyclin D₂ protein and mRNA in response to FSH plus activin is abrogated upon transduction (as an adenoviral vector) of a constitutively active FOXO1 mutant [51], in which the three Akt phosphorylation sites were mutated to Ala [82]. This constitutively active FOXO1 mutant readily bound cyclin D₂ promoter DNA, as detected in ChIP assays, and was not displaced in cells treated for 1h with FSH plus activin [51]. This result provides additional support for the hypothesis that nonphosphorylated FOXO1 functions to repress cyclin D₂ in granulosa cells and that Akt-dependent phosphorylation excludes FOXO1 from the cyclin D₂ promoter. Suppression of

cyclin D₂ promoter activity by constitutively active FOXO1 mutant in cells treated with FSH plus activin was prevented [51] by introduction of an additional mutation in the DNA binding domain of FOXO1 which prevented binding of FOXO1 to DNA [82], resulting in expression of cyclin D₂ protein. These results suggest that FOXO1 suppression of cyclin D₂ promoter requires binding of FOXO1 to the cyclin D₂ promoter and is not mediated via a protein–protein interaction.

The requirement for both activin and FSH to activate the cyclin D₂ gene indicates that acute relief from FOXO1 repression upon FSH activation of Akt is not sufficient and that additional positive signals from activin are necessary to activate the cyclin D₂ gene. We noted that while phosphorylation of Akt and FOXO1 in FSH-treated cells is transient and returns to basal levels of vehicle-treated cells by 24h, phosphorylation of Akt and FOXO1 is prolonged for at least 24h in the presence of FSH plus activin [51]. Transduction of granulosa cells with a dominant negative Smad3 mutant, in which the C-terminal activin type I receptor phosphorylation sites [83] are deleted, blocked the persistent phosphorylation of Akt and FOXO1 at 24h post addition of FSH plus activin and abrogated the induction of cyclin D₂ despite normal phosphorylation of FOXO1 at 1h [51]. These results suggest that persistent phosphorylation of FOXO1 is necessary for activation of the cyclin D₂ gene. However, persistent FOXO1 phosphorylation is not sufficient based on results showing that addition of constitutively active Akt (as a adenoviral vector) in the presence of FSH does not activate the cyclin D₂ gene [33]. It is likely that in addition to prolonged relief from FOXO1 repression, activation of the cyclin D₂ gene requires activin- dependent Smad3 binding to either the cyclin D₂ promoter or to regulate coactivator or transcription factor association [84] with the cyclin D₂ promoter. It is also likely that additional positive signals generated by FSH potentially via CREB [85] and/or pathways that lead to Myc expression [86] are required to activate cyclin D₂ gene expression, as occurs in other cells. However, signals from the ERK pathway, either acutely in response to FSH or more long term in response to increased expression of epiregulin and consequent activation of the EGFR (reviewed in [15]), do not contribute to increased protein expression of cyclin D₂, based on the inability of the EGFR inhibitor AG1478 (which also inhibits FSH-stimulated ERK activation [27]) to affect cyclin D₂ expression in cells treated with FSH plus activin [51].

While FSH alone promotes activation of a number of “differentiation” target genes in granulosa cells, such as aromatase, inhibin- α , epiregulin, LH receptor, and SCC, as previously reviewed, addition of activin plus FSH enhances expression of these genes over levels seen with FSH alone [51,78] and promotes expression of nuclear receptors SF-1 and LRH-1; and these effects are abrogated by transduction of cells with dominant negative Smad3 mutant [51]. In contrast to the induction of cyclin D₂, activation of the “differentiation” target genes (inhibin- α , aromatase, and the LH receptor) is mimicked by FSH plus constitutively active Akt [33], suggesting that persistent relief from FOXO1 repression, in addition to other positive signals initiated by FSH via CREB, etc., is sufficient and that Smad3 binding to promoters is not necessary to activate these genes. Rather, the activin/Smad3 contribution to activation of the “differentiation” target genes appears to be from prolonged Akt/FOXO1 phosphorylation. Indeed, our results show that active FOXO1 suppresses aromatase, inhibin- α , epiregulin, SCC, SF-1, and LRH-1, based on the ability of constitutively active FOXO1 to abrogate or reduce activation of these genes in granulosa cells treated with FSH plus activin [51]. It is not known if FOXO1 binds directly to promoters of these target genes or whether repression is indirect. However, enhanced expression of these genes in the presence of activin could reflect increased expression of the potentially limiting transcription activator SF-1, which is required to activate each of these targets [37,50–52]. Taken together, these results suggest FOXO1 is a general repressor of FSH target genes. The mechanism by which FOXO1 represses target genes other than cyclin D₂, and identification of additional FOXO1 targets in granulosa cells, awaits additional investigation. It is interesting that FOXO3a appears to serve an equivalent function in primordial follicles, based on evidence that FOXO3a null mice exhibit global maturation of

primordial follicles and resulting follicle depletion [87]. Unfortunately, FOXO1 null mice die at embryonic day 10.5 [88].

4.2.2. Tuberin—A second Akt target that regulates cell growth and proliferation is tuberin. Tuberin, also known as tuberous sclerosis complex 2 (TSC2), is complexed with hamartin, or TSC1, and the TSC complex is a negative regulator of cell growth and translation (reviewed in [89,90]). In the absence of Akt phosphorylation, tuberin functions as a GTPase activating protein (GAP) for the small G protein ras-homologue enriched in brain (Rheb), resulting in the accumulation of Rheb^{GDP} (reviewed in [89]). Upon Akt-dependent phosphorylation of tuberin, the GAP activity of tuberin is inhibited, Rheb^{GTP} accumulates, and Rheb^{GTP} via an unidentified mechanism promotes activation of the Ser/Thr kinase mammalian target of rapamycin (mTOR) (see Fig. 2). mTOR then phosphorylates both p70S6K and 4E-binding protein (BP)1 to enhance translation. Upon phosphorylation, p70S6K phosphorylates the 40S ribosomal protein S6, and 4E-BP1 releases eukaryotic initiation factor (eIF) 4E, the rate-limiting protein in translation of mRNAs with a 5' methyl cap structure (reviewed in [89]).

We have shown in granulosa cells that FSH stimulates the phosphorylation of tuberin, p70S6K, and S6 protein; phosphorylation is rapid and detected by 10min and peaks at 1h post FSH [16]. Phosphorylation of 4E-BP1 is also detected [16]. Phosphorylation of these proteins in granulosa cells is mimicked by forskolin and 8-chlorophenylthio-cAMP and by IGF-1 as well as by injection of PMSG into rats [16]. The linear order of the components of the pathway is evidenced by the abilities of the mTOR inhibitor rapamycin to abrogate and of the farnesyl transferase inhibitor FTI-277 of Rheb to reduce FSH-stimulated phosphorylation of p70S6K and S6 protein without affecting upstream protein phosphorylations [16].

Based on the functions of this pathway in other cells, it is expected that mTOR activation is required for granulosa cells to grow in size and mass following cell division and that this pathway enhances translation of newly synthesized mRNAs as well as of preexisting mRNAs. That the mTOR pathway is necessary at least for activation of a subset of FSH-regulated “differentiation” target genes is evidenced by results showing that the mTOR inhibitor rapamycin abrogates induction by FSH of MAP2D and RII β proteins as well as activation of LH receptor-, inhibin- α -, and VEGF-promoter-reporter activities [16].

One of the mRNAs whose translation is enhanced upon activation of the tuberin/mTOR pathway in granulosa cells is that for HIF-1 α [16]. HIF-1 α is the dimeric binding partner of HIF-1 β and together they form HIF-1, a member of the basic-loop-helix-Per/Arnt/Sim family of transcription factors (reviewed in [91,92]). HIF-1 β is constitutively expressed by most cells while HIF-1 α is generally translated but very rapidly degraded under normal oxygen concentrations ($T_{1/2}$ ~5min) via the proteosomal pathway following posttranslational modifications that are oxygen-regulated (reviewed in [91]). Under hypoxia, HIF-1 α protein is stabilized, HIF-1 α dimerizes with HIF-1 β to bind to hypoxia response elements (HREs) to activate a number of genes, such as VEGF, glucose transporters, IGF-BPs, lactic dehydrogenase, erythropoietin, etc., that allow cells to function with depressed levels of oxygen. However, under normal oxygen levels, growth factors can also enhance translation of HIF-1 α (reviewed in [91]) without affecting its degradation, resulting in accumulation of HIF-1 α .

We showed in granulosa cells that FSH stimulated an accumulation of HIF-1 α in a cycloheximide-dependent manner and activation of HIF-1 activity, evidenced by activation of a canonical HRE-promoter-reporter [16]. While the hypoxiametic CoCl₂ [93] was required to detect accumulation of HIF-1 α protein, HIF-1 activity was detected in the absence of CoCl₂, consistent with the notion that sufficient HIF-1 α is present in the nucleus of FSH-treated cells to activate HIF-1-responsive target genes. Activation of HIF-1 activity was reduced by

inhibitors of PI3K, Rheb, and mTOR, consistent with activation of HIF-1 α translation downstream of the tuberlin/mTOR pathway [16]. The ability of the mTOR inhibitor rapamycin to inhibit promoter-reporter assays for VEGF, the LH receptor and inhibin- α stimulated by FSH suggested that HIF-1 could regulate these FSH “differentiation” target genes. Co-transfection of granulosa cells with a dominant negative HIF-1 α that retained its ability to heterodimerize with HIF-1 β but no longer expressed its DNA binding or transactivation domains [94], significantly reduced FSH-stimulated activation of VEGF-, inhibin- α -, and LH receptor-luciferase reporters [16]. These results then suggest that HIF-1 is necessary for the activation of inhibin- α , VEGF, and LH receptor. Inhibin- α and the LH receptor constitute what appear to be unique HIF target genes. However, direct proof that these are direct HIF-1 targets awaits further studies. It is likely that additional FSH target genes are regulated by HIF-1, such as IGF-BP 3, which has recently been shown to be regulated by FSH in a PKA-, PI3K-, and ERK-dependent manner in porcine granulosa cells [66]. We do not know if accumulation of HIF-1 α in granulosa cells is also regulated independently of FSH by hypoxic conditions, but suspect this is not the case since granulosa cells are likely to be exposed to increasingly hypoxic conditions as follicles enlarge as a result of the absence of a direct vascular supply interior to the basal lamina. Since HIF-1 activity contributes to the expression of key target genes that define the mature granulosa cell, such as inhibin- α and LH receptor, regulated expression of HIF-1 α by FSH rather than hypoxia would appear to be a mechanism to prevent untimely expression of these genes.

Addition of exogenous IGF-1 to rat granulosa cells also activates the PI3K pathway to stimulate phosphorylation of tuberlin, p70S6K and S6 [16]. IGF-1 also stimulates accumulation of HIF-1 α protein; however, HIF-1 activity is not activated based on the inability of IGF-1 to activate either HRE- or VEGF-reporters [Alam and Hunzicker-Dunn, unpublished]. This result suggests that additional signaling pathways activated by FSH but not by IGF-1 converge to promote HIF-1 activity, and that the apparent dimerization of HIF-1 α and HIF-1 β is not sufficient to activate HIF-1. As HIF-1 activity is reportedly enhanced by ERK (reviewed in [95]), and IGF-1 [Alam and Hunzicker-Dunn, unpublished] unlike FSH [27] does not stimulate ERK, we investigated whether FSH-stimulated HIF-1 activity was dependent on ERK. Results showed that the MEK inhibitor PD98059 abrogated FSH-stimulated HIF-1 activity and that HIF-1 activity was increased by constitutively active MEK [Alam and Hunzicker-Dunn, unpublished]. However, constitutively active MEK did not rescue IGF-1-stimulated HIF-1 activity [Alam and Hunzicker-Dunn, unpublished], suggesting that contributions from additional pathways are required to activate HIF-1 in granulosa cells.

5. Conclusions and future directions

Ovarian granulosa cells in primary culture offer a unique, physiologically relevant cell model to map signaling pathways that regulate cellular proliferation, growth and differentiation. While it is clear that the PKA and downstream PI3K pathways are required to activate genes associated with cell growth and differentiation, additional pathways like that of the ERKs and p38 MAPK certainly modify and appear to also be required to elicit target gene activation. It is likely that contributions from additional pathways are also mandatory. While much has been learned over the past decade, the cross-talk among these pathways and regulated targets in transcription factors and coactivators and repressors offers many new avenues for investigation. Moreover, the combinatorial effects of transcription factors and coactivators plus regulation of repressor release from FSH target genes is incompletely understood and offers many new areas to explore. The apparent ability of PKA to direct signaling appears to be unique to granulosa cells but may be a more generalized phenomena applicable perhaps to other cells, such as neuronal, thyroid, and adrenal cortical cells. While we know that granulosa cells express a large number of AKAPs [96], which PKA substrates and other scaffolded proteins are associated with each of the AKAPs remains to be elucidated. Moreover, while we predict the

presence of cAMP microdomains generated by FSH receptor activation of adenylyl cyclase(s) and destroyed by PDEs, such domains have not been physically demonstrated. Thus, much remains to be learned regarding signaling pathways that regulate FSH target gene expression.

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Fig. 1.

FSH activates a complex program to promote of gene expression differentiation and proliferation of granulosa cells to achieve formation of the preovulatory follicle. This figure is a schematic diagram showing a subset of the FSH-regulated differentiation targets. In primary culture of rat granulosa cells, activin plus FSH are required to achieve initiation of the cell cycle.

Fig. 2.

FSH-regulated signaling pathways in granulosa cells. This figure is a schematic diagram of our current understanding of signaling pathways utilized by FSH to regulate target gene expression in estrogen-treated granulosa cells.