BIOLOGY OF REPRODUCTION **67,** 1313–1320 (2002)
DOI 10.1095/biolreprod.102.004747

Relationship Between Steroidogenic Acute Regulatory Protein Expression and Progesterone Production in Hen Granulosa Cells During Follicle Development[1]

A.L. Johnson,[2] E.V. Solovieva, and J.T. Bridgham

Department of Biological Sciences, The University of Notre Dame, Notre Dame, Indiana 46556

ABSTRACT

The present studies were conducted to address cellular mechanisms responsible for regulating steroidogenic acute regulatory protein (StAR) expression and progesterone synthesis at maturational stages corresponding to both the time of hen follicle selection, as well as before and after the LH surge in preovulatory follicle granulosa cells. A recently published report has established that mitogen-activated protein (MAP) kinase signaling induced by transforming growth factor α (TGFα) treatment blocks FSH-induced differentiation and StAR expression in cultured hen granulosa cells, whereas inhibitors of MAP kinase signaling enhance FSH-induced differentiation. The present in vitro studies demonstrate that in addition to MAP kinase signaling, activation of protein kinase C (PKC) blocks both FSH-induced StAR expression and the initiation of progesterone production in prehierarchal follicle granulosa cells, whereas the pharmacologic inhibitor of PKC, GF109203X, potentiates FSH-induced StAR expression and, as a consequence, the initiation of progesterone synthesis. Moreover, we demonstrate in granulosa cells collected from preovulatory follicles that although an acute increase in progesterone production in response to LH treatment requires rapid transcription and translation of StAR, the magnitude of progesterone production is rate-limited by one or more factors other than StAR (e.g., the P450 cholesterol side-chain enzyme). Finally, the rapid turnover of StAR protein, such as occurs following the withdrawal of LH, provides an additional mechanism for the tight regulation of progesterone production that occurs during the hen ovulatory cycle, and explains the rapid loss of steroidogenesis in the postovulatory follicle. In summary, data reported herein support the proposal that paracrine/autocrine factors (including but not necessarily limited to TGFα) prevent premature expression of StAR in prehierarchal follicle granulosa cells by more than one receptor-mediated signaling pathway. Furthermore, subsequent to follicle selection into the preovulatory hierarchy, StAR transcription and translation is necessary but not sufficient for the full potentiation of the preovulatory surge of serum progesterone.

follicular development, granulosa cells, growth factors, ovary, progesterone

INTRODUCTION

The appropriate expression of steroidogenic acute regulatory protein (StAR) represents an indispensable component of steroidogenesis within preovulatory follicles and the corpus luteum of the mammalian ovary [1–4]. This protein serves to facilitate transfer of cholesterol to the inner mitochondrial membrane where it undergoes conversion to pregnenolone by the P450 cholesterol side-chain enzyme ($P450_{scc}$, derived from the CYP11A1 gene). Considering recent evidence for the conservation of StAR and its tissue-specific expression in avian, fish, and amphibian species, one would predict the regulation and function of StAR to be highly conserved within the vertebrate ovary in general [5]. While it is well accepted that ovarian StAR expression in mammals is promoted by FSH and LH predominantly via protein kinase A (PKA) signaling [2], additional factors such as insulin-like growth factor I (IGF-I; [6]), estradiol [7], growth differentiation factor 9 [8], and arachidonic acid [9] may mediate the actions of gonadotropins, or potentiate them, or both. Agonist-induced stimulation of steroid biosynthesis is dependent on active transcription and translation of StAR, and recent efforts have been directed toward identifying the mechanisms by which trophic hormones promote StAR gene transcription [4]. On the other hand, it has been known for some time that activation of protein kinase C (PKC) signaling attenuates gonadotropin-induced steroidogenesis (e.g., [10]), and it is now recognized that this effect can at least in part be explained by PKC inhibition of StAR expression [11].

The cloning and characterization of the chicken StAR cDNA [5] has enabled us to document the induction of StAR mRNA and protein expression as a critical component of FSH-mediated differentiation in hen granulosa cells derived from immature, prehierarchal follicles [12]. This up-regulation of StAR is a prerequisite for the induction of granulosa cell progesterone production, and is associated with the selection of a follicle into the preovulatory hierarchy. Moreover, the full potentiation of FSH-induced StAR expression and progesterone production at this stage of development appears to be dependent on the inhibition of paracrine or autocrine factor-mediated mitogen-activated protein (MAP) kinase signaling (e.g., via transforming growth factor α). Once a follicle reaches the preovulatory stage of development, granulosa cells become LH receptor-dominant [13], and LH-mediated progesterone production occurs in association with the rapid induction of StAR mRNA (within 1 h) and protein (within 3 h) expression [12]. It is well established that a fully potentiated preovulatory surge of serum progesterone is obligatory for ovulation of the largest preovulatory follicle [14, 15].

Accordingly, the present studies were conducted to extend our understanding of mechanisms regulating StAR expression and function in hen granulosa cells both at approximately the time of follicle selection, as well as during the period leading up to and following an LH-induced progesterone surge in preovulatory follicles. A primary difference between these two stages of follicle development rests

[1]This work was supported by grant 99-35203-7736 from the U.S. Department of Agriculture and by grant HD36095 from the National Institutes of Health to A.L.J.
[2]Correspondence: A.L. Johnson, Department of Biological Sciences, P.O. Box 369, The University of Notre Dame, Notre Dame, IN 46556.
FAX: 574 631 7413; e-mail: johnson.128@nd.edu

Received: 25 February 2002.
First decision: 17 March 2002.
Accepted: 27 May 2002.

ISSN: 0006-3363. http://www.biolreprod.org

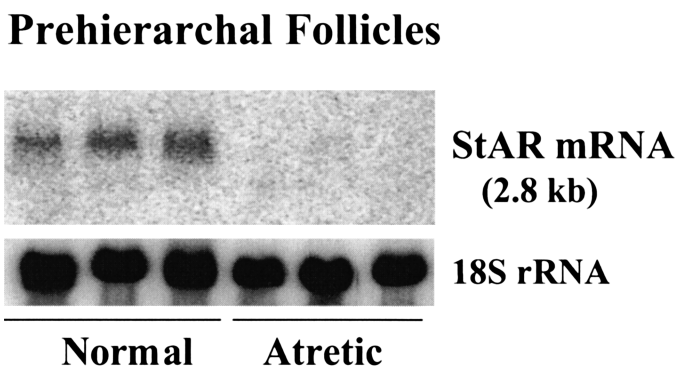

FIG. 1. Expression of the single 2.8-kilobase StAR transcript in three different normal or atretic prehierarchal (4–8 mm) follicles. Ribosomal (18S) RNA is presented as an indication of equal loading.

in the fact that undifferentiated cells from prehierarchal follicles fail to express StAR to any significant extent (despite FSH receptor expression), whereas differentiated granulosa cells from preovulatory follicles readily up-regulate the expression of StAR and produce large amounts of progesterone in response to gonadotropin (largely LH) treatment. Moreover, the working hypothesis tested herein is that the full potentiation of FSH-mediated differentiation in FSH receptor-dominant granulosa cells from prehierarchal follicles requires a concomitant removal of tonic inhibitory paracrine signaling. Specifically, experiments were designed to address 1) the role of PKC signaling in modulating FSH-induced differentiation in granulosa cells from prehierarchal follicles, and 2) the relationship between LH-induced StAR expression and the preovulatory progesterone surge in granulosa cells from hierarchal follicles.

MATERIALS AND METHODS

Animals and Reagents

Single-comb white Leghorn hens (Creighton Brothers, Warsaw, IN), 25–35 wk of age and laying regular sequences of more than six eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layena Mash; Purina Mills, St. Louis, MO) and water, and were exposed to a 15L:9D photoperiod, with lights-on at midnight. Ovulatory cycles were monitored by recording oviposition on a daily basis. Hens were killed approximately 16–18 h before a midsequence ovulation by cervical dislocation. All procedures described herein were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee, and were performed in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals.*

Recombinant human transforming growth factor α (TGFα) was obtained from PeproTech, Inc (Rocky Hill, NJ), whereas ovine LH (AFP-5551B) and recombinant human FSH (lot AFP-8468A) were provided by Dr. A.F. Parlow through the National Hormone and Peptide Program. The MAP kinase inhibitor, U0126, and PKC inhibitor, GF109203X, were from Biomol (Plymouth Meeting, PA), and phorbol 12-myristate 13-acetate (PMA; an activator of PKC), actinomycin D (an inhibitor of RNA transcription), and cycloheximide (a protein synthesis inhibitor) were from Sigma Chemical Co. (St. Louis, MO).

Tissue Collection and Granulosa Cell Cultures

Granulosa layers from the three largest preovulatory (F1, F2, and F3) follicles and undifferentiated prehierarchal (6–8 mm) follicles were collected, combined within their respective group, and dispersed in 0.3% collagenase (type 2; Worthington, Freehold, NJ) before treatment. An aliquot of cells from each group was frozen immediately at −70°C (T0 controls), whereas the remaining cells were plated at 40°C in an atmosphere containing 5% CO_2/95% O_2 in six-well polystyrene culture plates (Falcon 3046; Fisher Scientific, Pittsburgh, PA) at a density of approximately 10^6/well in 1 ml of M199-Hepes supplemented with Hanks salts

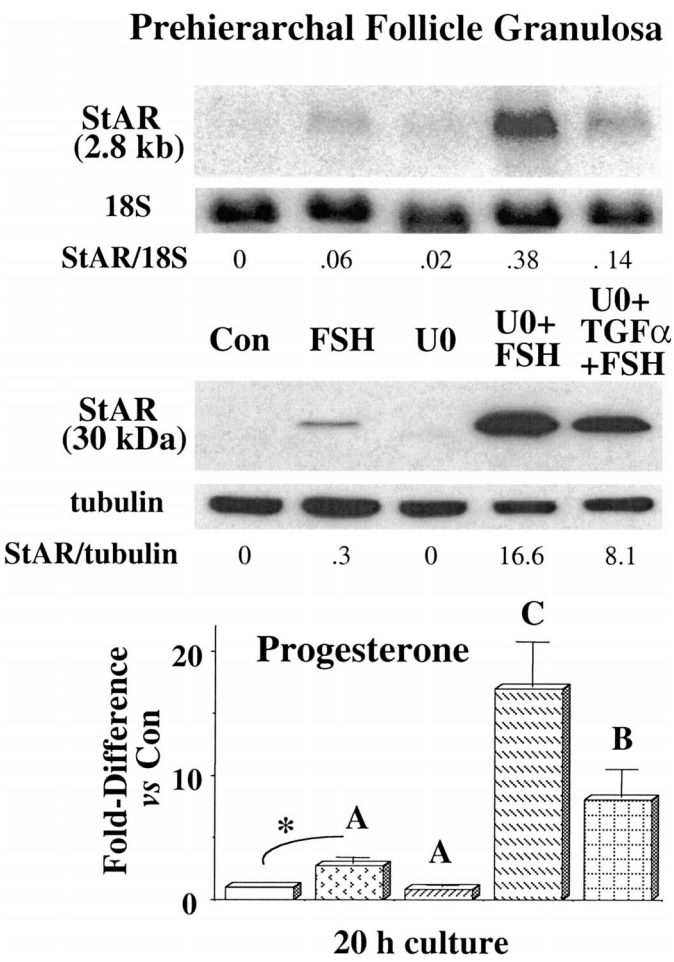

FIG. 2. Potentiation of FSH (100 ng/ml)-induced StAR mRNA, StAR protein, and progesterone production by the MAP kinase inhibitor U0126 (U0; 50 μM), and the ability of TGFα (50 ng/ml) to partially reverse this effect. Numbers below blots represent a relative ratio of StAR mRNA or protein to 18S ribosomal (r) RNA or α-tubulin, respectively. Northern and Western blots were replicated two additional times (here and in subsequent figures) with similar results. Progesterone is expressed as a fold-difference and compared with control (Con) cells cultured for 20 h. *$P <$ 0.05 versus Con by paired *t*-test; A,B,C $P < 0.05$; n = 5 replicate experiments.

(Gibco-BRL, Gaithersburg, MD) plus 1 ml of Dulbecco modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS; Gibco-BRL). In some instances, granulosa cells from the F1 follicle, or from the combined F2 plus F3 follicle layers, or both, were studied separately because it had previously been established that levels of StAR mRNA were dramatically higher in F1 follicles than in F2 or F3 follicles [5]. Morphologically normal and atretic prehierarchal follicles (3–8 mm) were collected and frozen without separating the granulosa and theca layers, as previously described [16].

In the first set of experiments, levels of StAR mRNA were compared in normal versus atretic prehierarchal follicles. In addition, we evaluated the involvement of MAP kinase signaling versus PKC signaling for initiation of StAR expression and progesterone production in functionally undifferentiated prehierarchal follicle granulosa cells. Granulosa cells were precultured for 1 h in the absence or presence of U0126 (50 μM), then cultured an additional 20 h with FSH (100 ng/ml), or TGFα (50 ng/ml) plus FSH. Doses used were based on previous experiments in hen granulosa cells [12, 17]. Following culture, media and plated cells were collected, cells were pelleted by centrifugation at 500 × *g* for 5 min, then media and cells were frozen separately at −70°C until media were assayed for progesterone and cells were prepared for total cellular mRNA or protein.

To explore alternative signaling pathways by which TGFα can affect StAR expression, prehierarchal follicle granulosa cells were cultured in the absence or presence of FSH, PMA [18] or FSH plus 162 nM PMA

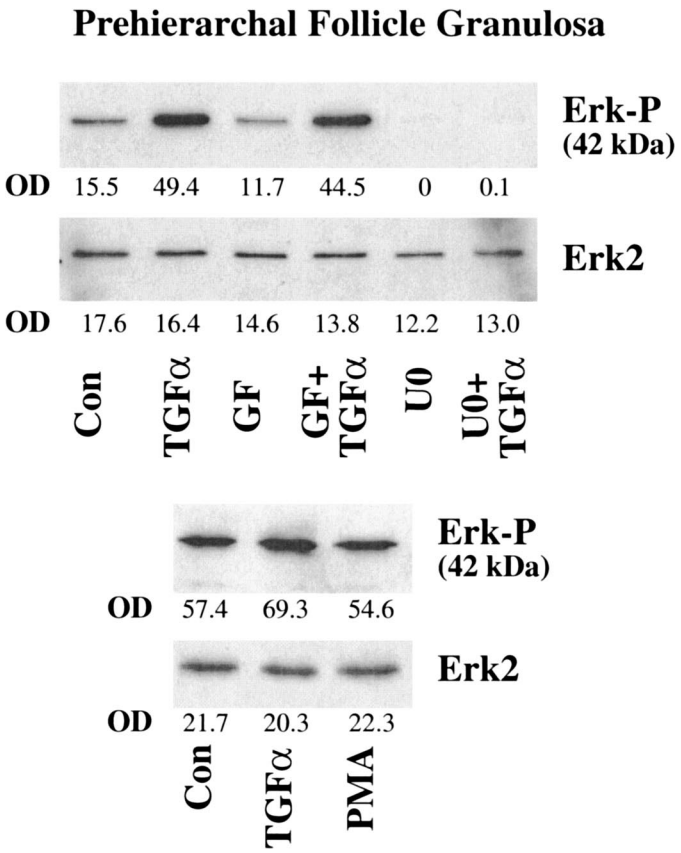

FIG. 3. The pharmacologic protein kinase C (PKC) inhibitor GF109203X (GF; 10 μM) fails to inhibit either TGFα-induced phosphorylation of Erk (Erk-P) or alter levels of Erk2 protein. By comparison, the MAP kinase inhibitor U0126 (U0; 50 μM) effectively prevents TGFα-induced Erk-P. PMA (162 nM) does not induce Erk-P or alter levels of Erk2. Numbers beneath blots represent arbitrary optical density (OD) units derived from densitometric scanning.

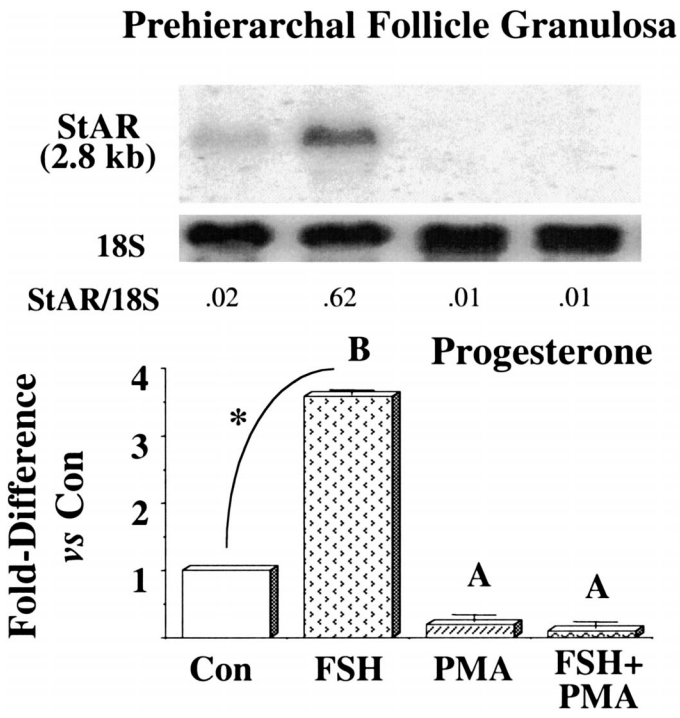

FIG. 4. PMA effectively blocks FSH-induced StAR expression and progesterone production in prehierarchal follicle granulosa cells cultured for 20 h. Numbers below blots represent a relative ratio of StAR mRNA to 18S rRNA. *$P < 0.05$ versus control by paired *t*-test; A,B $P < 0.05$; n = 6 replicate experiments.

for 20 h. In addition, cells were precultured for 1 h in the absence or presence of GF109203X (10 μM), then cultured an additional 20 h with FSH or TGFα plus FSH. The dose and preculture time for GF109203X was derived from previous studies of this pharmacologic inhibitor in granulosa/luteal cells [19, 20], and importantly, neither U0126 nor GF109203X induce cell death in hen granulosa cells after 24 h of culture ([12] and unpublished data). Media and cells were collected and processed as described above. In a related experiment, the effectiveness of each inhibitor to block MAP kinase signaling through extracellular signal-regulated kinases 1 and 2 (Erk1/2) was tested by pretreating freshly cultured granulosa cells for 1 h in the absence or presence of U0126 or GF109203X, then treating cells for an additional 20 min without or with PMA or TGFα. Samples were processed for levels of phosphorylated Erk (Erk-P). Treatment with this dose of TGFα has previously been demonstrated to be an effective inducer of Erk-P in hen granulosa cells [17].

In a second set of experiments, granulosa cells from F2 plus F3 follicles were precultured for 1 h in the absence and presence of actinomycin D (1 μg/ml) or cycloheximide (0.5 μg/ml), then cultured an additional 3 h without or with LH (100 ng/ml) to evaluate the requirement of StAR transcription, translation for progesterone production, or both. In addition, equal numbers of F1 or F2 plus F3 granulosa cells were incubated in the absence or presence of LH for 30 to 180 min to correlate levels of induced StAR protein with progesterone production according to stage within the hierarchy. For both of these experiments, media and cells were collected separately and frozen at −70°C until assayed for progesterone or prepared for total cellular RNA, protein levels, or both. For comparison, levels of $P450_{scc}$ mRNA in freshly collected granulosa cells from the F1 follicle were compared with those in F2 follicles.

To evaluate the turnover of StAR protein in cultured cells, granulosa cells from F1 follicles were pretreated with LH for 3 h, then medium was removed and cells were rinsed once with medium before culturing an additional 1 to 6 h in the absence of LH. Control cells included those that were untreated or that remained in the presence of LH for the entire 9-h experimental period. All granulosa cells were collected, frozen, and subsequently processed for protein. Finally, levels of StAR mRNA in freshly collected tissue and progesterone production from incubated (for 3 h) granulosa cells derived from the largest (F1) preovulatory follicle were compared with those collected from the most recent and second-most recent postovulatory follicles (POF1 and POF2, respectively; collected approximately 10 to 12 h following the most recent preovulatory LH surge). Granulosa cell layers from postovulatory follicles were collected by gently scraping the granulosa layer away from the theca layer with a scalpel blade.

Northern Blot Analysis

The chicken StAR cDNA used for probing Northern blots has been previously described [5], while the chicken $P450_{scc}$ probe consisted of a 569-base pair polymerase chain reaction (PCR) product corresponding to nucleotides 383 to 952 from chicken $P450_{scc}$ (GenBank accession number D49803). Separate blots were probed for StAR mRNA or $P450_{scc}$ mRNA, then each was reprobed for chicken 18S ribosomal RNA to standardize for equal loading of RNA samples. Conditions for conducting Northern blot analysis have been previously detailed [21], and all blots were visualized on phosphor screens using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Analysis of blots was conducted using the ImageQuant data reduction system (Molecular Dynamics).

Western Blot Analysis

The StAR polyclonal antibody was generously provided by Dr. D.B. Hales (University of Illinois-Chicago; [22]). The phospho-specific Erk1/2 monoclonal antiserum was from Upstate Biotechnology (Lake Placid, NY), the Erk2 polyclonal antiserum was from Santa Cruz Biotechnology (Santa Cruz, CA), and the α-tubulin monoclonal antibody used for standardization was from Sigma. Western blot analyses were conducted essentially as previously described [12, 17]. Incubations with primary antibodies (phospho-Erk at a 1:1000 dilution; Erk2 at a 1:1000 dilution; and StAR at a 1:5000 dilution) were conducted overnight at 4°C, whereas those for the horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig) G secondary antibody (for phospho-Erk; Pierce, Rockford, IL) or HRP-conjugated anti-rabbit IgG secondary antibody (for Erk2 and

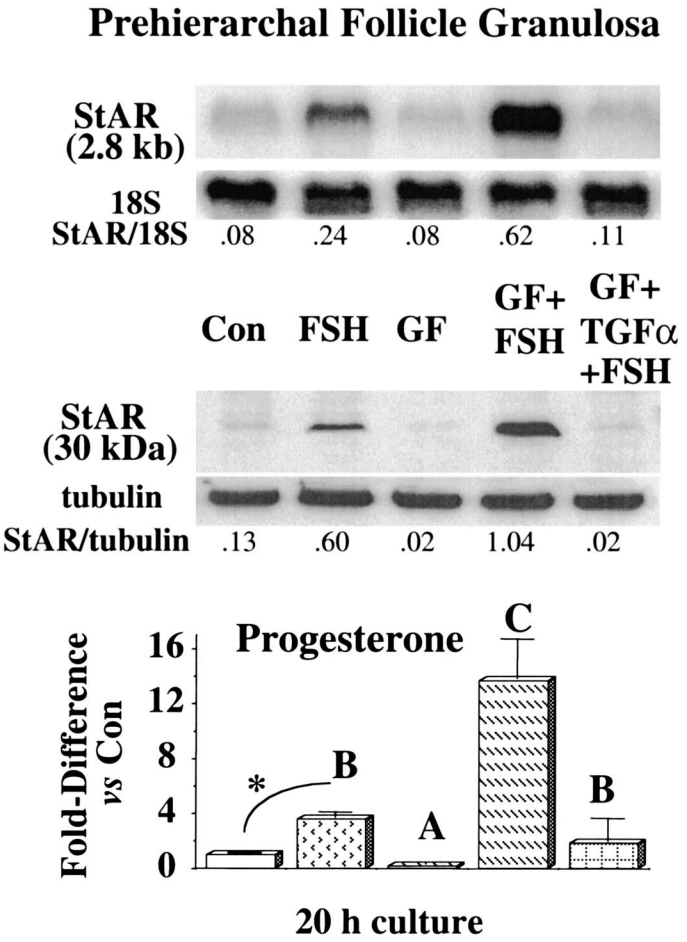

FIG. 5. Potentiation of FSH-induced StAR mRNA, StAR protein, and progesterone production by the PKC inhibitor GF109203X (GF; 10 μM) and the ability of TGFα to block this effect. Numbers below blots represent a relative ratio of StAR mRNA or protein to 18S rRNA or α-tubulin, respectively. Progesterone is expressed as a fold-difference compared with control (Con) cells cultured for 20 h. $^*P < 0.05$ versus Con by paired *t*-test; A,B,C $P < 0.05$; n = 4 replicate experiments.

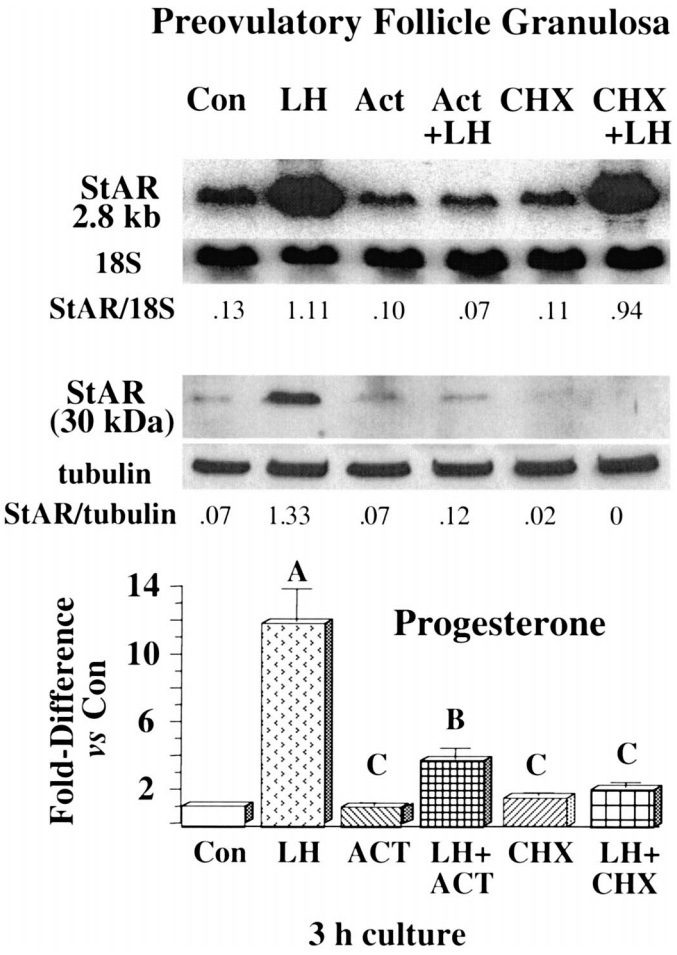

FIG. 6. Full potentiaton of LH-induced progesterone production in preovulatory follicle granulosa requires de novo StAR mRNA transcription and protein translation. Granulosa cells were plated and precultured for 1 h in the absence or presence of actinomycin D (1 μg/ml) or cycloheximide (0.5 μg/ml), then subsequently cultured an additional 3 h without or with LH (100 ng/ml). Numbers below blots represent a relative ratio of StAR mRNA or protein to18S rRNA or α-tubulin, respectively. A,B,C $P < 0.05$; n = 5 replicate experiments.

StAR; Pierce) were incubated for 1 h at room temperature. Blots were incubated with enhanced chemiluminescence Western blotting agent (Amersham Corp., Arlington Heights, IL) for 1 min and exposed to x-ray film for 1–15 min. The extent of antibody binding was quantitated by densitometry (UltraScan XL laser densitometer, Pharmacia LKB, Piscataway, NJ).

Progesterone Assays

Progesterone levels in media samples were quantified without extraction by radioimmunoassay according to previously published methods [18].

Data Analysis

All experiments were repeated a minimum of three times, unless otherwise stated. Summarized levels of StAR and $P450_{scc}$ mRNA and progesterone were expressed as fold-difference (mean ± SEM) versus a designated reference treatment such as freshly collected (T0) or cultured control cells (the value for the reference treatment was arbitrarily set at 1). Data were analyzed by *t*-test or by one-way ANOVA (analysis of fold-difference data did not include the reference treatment) followed by the Fisher protected least significant difference multiple range test. Post hoc analysis of selected data was conducted by paired *t*-test using original (nontransformed) data (e.g., Fig. 2, progesterone data).

RESULTS

Protein Kinase C Signaling in Prehierarchal Follicles

A single 2.8-kilobase transcript for StAR mRNA was detected in whole, morphologically normal prehierarchal follicles, but it was virtually undetectable in atretic follicles (Fig. 1). As recently reported [12], the MAP kinase inhibitor, U0126, potentiates FSH-induced StAR mRNA and protein expression plus progesterone production in cultured prehierarchal follicle granulosa cells. The novel finding is that TGFα treatment attenuates this stimulatory effect on StAR expression and progesterone production (Fig. 2) even though U0126 completely prevents TGFα-induced Erk-P (Fig. 3).

Similar to the effects of TGFα, cotreatment with PMA, a pharmacologic activator of PKC, effectively blocks the induction of FSH-induced StAR mRNA and progesterone production after 20 h in culture (Fig. 4), and this inhibitory effect is not mediated via Erk-P (Fig. 3). Furthermore, pretreatment of granulosa cells with the PKC inhibitor GF109203X potentiates FSH-induced StAR mRNA and protein expression plus progesterone production. However, under these conditions, cotreatment with TGFα completely

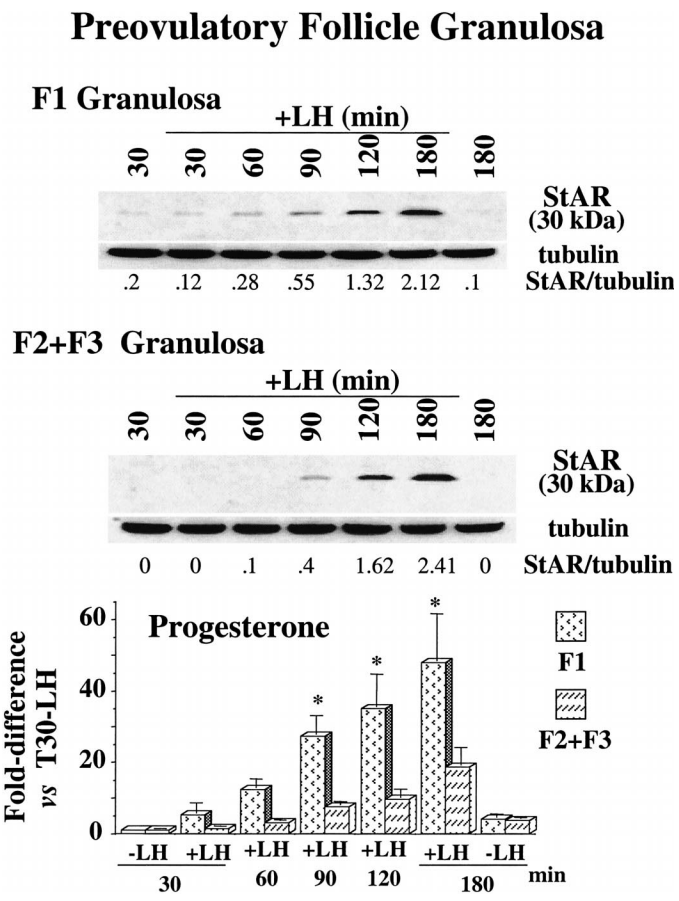

FIG. 7. LH-induced progesterone production in granulosa cells from the largest (F1) follicle greatly exceeds that from the second (F2) plus third (F3) largest preovulatory follicles, and in both instances is associated with increased StAR protein expression. Numbers below blots represent a relative ratio of StAR protein to α-tubulin. Progesterone is expressed as a fold-difference compared to granulosa cells incubated for 30 min in the absence of LH. $^*P < 0.05$ compared to F2+F3 granulosa; n = 3 replicate experiments.

reverses the potentiating effect of GF109203X (Fig. 5) and, importantly, GF109203X fails to attenuate TGFα-mediated Erk-P (Fig. 3).

Relationship Between StAR Expression and Progesterone Production in Preovulatory Follicles

Preculture of F2 plus F3 follicle granulosa cells with actinomycin D effectively blocks LH-induced StAR mRNA transcription and protein translation, and greatly attenuates LH-induced progesterone production after a 3-h culture (Fig. 6). By comparison, preculture with cycloheximide fails to alter LH-induced StAR mRNA, but completely inhibits any increase in StAR protein and, moreover, effectively blocks LH-induced progesterone production.

Although granulosa cells from the F1 follicle and F2 plus F3 follicles show comparable LH-induced responses after 90 to 180 min of treatment in terms of StAR protein expression, there is significantly greater progesterone production from F1 follicle granulosa cells at each interval (Fig. 7). To address this apparent discrepancy, levels of $P450_{scc}$ mRNA were compared in F1 versus F2 follicles, and were found to be significantly higher in granulosa cells from F1 follicles (Fig. 8).

Finally, levels of StAR protein are increased by 17.5 ± 4.4-fold after a 3-h pretreatment of cultured F1 follicle

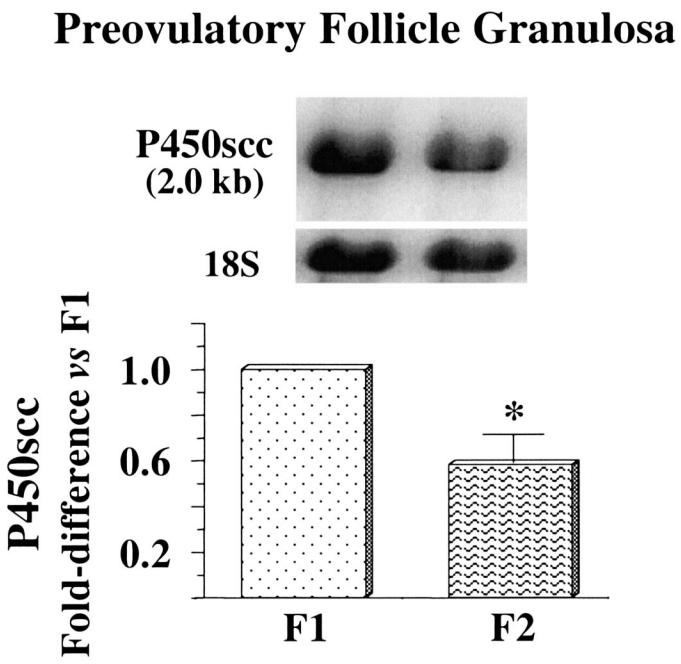

FIG. 8. Constitutive $P450_{scc}$ mRNA expression in granulosa cells from F1 compared with F2 follicles. Summarized data represent $P450_{scc}$ levels from three different ovaries standardized to 18S rRNA. $^*P < 0.05$ by paired *t*-test; n = 3 replicate experiments.

granulosa cells with LH (compared to control). Following the withdrawal of LH, StAR protein rapidly decreases to nondetectable levels within 6 h (Fig. 9). In an effort to relate this rapid loss of StAR expression to the interval following the preovulatory LH surge and early postovulatory period, in vivo, StAR mRNA (a more sensitive measure of detection than protein levels) was evaluated in freshly collected granulosa layers from the F1 and two most recent postovulatory follicles. Basal expression of the StAR transcript was readily detected in granulosa cells from the F1 follicle, but was decreased by more than 50% in granulosa cells from POF1 (collected 6 to 8 h following ovulation) and was virtually nondetectable in POF2. Similarly, basal progesterone produced after a 3-h incubation (expressed as nanograms of progesterone per micrograms of RNA) decreased from 4.9 ± 0.6 to 0.6 ± 0.1, respectively ($P < 0.01$; Fig. 10).

DISCUSSION

In hens, the selection of a single follicle into the final stages of growth and maturation before ovulation (the preovulatory hierarchy) occurs on a daily basis from a cohort of prehierarchal follicles that are 6–8 mm in diameter. Although the mechanism or mechanisms directly responsible for the recruitment of one prehierachal follicle over another are not currently known, the cellular events surrounding follicle selection continue to be a focus of investigation. One immediate consequence of selection entails the differentiation of the follicle granulosa cell layer (as measured by greater StAR expression and steroidogenic potential) promoted by FSH, whereas by contrast, the fate of a follicle not selected is likely to be atresia [23].

The ability to detect StAR mRNA in whole, nonatretic prehierarchal follicles likely represents expression almost entirely within the theca layer, as granulosa cells at this stage of development express little to no StAR [5] and produce essentially no progesterone [24]. Not unexpectedly,

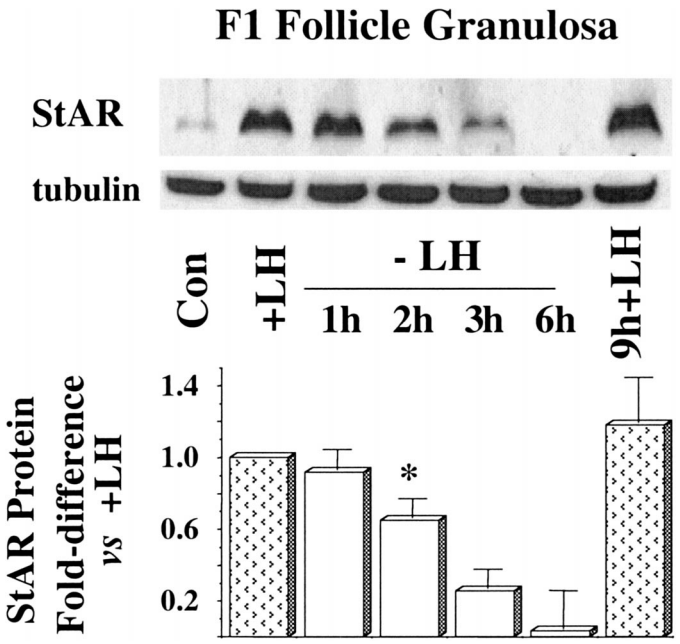

FIG. 9. Levels of StAR protein up-regulated by a prior 3-h treatment with LH rapidly decrease in cultured F1 follicle granulosa cells following the removal of LH. Summarized data represent levels of StAR protein (standardized to α-tubulin) compared to LH-induced levels (+LH). Con represents cells cultured without LH, whereas the 9h+LH group represents cells cultured with LH throughout the experimental period.

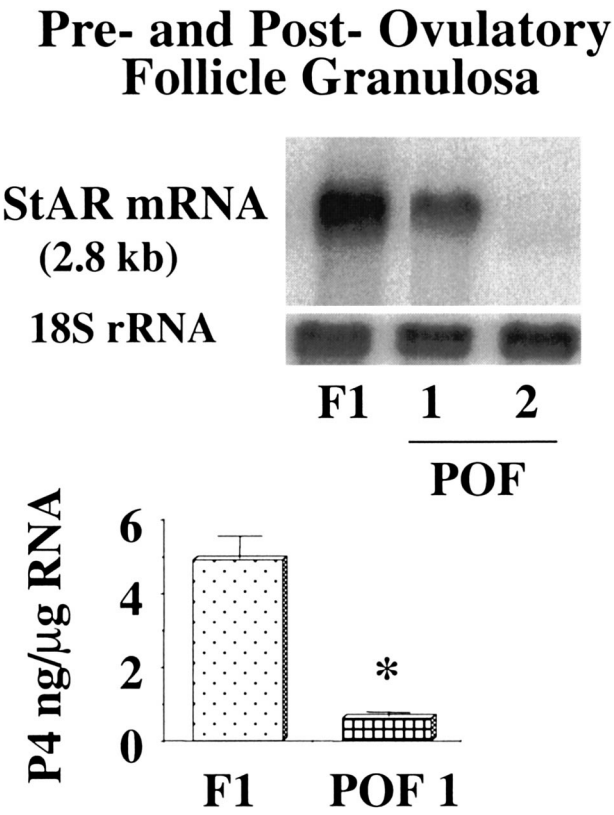

FIG. 10. Top panels show a representative blot of StAR mRNA from granulosa cells of the largest preovulatory (F1) follicle compared with the most recent and second-most recent postovulatory follicles (POF1 and POF2, respectively). Ribosomal (18S) RNA is presented as an indication of equal loading. This experiment was repeated once with similar results. Bottom panel shows summary of basal progesterone (P_4) production (n = 3 replicate experiments) from the F1 versus POF1 expressed as nanograms per 3-h incubation per microgram of RNA. $*P < 0.01$; n = 5 replicate experiments.

follicle atresia is associated with a significant decrease in LH and FSH receptor mRNA expression [13, 25] and, presumably due to the withdrawal of gonadotropin support, the loss of StAR mRNA expression. Although the theca layer from viable prehierarchal follicles is known to be steroidogenically active [26], it has not yet been established whether the loss of StAR expression, and as a result steroidogenic potential, is a contributing factor to the death of the granulosa layer or simply a result of the atretic process.

On the other hand, granulosa cells collected from normal prehierarchal follicles respond to FSH treatment with a modest but significant increase in StAR and $P450_{scc}$ expression plus progesterone production after 24 h of culture, yet coculture with TGFα blocks each of these effects [12, 27]. The MAP kinase inhibitors U0126 and PD98059 completely inhibit TGFα-induced Erk-P, and, importantly, they potentiate FSH-induced StAR expression and progesterone production (Fig. 2; [12]). These findings support the working hypothesis that tonic MAP kinase activity prevents premature differentiation of granulosa cells and, moreover, that the inhibition of such signaling may be a prerequisite for the full potentiation of steroidogenesis following follicle selection. It is worthy of note that the ability of the MAP kinase signaling via Erk to inhibit gonadotropin-stimulated steroidogenesis in human granulosa-derived cells has recently been reported [28, 29].

However, the present data also demonstrate that even in the absence of MAP kinase signaling via Erk-P, TGFα remains capable of attenuating FSH-induced StAR expression and progesterone production (Figs. 2 and 3). Previously published data have implicated PKC signaling as a negative modulator of steroid production in hen granulosa cells [18]. The present results confirm that this negative regulation is at least in part attributed to PKC-mediated inhibition of StAR transcription (Fig. 4), and this is further supported by the finding that preculture with the pharmacologic PKC inhibitor GF109203X, greatly potentiates FSH-induced StAR expression and progesterone induction (Fig. 5). Furthermore, this effect is not mediated via MAP kinase/Erk signaling (Fig. 3). Although neither the specific PKC isoforms involved nor the precise cellular mechanisms effecting negative regulation of StAR transcription are currently known, there is evidence from mammalian model systems for the involvement of the transcription factors DAX-1 and Yin Yang 1 [1, 30].

In contrast to the inability of TGFα to completely reverse the potentiating effects of U0126 on differentiation, TGFα effectively blocks GF109203X-potentiated differentiation, presumably through the alternative MAP kinase signaling pathway. This indicates that TGFα can independently signal via both the MAP kinase/Erk-P and PKC pathways in prehierarchal follicle granulosa cells, yet suggest that MAP kinase signaling may represent the more potent differentiation-inhibiting pathway. Furthermore, unlike gonadotropin-releasing hormone in human granulosa-luteal cells [19], TGFα-induced Erk-P is not dependent on PKC signaling (Fig. 3). The findings reported herein are similar to those using cultured bovine granulosa cells from which it was concluded that TGFα inhibits PKA-mediated progesterone and estradiol production via both MAP kinase and PKC signaling [31]. We found it interesting that in the same studies, inhibition of MAP kinase signaling (using genistein) was also found to potentiate FSH-induced estradiol production. We also note that both epidermal growth

factor and PMA have previously been found to reverse the phenotypic remodeling that occurs in porcine granulosa cells during FSH-induced differentiation and spontaneous luteinization in vitro [32, 33]. These results collectively indicate that the MAP kinase and PKC signaling pathways represent a conserved mechanism by which granulosa cell differentiation is tonically inhibited.

Once a follicle has been recruited into the preovulatory hierarchy, granulosa cells become competent to continuously secrete low levels of progesterone throughout the ovulatory cycle, plus respond to LH with a transient preovulatory surge of progesterone during the period 6 h through 2 h before ovulation [34]. Studies have previously demonstrated that the granulosa cell layer is the primary source of progesterone production during the preovulatory LH surge, and both in vivo and in vitro data demonstrate that LH-induced progesterone production is significantly greater within F1 follicles than it is in F2 and smaller preovulatory follicles [35]. The latter observation is confirmed in the present in vitro experiment (Fig. 7). Significantly, however, the enhanced potential for progesterone synthesis is not associated with a detectable difference in LH receptor mRNA levels [13] or LH-induced StAR expression between these two stages of development, but is associated with a higher level of $P450_{scc}$ expression in F1 follicle granulosa cells (Fig. 8). We conclude that the increase in StAR expression in F2 follicles is necessary for rapid, LH-induced progesterone production, but is not sufficient for producing surge levels of progesterone. Instead, the data support the longstanding proposal that levels of $P450_{scc}$ activity [36] represent the rate-limiting step in producing the preovulatory surge of progesterone primarily by the F1 follicle granulosa layer.

In contrast to StAR expression, which increases within 90 to 180 min following gonadotropin treatment (Fig. 7, [12]), significant increases in $P450_{scc}$ expression occur after 8 to 16 h of treatment [27]. It is interesting to speculate why the transient preovulatory surge of progesterone induced by LH might be regulated by the product of a gene that is not rapidly regulated (e.g., $P450_{scc}$) rather than one that can be acutely up-regulated (StAR). In this regard, there is evidence that progesterone, in the absence of gonadotropins, may directly affect the follicle to promote the rupture of the stigma at ovulation [37, 38]. These effects may be mediated via progesterone receptors that are known to be expressed in both the granulosa and theca layers of preovulatory follicles [39]. Therefore, from a physiologic perspective, the inability of the F2 follicle to produce sufficiently large amounts of progesterone in response to a preovulatory LH surge (due to limiting amounts of $P450_{scc}$) may preclude its ability to initiate the ovulatory process, and thus ensure ovulation of only the F1 follicle.

Similar to findings derived from porcine luteinized granulosa cells [11] and mouse Leydig tumor cells [40], PKA-induced StAR expression, and as a consequence enhanced progesterone production, is largely dependent on new transcription and translation (Fig. 6). In the presence of actinomycin D, there is a marked (but not complete) reduction in LH-induced progesterone production from hen granulosa cells. This suggests that StAR protein synthesis continues in the presence of actinomycin D, and that the low but detectable levels of StAR protein are capable of facilitating limited progesterone synthesis after a 3-h culture. On the other hand, although cycloheximide does not block LH-induced StAR mRNA expression (an indication that StAR transcription does not require new protein synthesis), granulosa cells treated with cycloheximide plus LH fail to respond with either increased StAR protein expression or progesterone synthesis compared with those treated with cycloheximide alone. This latter result demonstrates the virtual requirement for new protein synthesis to mediate agonist-induced steroid production.

Following the preovulatory release of LH in vivo, serum progesterone levels decline to presurge levels by approximately 2 h before ovulation [34]. Results from the present studies suggest that this decrease in circulating progesterone is associated with a rapid loss of StAR protein, as is observed following the withdrawal of LH in cultured granulosa cells (Fig. 9). The ability of StAR protein to be rapidly turned over as described here is consistent with the previously estimated half-life of StAR mRNA (3–3.5 h, [40]). Mechanisms responsible for terminating the transient preovulatory LH and progesterone surges have yet to be adequately defined.

Unlike mammalian species in which a steroidogenically active corpus luteum forms from the remaining follicle tissues following ovulation, the postovulatory follicle of the hen rapidly becomes steroidogenically inactive and is completely resorbed within a few days to a week [41, 42]. Not unexpectedly, this decline in basal progesterone production in hen postovulatory follicles is associated with the loss of StAR mRNA expression (Fig. 10).

In summary, the data reported herein support the proposal that paracrine/autocrine factors (including but not limited to TGFα) prevent premature expression of StAR in prehierarchal follicle granulosa cells by more than one receptor-mediated signaling pathway. Accordingly, in addition to ongoing efforts directed toward identifying the mechanisms by which trophic hormones promote StAR gene transcription, and by implication enhanced steroidogenic potential, emphasis should be also focused on downstream target regulatory proteins that can actively prevent StAR transcription. Subsequent to follicle selection into the preovulatory hierarchy, StAR transcription and translation can be rapidly increased within the granulosa layer in response to a preovulatory LH surge. Although greater StAR expression is clearly required to produce the preovulatory surge of circulating progesterone, it is evident that additional factors, such as levels of $P450_{scc}$ expression, ultimately determine the stage-dependent quantity of progesterone synthesis. Finally, in combination with the ability of StAR expression to be rapidly up-regulated by PKA signaling, the subsequent rapid loss of StAR protein following LH withdrawal provides for an ongoing, tightly controlled regulation of granulosa cell steroidogenesis.

ACKNOWLEDGMENTS

We thank J. Martin for excellent technical support and Dr. D.B. Hales for the StAR polyclonal antiserum.

REFERENCES

1. Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 2001; 63:193–213.
2. Miller WL, Strauss III JF. Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, StAR. J Steroid Biochem Mol Biol 1999; 69:131–41.
3. Stocco DM. Steroidogenic acute regulatory (StAR) protein: what's new? Bioessays 1999; 21:768–775.
4. Stocco DM, Clark BJ, Reinhart AJ, Williams SC, Dyson M, Dassi B, Walsh LP, Manna PR, Wang XJ, Zeleznik AJ, Orly J. Elements involved in the regulation of the StAR gene. Mol Cell Endocrinol 2001; 177:55–59.

5. Bauer MP, Bridgham JT, Langenau DM, Johnson AL, Goetz FW. Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. Mol Cell Endocrinol 2000; 168:119–125.
6. LaVoie HA, Garmey JC, Veldhuis JD. Mechanisms of insulin-like growth factor I augmentation of follicle-stimulating hormone-induced porcine steroidogenic acute regulatory protein gene promoter activity in granulosa cells. Endocrinology 1999; 140:146–153.
7. Townson DH, Wang XJ, Keyes PL, Kostyo JL, Stocco DM. Expression of the steroidogenic acute regulatory protein in the corpus luteum of the rabbit: dependence upon the luteotropic hormone, estradiol-17 beta. Biol Reprod 1996; 55:868–874.
8. Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol Endocrinol 1999; 13:1035–1048.
9. Wang X, Walsh LP, Reinhart AJ, Stocco DM. The role of arachidonic acid in steroidogenesis and steroidogenic acute regulatory (StAR) gene and protein expression. J Biol Chem 2000; 275:20204–20209.
10. Wiltbank MC, Belfiore CJ, Niswender GD. Steroidogenic enzyme activity after acute activation of protein kinase (PK) A and PKC in ovine small and large luteal cells. Mol Cell Endocrinol 1993; 97:1–7.
11. Pescador N, Houde A, Stocco DM, Murphy BD. Follicle-stimulating hormone and intracellular second messengers regulate steroidogenic acute regulatory protein messenger ribonucleic acid in luteinized porcine granulosa cells. Biol Reprod 1997; 57:660–668.
12. Johnson AL, Bridgham JT. Regulation of steroidogenic acute regulatory protein and luteinizing hormone receptor messenger ribonucleic acid in hen granulosa cells. Endocrinology 2001; 142:3116–3124.
13. Johnson AL, Bridgham JT, Wagner B. Characterization of a chicken luteinizing hormone receptor (cLH-R) cDNA, and expression of cLH-R mRNA in the ovary. Biol Reprod 1996; 55:304–309.
14. Lang GF, Walton JS, Etches RJ. The effect of aminoglutethimide on steroid secretion, ovulation, and luteinizing hormone release in the hen. Poult Sci 1984; 63:1861–1871.
15. Johnson AL, van Tienhoven A. Effects of aminoglutethimide on luteinizing hormone and steroid secretion, and ovulation in the hen, *Gallus domesticus*. Endocrinology 1984; 114:2276–2283.
16. Johnson AL, Bridgham JT, Witty JP, Tilly JL. Susceptibility of avian ovarian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of bcl-xlong gene expression. Endocrinology 1996; 137:2059–2066.
17. Johnson AL, Bridgham JT, Swenson JA. Activation of the AKT/protein kinase B signaling pathway is associated with granulosa cell survival. Biol Reprod 2001; 64:1566–1574.
18. Tilly JL, Johnson AL. Attenuation of hen granulosa cell steroidogenesis by a phorbol ester and 1-oleoyl-2-acetylglycerol. Biol Reprod 1988; 39:1–8.
19. Kang SK, Tai CJ, Nathwani PS, Choi KC, Leung PC. Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone in human granulosa-luteal cells. Endocrinology 2001; 142:671–679.
20. Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS. Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 2000; 14: 1283–1300.
21. Johnson AL, Bridgham JT, Witty JP, Tilly JL. Expression of bcl-2 and nr-13 in hen ovarian follicles during development. Biol Reprod 1997; 57:1096–1103.
22. Held Hales K, Diemer T, Ginde S, Roberts MR, Shankar BK, Bosmann HB, Hales DB. Diametric effects of bacterial endotoxin lipopolysaccharide on adrenal and Leydig cell steroidogenic acute regulatory protein (StAR). Endocrinology 2000; 141:4000–4012.
23. Johnson AL. The avian ovarian hierarchy: a balance between follicle differentiation and atresia. Poult Avian Biol Rev 1996; 7:99–110.
24. Tilly JL, Kowalski KI, Johnson AL. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod 1991; 44:305–314.
25. You S, Bridgham JT, Foster DN, Johnson AL. Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. Biol Reprod 1996; 55:1055–1062.
26. Kowalski KI, Tilly JL, Johnson AL. Cytochrome P450 side-chain cleavage ($P450_{scc}$) in the hen ovary. I. Regulation of $P450_{scc}$ messenger RNA levels and steroidogenesis in theca cells of developing follicles. Biol Reprod 1991; 45:955–966.
27. Li Z, Johnson AL. Regulation of P450 cholesterol side-chain cleavage messenger ribonucleic acid expression and progesterone production in hen granulosa cells. Biol Reprod 1993; 49:463–469.
28. Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss JF III, Amsterdam A. The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. J Biol Chem 2001; 276:13957–13964.
29. Tai CJ, Kang SK, Choi KC, Tzeng CR, Leung PC. Role of mitogen-activated protein kinase in prostaglandin F2α action in human granulosa-luteal cells. J Clin Endocrinol Metab 2001; 86:375–380.
30. Nackley AC, Shea-Eaton W, Lopez D, McLean MP. Repression of the steroidogenic acute regulatory gene by the multifunctional transcription factor yin yang 1. Endocrinology 2002; 143:1085–1096.
31. Legault S, Bailey JL, Fortier MA, Rouillier P, Guilbault LA. Intracellular regulation of estradiol and progesterone production by cultured bovine granulosa cells. Mol Reprod Dev 1999; 54:371–378.
32. Hatey F, Mulsant P, Bonnet A, Benne F, Gasser F. Protein kinase C inhibition of in vitro FSH-induced differentiation in pig granulosa cells. Mol Cell Endocrinol 1995; 107:9–16.
33. Pescador N, Stocco DM, Murphy BD. Growth factor modulation of steroidogenic acute regulatory protein and luteinization in the pig ovary. Biol Reprod 1999; 60:1453–1461.
34. Johnson AL, van Tienhoven A. Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen, *Gallus domesticus*. Biol Reprod 1980; 23:386–393.
35. Etches RJ, Petitte JN. Reptilian and avian follicular hierarchies: models for the study of ovarian development. J Exp Zool Suppl 1990; 4: 112–122.
36. Asem EK, Hertelendy F. Influence of follicular maturation on luteinizing hormone-, cyclic 3′,5′-adenosine monophosphate-, forskolin- and cholesterol-stimulated progesterone production in hen granulosa cells. Biol Reprod 1985; 32:257–268.
37. Tanaka K, Li ZD, Ataka Y. Studies of ovulation in the perfused ovary of the fowl (*Gallus domesticus*). J Reprod Fertil 1987; 80:411–416.
38. Nakada T, Koja Z, Tanaka K. Effect of progesterone on ovulation in the hypophysectomised hen. Br Poult Sci 1994; 35:153–156.
39. Yoshimura Y, Bahr JM. Localization of progesterone receptors in pre- and postovulatory follicles of the domestic hen. Endocrinology 1991; 128:323–330.
40. Clark BJ, Combs R, Hales KH, Hales DB, Stocco DM. Inhibition of transcription affects synthesis of steroidogenic acute regulatory protein and steroidogenesis in MA-10 mouse Leydig tumor cells. Endocrinology 1997; 138:4893–4901.
41. Calvo FO, Wang SC, Bahr JM. LH-stimulable adenylyl cyclase activity during the ovulatory cycle in granulosa cells of the three largest follicles and the postovulatory follicle of the domestic hen (*Gallus domesticus*). Biol Reprod 1981; 25:805–812.
42. Dick HR, Culbert J, Wells JW, Gilbert AB, Davidson MF. Steroid hormones in the postovulatory follicle of the domestic fowl (*Gallus domesticus*). J Reprod Fertil 1978; 53:103–107.