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Cellular Mechanisms and Modulation of Activin A- and Transforming Growth Factor β-Mediated Differentiation in Cultured Hen Granulosa Cells[1]

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ABSTRACT

Undifferentiated granulosa cells from prehierarchal (6- to 8-mm-diameter) hen follicles express very low to undetectable levels of LH receptor (LH-R) mRNA, P450 cholesterol side chain cleavage (P450scc) enzyme activity, and steroidogenic acute regulatory (StAR) protein, and produce negligible progesterone, in vitro, following an acute (3-h) challenge with either FSH or LH. It has previously been established that culturing such cells with FSH for 18–20 h induces LH-R, P450scc, and StAR expression, which enables the initiation of progesterone production. The present studies were conducted to characterize the ability of activin and transforming growth factor (TGF) β, both alone and in combination with FSH, to promote hen granulosa cell differentiation, in vitro. A 20-h culture of prehierarchal follicle granulosa cells with activin A or transforming growth factor β (TGFβ)1 increased LH-R mRNA levels compared with control cultured cells. Activin A and TGFβ1 also promoted FSH-receptor (FSH-R) mRNA expression when combined with FSH treatment. Neither activin A nor TGFβ1 alone stimulated progesterone production after 20 h culture. However, preculture with either factor for 20 h (to induce gonadotropin receptor mRNA expression) followed by a 3-h challenge with FSH or LH potentiated StAR expression and progesterone production compared with cells challenged with gonadotropin in the absence of activin A or TGFβ1 preculture. Significantly, activation of the mitogen-activated protein (MAP) kinase pathway with transforming growth factor α (TGFα) (monitored by Erk phosphorylation) blocked TGFβ1-induced LH-R expression, and this effect was associated with the inhibition of Smad2 phosphorylation. We conclude that a primary differentiation-inducing action of activin A and TGFβ1 on hen granulosa cells from prehierarchal follicles is directed toward LH-R expression. Enhanced LH-R levels subsequently sensitize granulosa cells to LH, which in turn promotes StAR plus P450scc expression and subsequently an increase in P4 production. Significantly, the finding that TGFβ signaling is negatively regulated by MAP kinase signaling is proposed to represent a mechanism that prevents premature differentiation of granulosa cells.

activin, follicle-stimulating hormone receptor, granulosa, granulosa cells, hen ovary, LH receptor, luteinizing hormone, ovary, StAR, steroidogenesis, TGFβ

INTRODUCTION

In the hen ovary, the selection of a single follicle per day into the preovulatory hierarchy (which consists of the 5–9 largest yolk-filled follicles destined for ovulation) occurs from a small cohort of 8–12 prehierarchal (6- to 8-mm-diameter) follicles. Prior to selection, granulosa cells from such follicles express very low levels of LH-receptor (LH-R), cytochrome P450 cholesterol side chain cleavage (P450scc) enzyme activity, and steroidogenesis acute regulatory (StAR) protein, and produce negligible amounts of progesterone [1–3]. Although the precise mechanisms by which a single follicle per day is selected (and associated granulosa cell differentiation is initiated) have not been elucidated, studies with cultured cells have determined that FSH treatment is capable of inducing each of the aforementioned parameters. Just as important, concurrent activation of mitogen-activated protein (MAP) kinase signaling via Erk can block these FSH-induced effects [1, 4].

Transforming growth factor (TGF) β and activin belong to a large superfamily of extracellular regulatory proteins. A number of such family members are expressed within the mammalian ovary, including TGFβ (TGFβ1, β2, β3), activins (activin A, AB, B), growth differentiation factor-9 (GDF-9), inhibins, anti-Mullerian hormone, and at least five bone morphogenic proteins (BMP-2, -4, -6, -7, and -15) [5–8]. To date, various members of the TGFβ superfamily have been found to act in the mammalian ovary either as positive or negative regulators of granulosa cell differentiation [9–12].

Both activin and TGFβ induce their biological effects by forming a heterodimeric complex with two type II and two type I serine and threonine kinase receptors. Activation of the type I receptor results in the phosphorylation of one or more receptor (r)-Smads (e.g., Smad2, -3) [8, 13]. Activated r-Smad subsequently associates with a co-Smad (e.g., Smad4) and is translocated to the nucleus to initiate gene transcription. On the other hand, negative modulation of activin and TGFβ signaling can occur via inhibitory (i)-Smads (e.g., Smad6, -7), which act by inhibiting r-Smad phosphorylation and preventing r-Smad/co-Smad complexes from translocating to the nucleus [13].

Studies to date have documented the presence of TGFβ expression in both the theca layer from prehierarchal and preovulatory hen follicles and within granulosa cells from the largest preovulatory follicle [14], and recent studies have identified a chicken TGFβ4 hypothesized to represent the avian homolog to mammalian TGFβ1 [15]. Immunoreactive activin A is localized mainly within the theca layer throughout follicle development, with lesser amounts detected within granulosa cells of preovulatory follicles. There is a significant increase in the theca content of activin A found within the most recently selected (9-mm-diameter)

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follicle [16], suggesting the potential for paracrine signaling at the time of follicle selection. Moreover, avian homologs to type I and type II TGFβ and activin receptors as well as their associated cell signaling components (Smad2, 3, 4, and 7) have been identified [17, 18; GenBank accession AF230192], and several of these have been described within hen granulosa cells [19–21]. Such findings support the possibility of a paracrine and/or autocrine function for activin and TGFβ within hen follicles.

Previously published data demonstrate that activin A treatment promotes Smad2 phosphorylation and nuclear translocation in hen granulosa cells from preovulatory follicles [21]. Additionally, both FSH and TGFβ can upregulate levels of *smad2* (but not *smad3*) mRNA expression [19, 21]. Combination treatment with FSH and activin A has been reported to stimulate or inhibit cell proliferation and to decrease LH-R mRNA expression in preovulatory follicle granulosa cells [22, 23]. To date, however, the actions of activin A and TGFβ in prehierarchal follicles have yet to be studied, particularly with regard to their potential involvement in the initiation of granulosa cell differentiation.

Accordingly, the present studies were designed to evaluate the role and cellular mechanism(s) of action for activin- and TGFβ-mediated differentiation of granulosa cells from prehierarchal follicles. In addition, in an attempt to explain the absence of granulosa cell differentiation in all but the single selected follicle per day, studies were initiated to identify factors that can negatively modulate activin and TGFβ signaling.

MATERIALS AND METHODS

Animals and Reagents

Single-comb white Leghorn hens (Creighton Bros., Warsaw, IN), 25–35 wk of age and laying regular sequences of five or more 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 photoperiod of 15L:9D, with lights on at midnight. Laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h prior to 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 (rh) TGFβ1 and rh transforming growth factor α (TGFα) were purchased from PeproTech, Inc. (Rocky Hill, NJ) while rh activin A was from R&D Systems (Minneapolis, MN). The deduced amino acid sequence for the mature form of chicken TGFβ4 is predicted to be 82% identical (89% similar) to human TGFβ1 [15, 24], with all nine Cys residues and conserved domains maintained. The chicken processed form of activin A is predicted to be 98% similar to human activin A [25]. Ovine LH (lot 26) and rhFSH (lot AP8468A) were obtained from Dr. A.F. Parlow and the National Hormone and Pituitary Program. Maximally effective doses of TGFα (50 ng/ml), FSH (100 ng/ml), and LH (100 ng/ml) used in the present studies were based on results from previous studies [1]. The MAP kinase inhibitor, U0126, was from BioMol (Plymouth Meeting, PA) and has previously been found to inhibit Erk (but not Akt) phosphorylation [1]. The Smad2 and phospho-specific Smad 2 antibodies, which selectively recognize activin and TGFβ receptor-mediated phosphorylation sites, were from Zymed Laboratories and Upstate Biotechnology, respectively. The StAR antibody was generously provided by Dr. D.B. Hales (University of Illinois, Chicago, IL), while α-tubulin monoclonal antibody used for standardization of StAR was from Sigma Chemical Co. (St. Louis, MO).

Granulosa Cell Cultures

Granulosa cells from prehierarchal (6- to 8-mm-diameter) follicles were collected and prepared for culture as previously described [1, 2]. Cells were plated in six-well polystyrene culture plates (Falcon 3046; Fisher Scientific, Hanover Park, IL) at a density of approximately 10^6/well in 2 ml Dulbecco modified Eagle medium (DMEM) containing 2.5% fetal bovine serum (FBS; Gibco-BRL). Where appropriate, an aliquot of dispersed cells was immediately frozen at −70°C to serve as an uncultured (T_0) control.

In the first experiment, prehierarchal follicle granulosa cells were plated in the absence or presence of FSH, activin A (1–50 ng/ml) or TGFβ1 (1–25 ng/ml), then cultured for 20 h. Cells were subsequently collected and frozen at −70°C until processed and probed for LH-R mRNA. In a related experiment, cells were cultured for 20 h in the absence or presence of FSH, activin A (25 ng/ml), TGFβ1 (10 ng/ml), FSH plus activin A (1–50 ng/ml), or FSH plus TGFβ1 (1–25 ng/ml). Media and cells were collected separately and frozen at −70°C until assayed for progesterone (media), and LH-R, P450scc, and StAR mRNA (cell pellet). Prehierarchal follicle granulosa cells were also treated without or with FSH, TGFβ1 (10 ng/ml), and/or activin A (25 ng/ml) for 20 h to determine effects on FSH-receptor (FSH-R) mRNA expression. In a fourth experiment, granulosa cells were preplated for 20 h in the absence or presence of TGFβ1 (10 ng/ml) or activin A (25 ng/ml), then challenged for an additional 3 h without or with FSH or LH. Media and cells were collected and frozen at −70°C for subsequent analysis of LH-R mRNA, StAR protein, and progesterone.

To evaluate the potential for interaction between MAP kinase signaling and TGFβ1-mediated signaling, granulosa cells were precultured for 30 min without or with TGFα, then treated with FSH or TGFβ1 (10 ng/ml) for 20 h. Cells were collected and subsequently probed for LH-R mRNA. Signaling via Smad2 was evaluated by preculturing cells without or with TGFα for 30 min, then treating for an additional 2 h with TGFβ1 or activin A. A recently published study reported that the stimulatory effects of activin A on Smad2 mRNA levels in hen granulosa cells requires a minimum of 6 h [21]. Finally, to specifically assess MAP kinase involvement in Smad2 signaling, cells were precultured for 1 h with U0126, then treated for 2 h with TGFα and TGFβ1. Cellular proteins were collected and analyzed for phosphorylated Smad2 and total Smad2 protein by Western blot analysis.

Northern Blot Analysis

The chicken LH-R, FSH-R, and StAR cDNAs used for probing Northern blots have been previously described [3, 26, 27]. Although both gonadotropin receptors have been reported to express multiple transcripts, only the predominant transcript for LH-R (3.0 kilobases [kb]) and FSH-R (2.5 kb) were quantitated in the present studies. A 522-nucleotide chicken P450scc probe corresponding to base pairs 269–790 of the predicted coding region was generated from the chicken P450scc cDNA sequence (GenBank accession D49803) by reverse transcriptase-polymerase chain reaction, and the cloned sequence was subsequently confirmed by sequence analysis. All Northern blots were reprobed with a chicken 18S ribosomal RNA cDNA to standardize for equal loading of RNA samples. Conditions for conducting Northern blot analysis have been previously detailed [28], and all blots were visualized using a Storm 840 Phosphorimager and ImageQuant analysis (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis

Western blot analyses were conducted essentially as described [1, 28]. Briefly, tissues were homogenized in a protein lysis buffer containing a cocktail of enzyme (including phosphatase) inhibitors (Sigma). Proteins were separated on a 10% polyacrylamide gel under denaturing conditions, then transferred to a polyvinylidene fluoride membrane (Millipore Immobilon-P; Fisher Scientific). Incubations with the StAR, phospho-specific Erk, or phospho-specific Smad2 primary antibodies were conducted overnight at 4°C, while those for horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Pierce, Rockford, IL) were for 1 h. Blots were incubated with enhanced chemiluminescence Western blotting agent (Amersham Corp., Arlington Heights, IL) for 1 min and exposed to x-ray film for 1–5 min. Membranes were subsequently reblotted for levels of α-tubulin, Erk2, or total Smad2 protein to enable standardization. Western data were quantitated by densitometry using an UltraScan XL laser densitometer (Pharmacia LKB, Piscataway, NJ).

Progesterone Assays

Levels of media progesterone were quantitated by radioimmunoassay as previously described [29] and expressed as mean nanogram per well ± SEM for the replicate experiments.

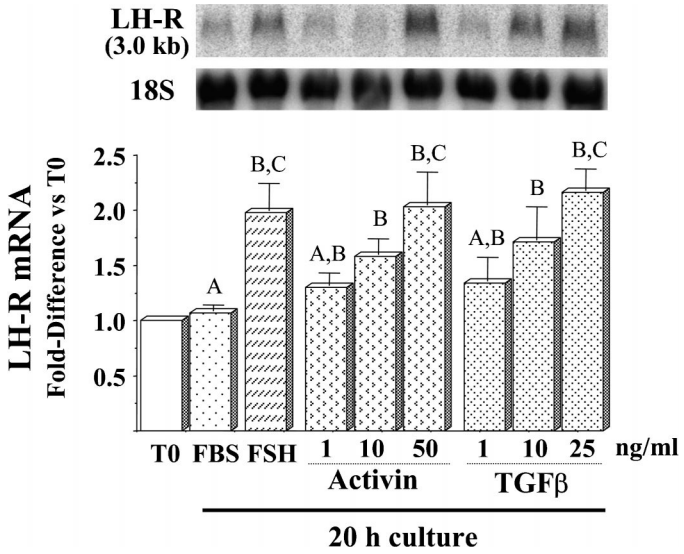

FIG. 1. FSH (100 ng/ml), activin A, and TGFβ1 promote LH-R mRNA expression in granulosa cells from prehierarchal follicles following 20 h of culture. T_0 represents cells collected immediately after dispersion. A, B, C) $P < 0.05$.

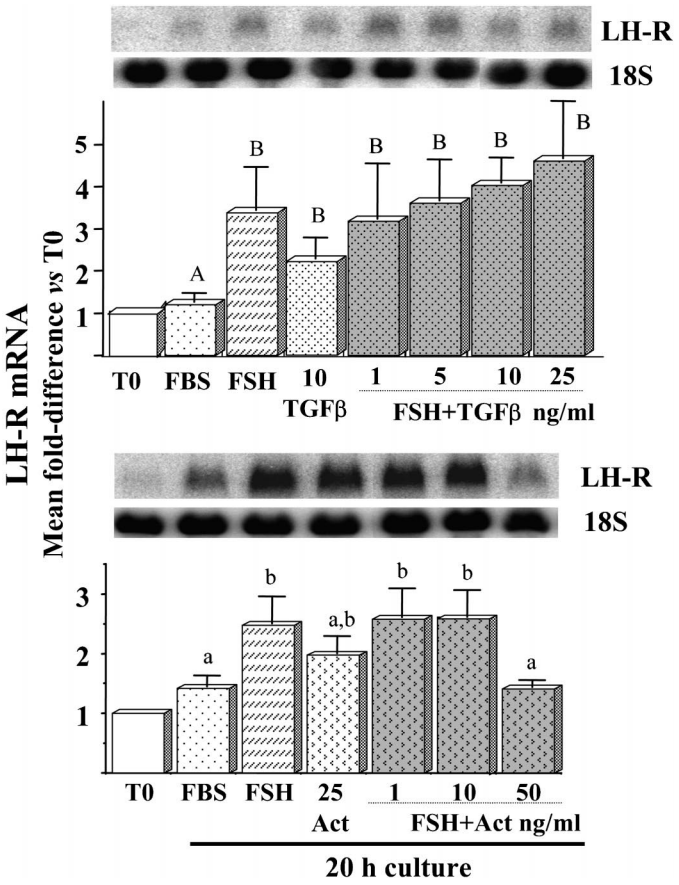

FIG. 2. FSH (100 ng/ml) fails to induce a significant additive effect in combination with activin A (Act) or TGFβ1 in promoting LH-R mRNA expression after 20 h of culture. T_0, freshly collected cells. A, B: a, b) $P < 0.05$.

Data Analysis

Experiments were independently replicated a minimum total of three times unless otherwise noted. Northern blot data were standardized to 18S ribosomal (r) RNA while StAR, phospho-Erk, and phospho-Smad2 protein levels were standardized to α-tubulin, Erk, and Smad2, respectively. Standardized data were expressed as a fold-change compared with the appropriate control (freshly collected [T_0] or FBS-cultured cells). Data were analyzed by one-way analysis of variance (without including the reference value, which was set to 1.0) and the Fisher protected least significant difference multiple range test.

RESULTS

Activin A- and TGFβ1-Induced Differentiation in Granulosa Cells from Prehierarchal (6- to 8-mm-Diameter) Follicles

Levels of LH-R mRNA were increased above FBS control levels following treatment with 10 and 50 ng/ml activin A and 10 and 25 ng/ml TGFβ1 (Fig. 1). There was no significant additive effect found for LH-R mRNA expression when cells were cultured for 20 h with FSH plus activin A or TGFβ1 (Fig. 2). Interestingly, the highest (50 ng/ml) dose of activin plus FSH failed to increase LH-R expression above control levels. A related set of experiments was conducted to evaluate the ability of activin A and TGFβ1 to influence cell differentiation at sites other than the LH-R. Culture for 20 h with activin A or TGFβ1 alone failed to increase P450scc or StAR mRNA above levels in control cultured cells, and FSH combined with activin A or TGFβ1 resulted in no greater increase in P450scc or StAR mRNA expression compared with FSH treatment alone after 20 h of culture (Fig. 3). TGFβ1 or activin A alone also had no direct stimulatory effect on progesterone production (Fig. 4). However, in combination with FSH, activin A (10 and 50 ng/ml) and TGFβ1 (10 and 25 ng/ml) stimulated significantly greater progesterone production compared with the FSH treatment. Levels of FSH-R mRNA were also significantly increased by TGFβ1, but not by activin A or FSH, when compared with FBS control cells following 20 h of culture (Fig. 5). Furthermore, there was a stimulatory effect of activin A and an additive effect of TGFβ1 on FSH-R mRNA levels when combined with FSH treatment.

Subsequent experiments were designed to determine whether increased levels of gonadotropin receptor mRNA induced by activin A and TGFβ1 resulted in the expression of functional gonadotropin receptors. To accomplish this, cells were precultured for 20 h in the absence or presence of TGFβ1 or activin A, and then challenged an additional 3 h without or with FSH or LH. Consistent with results from the first experiment, TGFβ1 and activin A preculture increased LH-R mRNA expression (by 1.6 ± 0.1- and 2.8 ± 0.3-fold, respectively) compared with control cultured cells ($P < 0.05$; data not shown). Evaluation of StAR protein and progesterone production determined that preculture with TGFβ1 or activin A followed by gonadotropin challenge produced significantly higher StAR protein levels and progesterone production compared with comparable treatments in cells precultured with media alone (Fig. 6).

Influence of the MAP Kinase Pathway on TGFβ1- and Activin A-Induced Signaling

The next set of experiments evaluated the potential for TGFα acting via MAP kinase signaling to modulate TGFβ1 and activin A signaling. Consistent with results from a previous study [1], a 20-min treatment with TGFα induced a significant increase in levels of phosphorylated Erk (Erk-P). In the present studies, TGFβ1 and activin A neither stimulated an increase in Erk-P nor inhibited TGFα-induced Erk-P ($P > 0.5$ for TGFβ1 and activin A versus

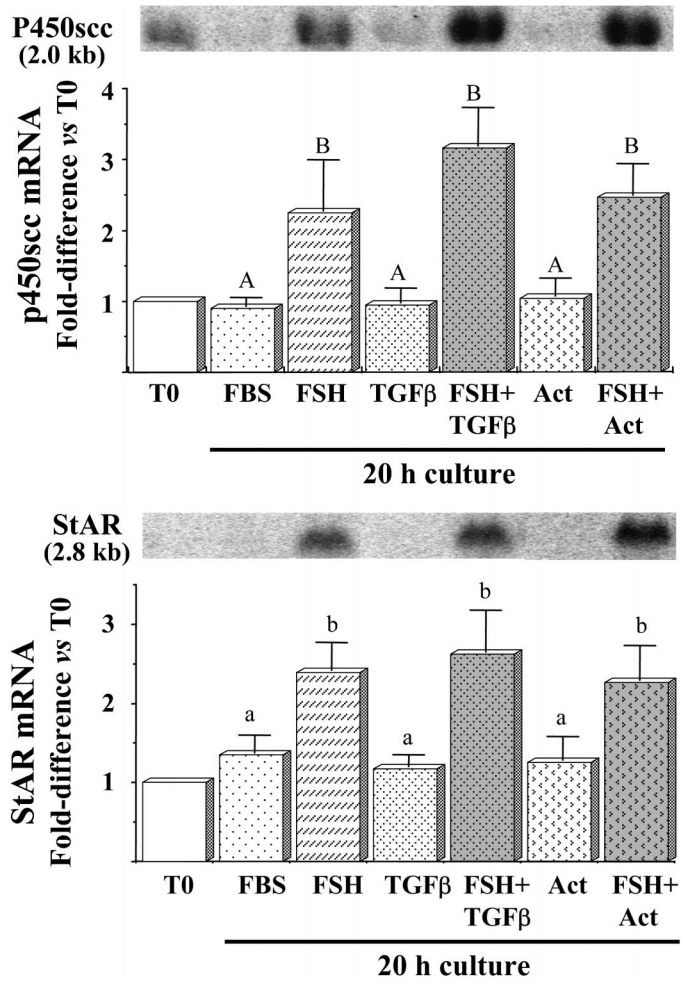

FIG. 3. Neither TGFβ1 (10 ng/ml) nor activin A (25 ng/ml) directly induces P450scc or StAR mRNA expression after 20 h of culture. In addition, coculture with FSH (100 ng/ml) fails to induce a significant additive effect with TGFβ1 or activin A in promoting P450scc or StAR mRNA expression. A, B: a, b) $P < 0.05$.

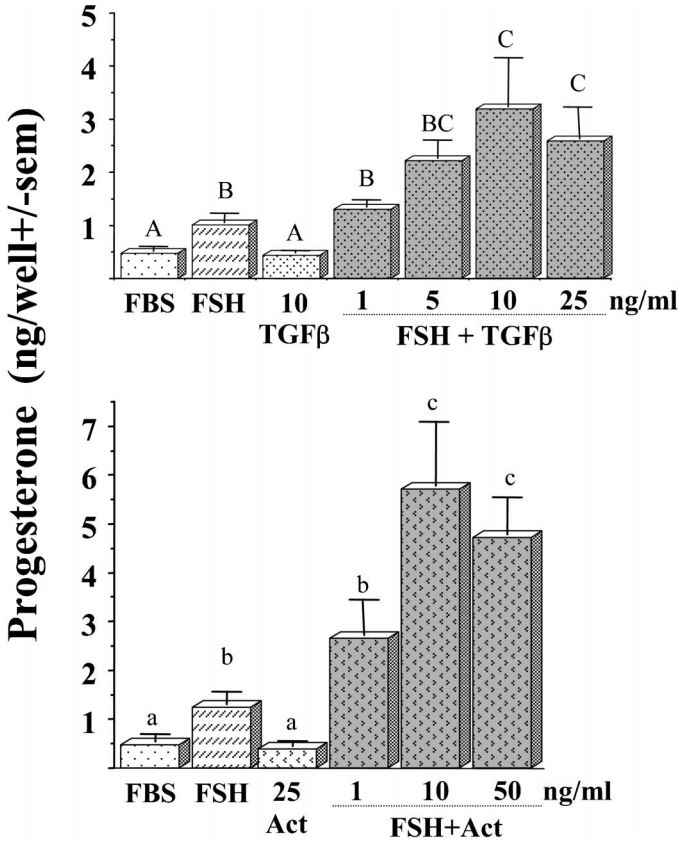

FIG. 4. TGFβ1 and activin A do not directly induce progesterone production. However, activin A and TGFβ1 do provide an additive effect on progesterone production when cocultured in the presence of FSH (100 ng/ml). A, B, C: a, b, c) $P < 0.05$.

TGFα alone; data not shown). However, with regard to effects on granulosa cell differentiation, TGFα treatment completely blocked TGFβ1-induced LH-R expression after 20 h of culture (Fig. 7). Finally, both TGFβ1 and activin A induced Smad2 phosphorylation (Smad2-P) following a 2-h culture, while levels of total Smad2 protein were unchanged (Fig. 8, top panel). Significantly, pretreatment for 15 min with TGFα completely blocked TGFβ1- and activin A-induced Smad-P. This inhibitory effect could be prevented by a prior treatment with the MAP kinase inhibitor, U0126 (Fig. 8, bottom panel).

DISCUSSION

The present studies demonstrate for the first time in cultured, undifferentiated hen granulosa cells that the related family members, TGFβ and activin, promote LH-R expression, but to varying levels of effectiveness. In addition, when combined with FSH, both TGFβ1 and activin A can maintain steady-state levels or promote FSH-R mRNA expression. Based on previously published patterns of TGFβ [14] and activin A [16] expression within hen follicles, it is proposed that this effect can be mediated in a paracrine or autocrine fashion in vivo. Furthermore, similar to the previous finding that activation of the MAP kinase pathway

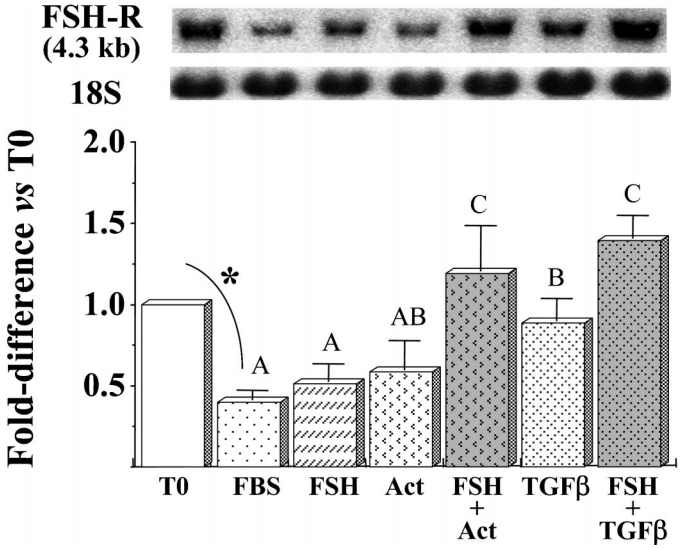

FIG. 5. TGFβ1 (10 ng/ml), but not FSH (100 ng/ml) or activin A (25 ng/ml), increases levels of FSH-R mRNA compared with controls (FBS) in granulosa cells from prehierarchal follicles. Moreover, FSH combined with activin A or TGFβ1 increased FSH-R mRNA levels compared with any of these factors alone. A, B, C) $P < 0.05$. Note also that FSH-R mRNA levels are decreased in control cells after 20 h of culture ($P < 0.05$ by paired *t*-test using original data). N = 5 replicate experiments.

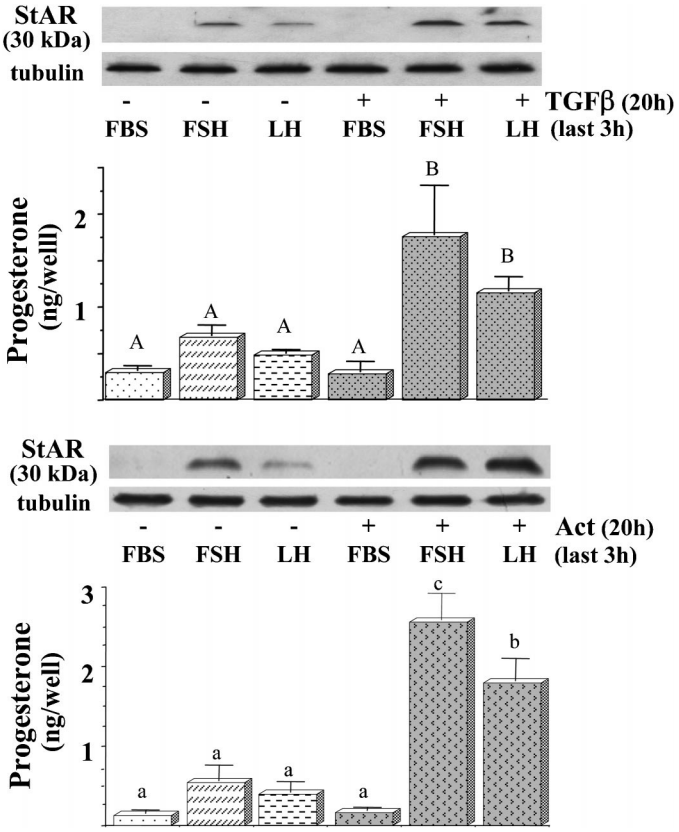

FIG. 6. Preculture with TGFβ1 (10 ng/ml) or activin A (25 ng/ml) for 20 h enhances StAR protein expression and progesterone production following a 3-h challenge with FSH (100 ng/ml) or LH (100 ng/ml). A, B: a, b, c) $P < 0.05$. The StAR Western depicts a representative blot from a total of three experiments.

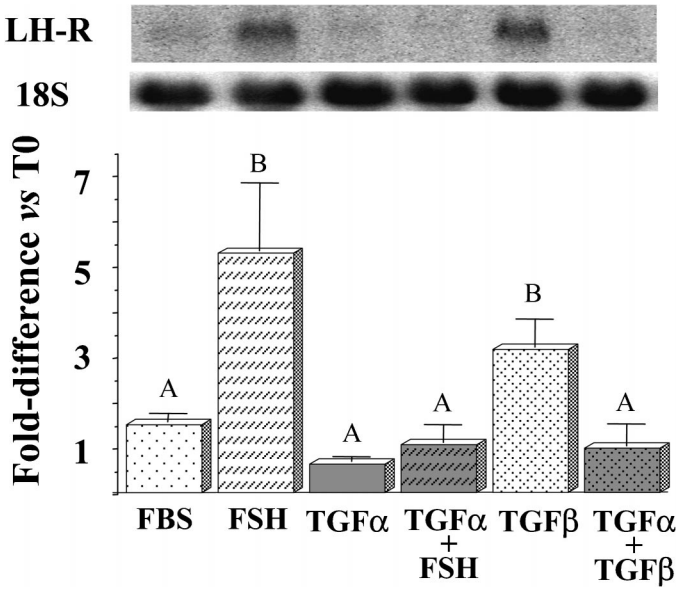

FIG. 7. Active stimulation of MAP kinase signaling using TGFα effectively prevents both FSH- (100 ng/ml) and TGFβ1 (10 ng/ml)-induced LH-R mRNA expression. A, B, C) $P < 0.05$ versus FBS control cells.

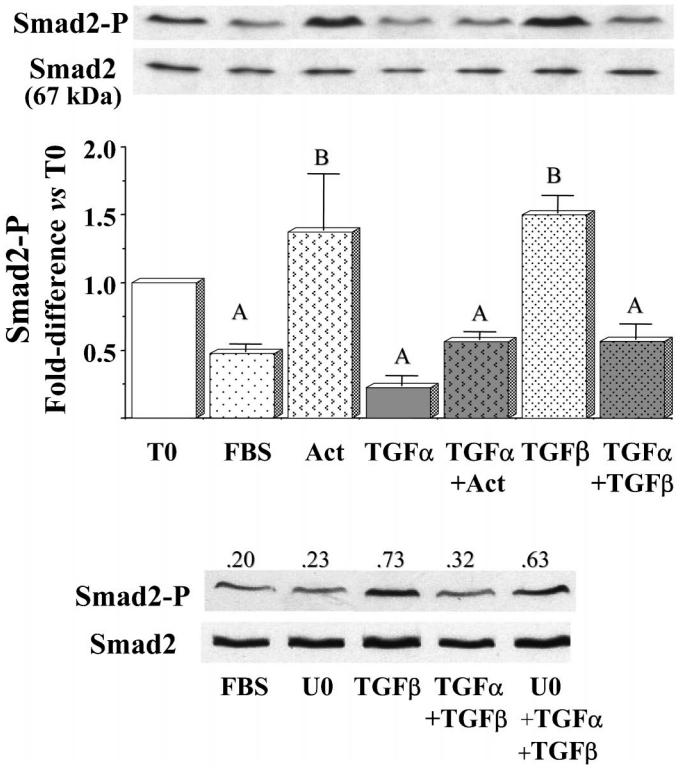

FIG. 8. Top panel: Activin A (Act; 25 ng/ml) and TGFβ1 (10 ng/ml) signal via the activation of the regulatory Smad, Smad2, as shown by increased Smad2 phosphorylation (Smad2-P) following a 2-h treatment. Significantly, TGFα effectively prevents activin A- and TGFβ1-induced Smad2-P. Bottom panel: The MAP kinase inhibitor, U0126 (50 μM), blocks the inhibitory effect of TGFα. Accordingly, MAP kinase signaling is implicated in preventing premature differentiation by TGFβ1 and activin in granulosa cells from prehierarchal follicles. Numbers over the densitometric scan represent levels of Smad2-P standardized to Smad2 (replicated once, with similar results). A, B) $P < 0.05$.

can block FSH-stimulated LH-R expression [1], TGFα-induced MAP kinase/Erk signaling is also shown to prevent TGFβ1- and activin A-induced LH-R expression. A summary of events describing activin A- and TGFβ1-mediated signaling and their proposed relationship to the initiation of steroidogenesis in undifferentiated granulosa cells from prehierarchal (6- to 8-mm-diameter) follicles is depicted in Figure 9.

Collectively, the data reported herein demonstrate that activin A and TGFβ1 directly induce LH-R expression and indirectly promote P450scc and StAR expression plus progesterone production following LH-R induction. This conclusion is supported by the apparent inability of activin A or TGFβ1 to induce P450scc mRNA expression or StAR mRNA or protein expression and the absence of any additive effect on either parameter in combination with FSH (Fig. 3). The inability of activin A and TGFβ1 to directly promote progesterone production or enhance production when combined with gonadotropin treatment is also consistent with previous reports using granulosa cells from preovulatory follicles [23, 30, 31], a stage of development where comparatively higher levels of P450scc and StAR expression are not limiting factors to progesterone production. On the other hand, these data contrast with those reported using undifferentiated granulosa cells from the rat in which activin A failed to increase LH-R mRNA expression and exhibited an additive effect on both LH-R and P450scc mRNA expression when combined with FSH pretreatment [32]. These differences may reflect a difference in the stage of differentiation for cultured granulosa cells

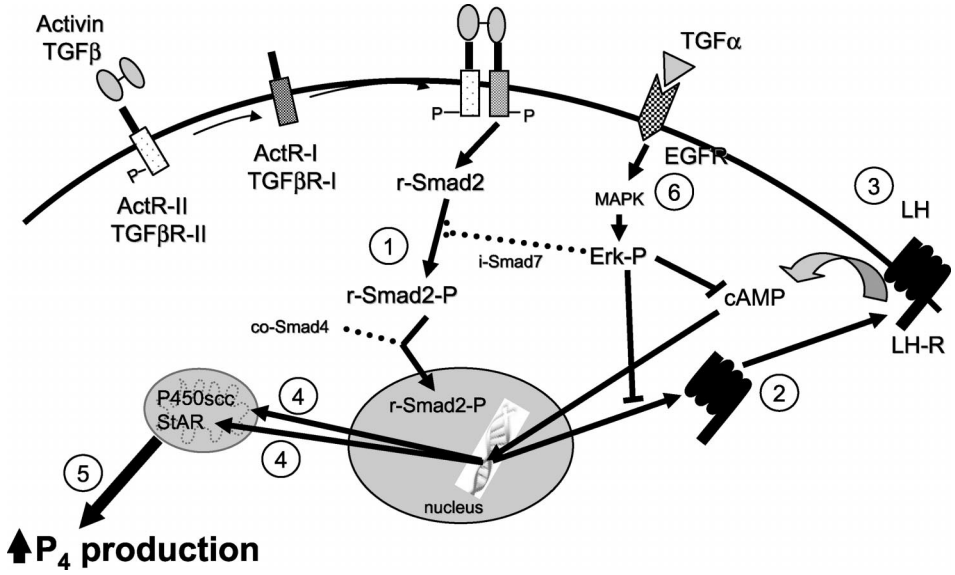

FIG. 9. Proposed events related to activin A- and TGFβ1-induced granulosa cell differentiation and prevention of LH-R expression via MAP kinase signaling. **1**) Receptor activation in response to activin A or TGFβ1 induces Smad2 phosphorylation. **2**) Transcriptional activity in response to activin A or TGFβ1 results in the synthesis of LH-R mRNA, independent of FSH signaling. In response to a subsequent exposure to LH (**3**), expression of StAR and P450scc activity is increased (**4**) and progesterone production can be initiated (**5**). On the other hand, activation of MAP kinase signaling through Erk blocks Smad2 phosphorylation (**6**) and effectively prevents the premature differentiation of granulosa cells induced by activin A and TGFβ1. Evidence for cAMP-mediated P450scc and StAR expression and the ability of MAP kinase (MAPK) signaling to inhibit this effect has been published previously [1, 4]. Additional implied factors and pathways are indicated by italics and dotted lines.

used and/or species-related responses between the two studies.

Constitutive expression of FSH-R mRNA during follicle development is highest in granulosa cells from 6- to 8-mm-diameter follicles [27]. It is interesting to note that FSH-R mRNA levels significantly decrease (by greater than 50%) in control cultured cells following 20 h of culture (Fig. 5). This suggests that the steady-state level of FSH-R mRNA expression in vivo requires tonic stimulation by endocrine or paracrine factors. While neither FSH nor activin A alone prevented this decline in FSH-R expression, treatment with TGFβ1 maintained FSH-R expression compared with T_0 levels. The variability and comparative ineffectiveness of activin A to promote gonadotropin receptor expression is likely related to granulosa cell production of follistatin [16]. In support of this proposal, we have recently determined that the addition of an antifollistatin serum effectively enables activin to promote FSH-R expression in vitro (Woods and Johnson, unpublished data). By comparison, both TGFβ1 and activin A produced a greater stimulatory effect on FSH-R mRNA levels when combined with FSH; thus, it appears that activin treatment still exerts some level of sensitization. In any event, it is likely that some combination of FSH, TGFβ, and activin are among the factors normally required to maintain FSH-R expression in granulosa cells at this stage of development in vivo.

Additional evidence for the ability of activin and TGFβ to induce not only increased levels of receptor mRNA but also translated and functionally coupled FSH-R and LH-R is provided by the finding that preculture for 20 h with activin A or TGFβ1 significantly enhanced StAR expression and progesterone production following a 3-h challenge with FSH or LH compared with control precultured cells (Fig. 6). Similar results for StAR have been reported after a 24-h TGFβ preculture in rat granulosa cells [33].

The only other report to date regarding effects on gonadotropin receptors in the hen ovary determined that activin A either had no effect on LH-R and FSH-R mRNA expression or a slight inhibitory effect [23]. However, these studies were conducted in granulosa cells collected from preovulatory follicles. The difference between such results and those reported herein indicate a role of activin and TGFβ in the process of early granulosa cell differentiation rather than the facilitation of short-term secretion of progesterone (as occurs during the preovulatory surge of progesterone) at a stage of development when cells already express comparatively high levels of LH-R [3]. Interestingly, FSH plus the highest level of activin A utilized (50 ng/ml) inhibited LH-R mRNA levels (Fig. 2) but stimulated progesterone production (Fig. 4). As speculated above, this result may represent a sensitizing effect of activin A at the level of postreceptor signaling.

While neither activin A nor TGFβ1 directly induces progesterone production, the higher doses of each factor in combination with FSH treatment did produce an additive effect on progesterone secretion compared with the FSH treatment alone (Fig. 4). This response may be related to the additive effect of FSH combined with activin A and TGFβ1 observed specifically for FSH-R expression (Fig. 5) because no additive effects of these treatments were found for P450scc and StAR expression (Fig. 3). It is possible that the increase in functional FSH-R expression may provide for somewhat higher and sustained levels of cAMP-mediated steroidogenesis without a requirement for a further increase in P450scc or StAR expression.

Significantly, TGFα-induced MAP kinase/Erk signaling was found to block TGFβ1-induced LH-R mRNA expression (Fig. 7). The inhibitory effect of TGFα was associated with a prevention of Smad2 phosphorylation and, importantly, was reversed by pretreatment with the MAP kinase inhibitor, U0126 (Fig. 8, lower panels). Previous reports from the rat have determined that EGF treatment can induce expression of i-Smad7 mRNA [13, 34] and that the Smad7 promoter region contains several transcription factor-binding sites that may represent the target of EGF actions [35]. In our ongoing studies of hen granulosa cells, we routinely utilize recombinant human TGFα to promote EGF receptor activation because human TGFα has been reported to bind the chicken EGF receptor with a considerably higher affinity compared with human EGF [36]. Accordingly, we speculate that the inhibitory effects of MAP kinase signaling on TGFβ1- and activin A-induced Smad2-P may result from increased i-Smad7 expression (Fig. 9).

One approach to understanding the process of follicle selection and why but a single follicle from a cohort of 8–12 prehierarchal follicles is recruited per day into the pre-

ovulatory hierarchy is to elucidate mechanisms that can prevent premature differentiation of the granulosa layer. This concept of release from inhibition is relevant because it has recently been established that all hen follicles within the cohort from which selection occurs (both whole follicles as well as the isolated granulosa layers from these follicles) express FSH-R mRNA (Woods and Johnson; unpublished data). Assuming similar levels of receptor translation and functional coupling to signaling pathways prior to the selection process, one would expect equal follicle exposure to circulating FSH [37], and thus the potential for multiple follicles to simultaneously begin FSH-promoted differentiation. Results presented previously and herein are consistent with MAP kinase/Erk activity serving as an effective inhibitor of premature granulosa cell differentiation. Specifically, it has been determined that TGFα-induced MAP kinase/Erk signaling can block FSH-induced P450scc, LH-R, and StAR expression in cultured, undifferentiated granulosa cells [1, 4]. Moreover, inhibition of constitutive MAP kinase activity using the selective pharmacologic inhibitors, U0126 or PD98059, promotes LH-R expression in cultured cells while the combination treatment of either MAP kinase inhibitor plus FSH results in an additive increase in LH-R expression [1]. Novel results from the present report are consistent with this hypothesis in that TGFβ- and activin A-mediated LH-R expression is also blocked by active MAP kinase/Erk signaling (Fig. 8).

Finally, it is important to consider potential interrelationships with additional factors proposed to regulate granulosa cell function during early follicle growth. For instance, Lovell et al. [16] have recently quantitated levels of inhibin A, inhibin B, follistatin, and activin A in granulosa and theca cells during hen follicle development. Because both inhibins and follistatin are proposed to antagonize the actions of activin A, they propose activin tone as an estimate of activin bioavailability with changing levels of the related antagonists. According to this concept, they predict that activin tone progressively decreases as prehierarchal follicles grow from 1–2 mm to approximately 9 mm, the stage of follicle selection. Clearly, it is difficult to predict the physiological consequences of such interrelationships, yet interestingly, they suggest that high activin tone in 1- to 2-mm follicles may aid in predisposing such follicles to the subsequent acquisition of LH responsiveness.

In summary, the related paracrine/autocrine factors, activin A and TGFβ1, represent additional factors (together with circulating FSH and possibly paracrine-acting vasoactive intestinal peptide) [38] that are proposed to initiate hen granulosa cell differentiation and, by implication, follicle selection into the preovulatory hierarchy. Accordingly, a current focus is to investigate additional cellular sites of MAP kinase inhibition together with mechanisms that are ultimately responsible for releasing granulosa cells from such inhibition within those follicles selected for ovulation.

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