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Role of transforming growth factor-β1 in gene expression and activity of estradiol and progesterone-generating enzymes in FSH-stimulated bovine granulosa cells

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Abstract

Survival and inhibitory factors regulate steroidogenesis and determine the fate of developing follicles. The objective of this study was to determine the role of transforming growth factor-β1 (TGFB1) in the regulation of estradiol-17β (E_2) and progesterone (P_4) secretion in FSH-stimulated bovine granulosa cells. Granulosa cells were obtained from 2 to 5 mm follicles and cultured in serum-free medium. FSH dose (1 and 10 ng/ml for 6 days) and time in culture (2, 4, and 6 days with 1 ng/ml FSH) increased E_2 secretion and mRNA expression of E_2-related enzymes cytochrome P450 aromatase (*CYP19A1*) and 17β-hydroxysteroid dehydrogenase type 1 (*HSD17B1*), but not *HSD17B7*. TGFB1 in the presence of FSH (1 ng/ml) inhibited E_2 secretion, and decreased mRNA expression of FSH receptor *(FSHR)*, *CYP19A1*, and *HSD17B1*, but not *HSD17B7*. FSH dose did not affect P_4 secretion and mRNA expression of 3β-hydroxysteroid dehydrogenase (*HSD3B*) and α-glutathione *S*-transferase (*GSTA*), but inhibited the amount of steroidogenic acute regulatory protein *(STAR)* mRNA. Conversely, P_4 and mRNA expression of *STAR*, cytochrome P450 side-chain cleavage *(CYP11A1)*, *HSD3B*, and *GSTA* increased with time in culture. TGFB1 inhibited P_4 secretion and decreased mRNA expression of *STAR*, *CYP11A1*, *HSD3B*, and *GSTA*. TGFB1 modified the formation of granulosa cell clumps and reduced total cell protein. Finally, TGFB1 decreased conversion of androgens to E_2, but did not decrease the conversion of estrone (E_1) to E_2 and pregnenolone to P_4. Overall, these results indicate that TGFB1 counteracts stimulation of E_2 and P_4 synthesis in granulosa cells by inhibiting key enzymes involved in the conversion of androgens to E_2 and cholesterol to P_4 without shutting down HSD17B reducing activity and HSD3B activity.
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Introduction

In cattle, follicle development occurs in waves consisting of rhythmic emergence and growth of a new cohort of antral follicles (Ireland *et al.* 2000). During this process, the proportion of follicles undergoing atresia increases dramatically with follicular diameter and the rate of atresia doubles between 2 and 8 mm (Lussier *et al.* 1987). At the final growth phase, most follicles are atretic and only one follicle (exceptionally two) is selected to become dominant and continue to grow until ovulation (Fortune *et al.* 2001). Many factors are involved in follicular development, including gonadotropins, steroid hormones, cytokines, and other endocrine, paracrine, and autocrine factors. These factors induce cell death or survival and thus determine the fate of the growing follicle.

Gonadotropin stimulation of responsive follicles is associated with increased synthesis of estradiol-17β (E_2) and progesterone (P_4), the two key steroid hormones associated with the development of the ovulatory follicle (Price *et al.* 1999, Kolibianakis *et al.* 2005). E_2 is produced by granulosa cells and is important for granulosa cell growth, attenuation of granulosa cell apoptosis, and positive and negative feedback regulation of the hypothalamic–pituitary–ovarian axis (Kolibianakis *et al.* 2005). In ruminants and humans, gonadotropin stimulation of E_2 requires luteinizing hormone (LH) stimulation of theca cells to produce androgens, mostly as androstenedione (A_4), and follicle-stimulating hormone (FSH)-mediated conversion of theca-derived A_4 to E_2 in granulosa cells (Fortune 1986, Hillier *et al.* 1994). A_4 is converted to estrone (E_1) by cytochrome P450 aromatase (CYP19A1) and then into E_2 by 17β-hydroxysteroid dehydrogenase (HSD17B) reducing enzymes (Hillier *et al.* 1994, Mindnich *et al.* 2004). Granulosa cells also produce P_4, which is necessary for the induction of ovulation (Drummond 2006). The production of P_4 involves transformation of cholesterol to pregnenolone (P_5) by the cytochrome P450 side-chain cleavage (CYP11A1) followed by the conversion of P_5 to P_4 by 3β-hydroxysteroid dehydrogenase (HSD3B). In addition, α-glutathione *S*-transferase (GSTA) is

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expressed in bovine granulosa cells and codes for a protein with high HSD3B activity (Rabahi *et al.* 1999, Raffalli-Mathieu *et al.* 2007). *In vivo*, most of the steroidogenic enzyme genes are not expressed in granulosa cells of small bovine antral follicles (0.4–4 mm; Bao & Garverick 1998). In granulosa cells, expression of *CYP19A1* and *CYP11A1* gradually increases during follicle growth, and *HSD3B* is first observed in non-atretic follicles at ~8 mm (Xu *et al.* 1995, Bao *et al.* 1997). These data indicate that the timely expression of steroidogenic enzymes at distinct periods of follicular development is important for the selection of the dominant follicle and successful ovulation. A better characterization of the regulation of E_2 and P_4 synthesis in granulosa cells will improve our understanding of follicle development.

In recent years, much attention has focused on the role of the members of the transforming growth factor-β (TGFB) superfamily acting as paracrine and autocrine factors to modulate ovarian function and fertility (Knight & Glister 2006). TGFB1, which is the most extensively studied factor of this family, is present in granulosa and theca cells and in the vascular system of the ovary in many species (Nilsson *et al.* 2003, Juengel & McNatty 2005), and has been shown to either stimulate or inhibit E_2 and P_4 synthesis. For instance, TGFB1 stimulated E_2 and P_4 secretion from rodent granulosa cells (Zachow *et al.* 1999, Knight & Glister 2006), whereas TGFB1 inhibited granulosa cell secretion of E_2 and P_4 from pigs and ruminants (Chang *et al.* 1996, Wandji & Fortune 1996, Ford & Howard 1997, Juengel *et al.* 2004, Ouellette *et al.* 2005). These reports detailing the effect of TGFB1 on ovarian cells appear to be highly dependent on the species studied, stage of follicle differentiation, and the presence of different growth factors as co-treatments, and the mechanism of TGFB1 action in granulosa cells is not clear. *In vivo*, the complexities of hormonal interactions with the cytokines produced by ovarian cells limit our investigations of steroidogenesis in follicle development. Fortunately, E_2 secretion can be maintained for several days in bovine granulosa cells cultured *in vitro* with FSH in serum-free conditions (Gutierrez *et al.* 1997). This *in vitro* model mimics the gradual increase in E_2 secretion seen in the growing follicles and can be used to identify the key factors regulating steroidogenesis in granulosa cells. Therefore, the objective of the present study was to determine the effect of TGFB1 on E_2 and P_4 synthesis and the corresponding expression and activity of key steroidogenic enzymes in FSH-stimulated bovine granulosa cells.

Results

Effect of FSH dose and time in culture

In granulosa cells cultured for 6 days, FSH at 1 and 10 ng/ml had no effect on P_4 synthesis, but significantly increased E_2 secretion (Fig. 1A and F). FSH decreased steroidogenic acute regulatory protein *(STAR)* mRNA level, increased *CYP11A1* (only at the 10 ng/ml dose), and did not affect *HSD3B* and *GSTA* mRNA levels (Fig. 1B–E). For E_2 secretion, FSH had no effect on *HSD17B7* mRNA level but increased *CYP19A1* and *HSD17B1* mRNA levels (Fig. 1H–J). In the presence of FSH at 1 ng/ml, both P_4 and E_2 accumulation increased from days 2 to 6 (Fig. 2A and F). In agreement with increased secretion of P_4 with time in culture, mRNA expression of the P_4-related enzymes *STAR*, *CYP11A1*, *HSD3B*, and *GSTA* also increased with time (Fig. 2B–E). For E_2-related enzymes, time in culture had no effect on *HSD17B7* mRNA level but *CYP19A1* and *HSD17B1* mRNA levels increased with time (Fig. 2H–J).

Effects of TGFB1 on secretion of steroid hormones and mRNA expression and activity of steroidogenic enzymes

Since P_4 and E_2 secretion was stimulated in granulosa cells cultured for 6 days with 1 ng/ml FSH (see above), we examined the effect of TGFB1 on steroid hormones and mRNA expression of steroidogenic enzymes under this condition. Addition of TGFB1 caused a significant dose-dependent inhibition of P_4 and E_2 secretion (Fig. 3). To determine enzymatic activities, the tritiated steroid hormone precursors of A_4, testosterone (T), E_1, and P_5 were converted to the corresponding radiolabeled product (E_2 and P_4) confirming that the CYP19A1, reducing HSD17B, and HSD3B activities measured were specific. When granulosa cells were cultured with 0.5 ng/ml TGFB1, there was a significant inhibition of combined CYP19A1 and HSD17B activity as measured by the conversion of $[^3H]A_4$ to $[^3H]E_2$ (Fig. 4A), although the intermediary product $[^3H]E_1$ was not detected. Both doses of TGFB1 caused a significant inhibition in the CYP19A1 activity measured by the conversion of $[^3H]T$ to $[^3H]E_2$ (Fig. 4B). TGFB1 did not alter HSD17B reducing activity or HSD3B activity (Fig. 5). Corresponding to the decreased secretion of P_4 and E_2, TGFB1 caused a significant inhibition in the mRNA expression of *STAR*, *CYP11A1*, *HSD3B*, *GSTA*, *CYP19A1*, *HSD17B1*, and *FSHR* (Fig. 6). However, *HSD17B7* mRNA was not changed by the treatment of TGFB1 (Fig. 6I).

Cell morphology and total cell protein

Granulosa cells were seeded onto wells and cultured for 6 days in serum-free medium. The granulosa cells initially formed tightly packed aggregates from day 2, which enlarged with time in culture (data not shown). When TGFB1 was added to FSH-stimulated cells, granulosa cell clumps were smaller and appeared more spherical than control cells (Fig. 7D). Total cell protein was 30 and 38% higher in the 1 and 10 ng/ml FSH-treated groups compared with the control group without FSH (Fig. 7A). By contrast, in the presence of 1 ng/ml FSH, the highest dose of TGFB1 (0.5 ng/ml) decreased total cell protein by 23% compared

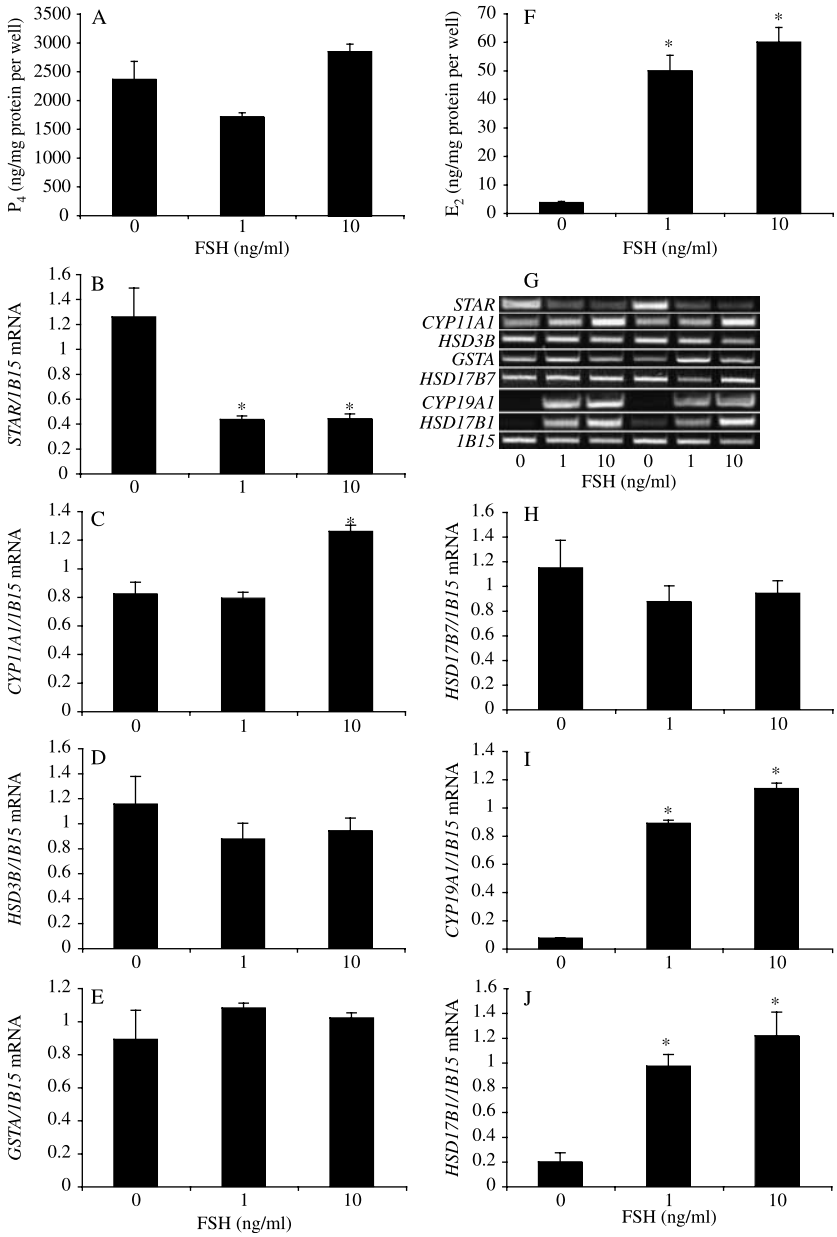

Figure 1 Effect of FSH dose (0, 1, and 10 ng/ml) on secretion of estradiol (E_2) and progesterone (P_4) and mRNA expression of steroidogenic enzymes in bovine granulosa cells. The cells were cultured for 6 days without serum in the presence of 0, 1, and 10 ng/ml FSH starting at day 0. The culture medium and cellular RNA were collected on day 6. (A and F) The data represent secretion of E_2 and P_4 during the last 48 h of culture. Levels of *STAR*, *CYP11A1*, *HSD3B*, *GSTA*, *HSD17B7*, *CYP19A1*, and *HSD17B1* mRNA expression were measured by semi-quantitative RT-PCR. (B–E and H–J) Data are presented as the amount of steroidogenic enzyme expressed relative to the amount of expression of the constitutively expressed gene *1B15*. Data are means±S.E.M. of three separate culture replicates. Asterisk indicates that the mean is significantly different from the 0 dose control ($P<0.05$, one-way ANOVA, with Dunnett's test). (G) Representative agarose gel from two replicates showing PCR products for each steroidogenic enzyme and constitutively expressed housekeeping gene *1B15*.

with the control group without TGFB1 (Fig. 7B). Furthermore, in the absence of FSH, 6 days of treatment with 0.1, 0.5, or 1 ng/ml TGFB1 had no significant effect on total cell protein (data not shown).

Discussion

The regulation of E_2 and P_4 production in granulosa cells is critical for ovarian follicle growth. The synthesis of E_2 from androgens requires aromatization by *CYP19A1*. In agreement with previous studies (Gutierrez *et al.* 1997, Silva & Price 2000, Sahmi *et al.* 2004), the physiological dose of FSH used in the present study (1 ng/ml) stimulated E_2 secretion and abundance of *CYP19A1* and *HSD17B1* mRNA. Under this stimulatory condition, TGFB1 caused a marked inhibition of E_2 secretion and CYP19A1 activity, due to decreased expression of *CYP19A1* mRNA. These findings concur with those obtained in extragonadal tissues where TGFB1 was shown to inhibit CYP19A1 activity, *CYP19A1* mRNA, and CYP19A1 protein levels in cultured human fetal hepatocytes, trophoblast cells, and adipose stroma cells (Simpson *et al.* 1989, Rainey *et al.* 1992, Luo *et al.* 2002). The conversion of A_4 to E_2 also requires HSD17B reducing activity; however, TGFB1 did not affect HSD17B activity in the present study. TGFB1 did inhibit *HSD17B1* expression, suggesting that another enzyme may be contributing to total HSD17B activity. Another enzyme known to convert E_1 to E_2 is *HSD17B7* (Krazeisen *et al.* 1999, Krusche *et al.* 2001), and in the

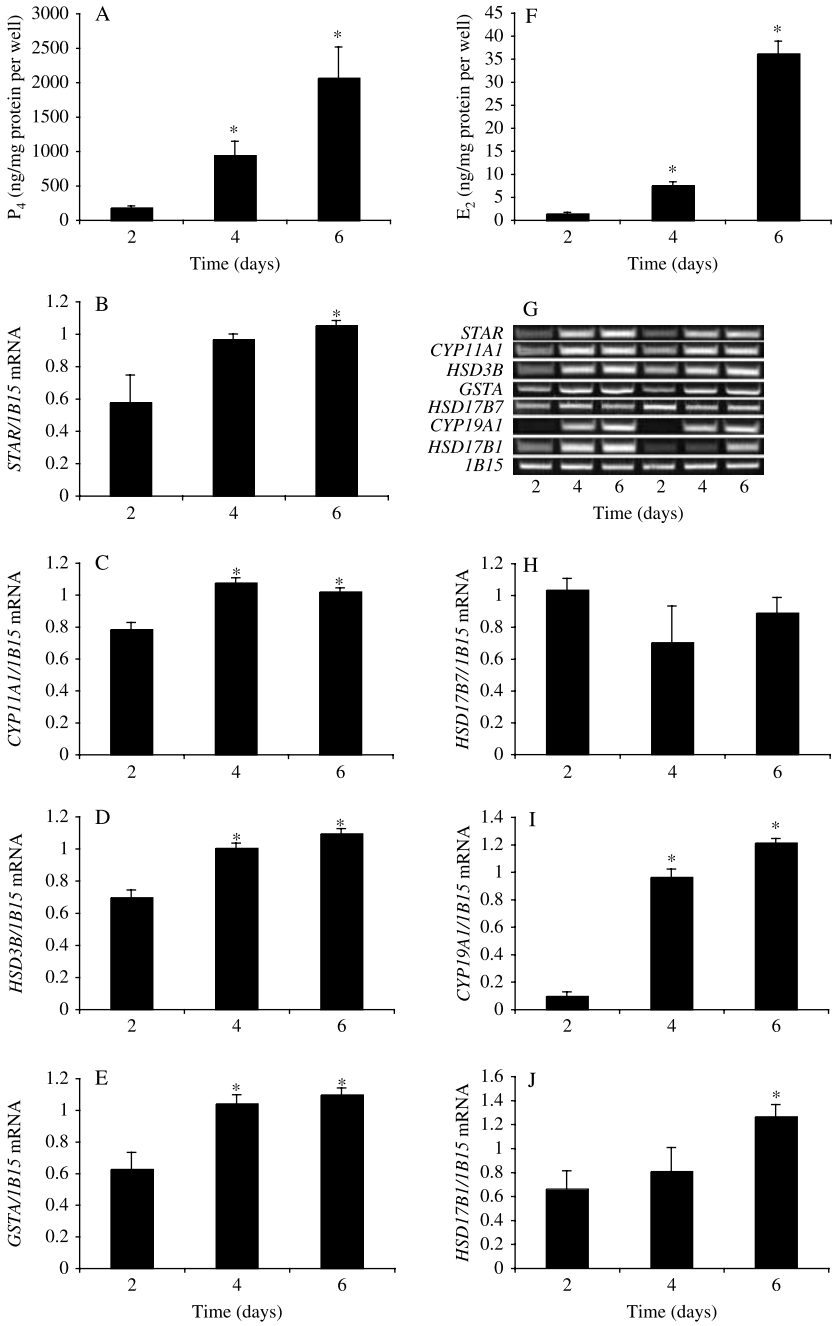

Figure 2 Effect of time in culture (2, 4, and 6 days) on secretion of E_2 and P_4 and mRNA expression of steroidogenic enzymes in bovine granulosa cells. Granulosa cells were cultured in serum-free medium with 1 ng/ml FSH starting at day 0. (A and F) The data represent secretion of E_2 and P_4 during the last 48 h of culture. (B–E and H–J) Abundance of *STAR*, *CYP11A1*, *HSD3B*, *GSTA*, *HSD17B7*, *CYP19A1*, and *HSD17B1* mRNA was measured by semi-quantitative RT-PCR and normalized to the housekeeping gene *1B15*. Data are means±S.E.M. of three separate culture replicates. Asterisk indicates that the mean is significantly different from control at day 2 ($P<0.05$, one-way ANOVA, with Dunnett's test). (G) Representative agarose gel from two replicates showing PCR products for each steroidogenic enzyme and constitutively expressed housekeeping gene *1B15*.

present study this isoform was detected in bovine granulosa cells and was not inhibited by TGFB1. In rodent and rabbit ovaries, this isoform is present only in the corpus luteum and was first reported as the prolactin receptor-associated protein (Nokelainen *et al.* 1998, Krusche *et al.* 2001, Risk *et al.* 2005). In humans, *HSD17B7* transcript was found in the ovaries of non-pregnant, but not pregnant, women (Krazeisen *et al.* 1999). The mRNA expression of *HSD17B7* was not affected by TGFB1 in agreement with a lack of the effect of TGFB1 on HSD17B activity. FSH dose and time in culture also did not affect the expression of *HSD17B7* mRNA in the present study. For HSD17B activity, a 1000-fold excess of unlabeled E_1 had to be added and incubation time had to be shortened to obtain comparable conditions of substrate excess, indicating that HSD17B reducing activity is very high in cultured bovine granulosa cells. The high HSD17B activity could explain why the CYP19A1 product [^{3}H]E_1 was undetectable in the presence or absence of TGFB1.

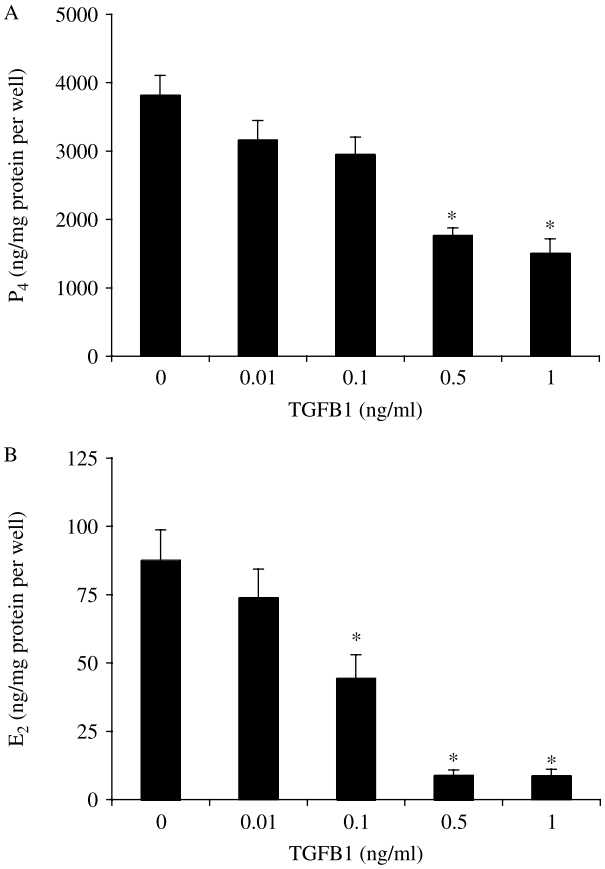

Figure 3 Effect of TGFB1 on E_2 and P_4 secretion from cultured bovine granulosa cells. The cells were cultured for 6 days without serum in the presence of 1 ng/ml FSH and were treated with TGFB1 beginning on the first day of culture. The medium was collected on day 6 and the data represent steroid produced during the last 48 h of culture. Data were corrected for total cell protein and represent means±S.E.M. derived from four different pools of ovaries. Asterisk indicates that steroid hormone secretion was significantly different from the 0 dose control group ($P<0.05$, $n=4$, one-way ANOVA, with Dunnett's test).

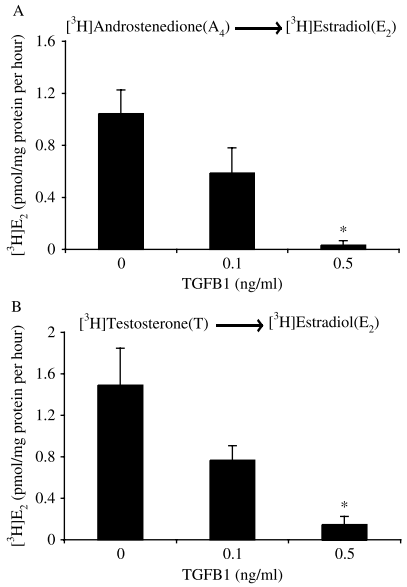

Figure 4 Effect of TGFB1 on conversion of androgens to E_2. Bovine granulosa cells were cultured for 6 days in serum-free medium. Labeled steroid precursor was added on day 6 and incubated for 3 h and enzymatic activity assays were conducted as described in Materials and Methods. Data are means±S.E.M. of three separate culture replicates. (A) Conversion of [^{3}H]A_4 to [^{3}H]E_2 (CYP19A1+HSD17B reducing activity). (B) [^{3}H]T conversion to [^{3}H]E_2 (CYP19A1 activity). Asterisk indicates that enzyme activity is significantly different from the 0 dose control group ($P<0.05$, $n=3$, one-way ANOVA, with Dunnett's test).

In developing antral follicles, a positive E_2/P_4 ratio must be maintained and it is critical to limit P_4 secretion until the time of ovulation induction, because premature increase in P_4 is associated with follicular atresia (Ireland & Roche 1982, Irving-Rodgers *et al.* 2003). In agreement with the above, a low dose of FSH stimulated E_2 but not P_4 in the present study and *CYP11A1* was only stimulated by the highest dose of FSH (10 ng/ml). Additionally, the present study is the first report showing that FSH down-regulates the expression of *STAR in vitro*. This finding may explain why *STAR* is undetectable in the granulosa of healthy antral follicles at any size *in vivo* (Soumano & Price 1997, Bao *et al.* 1998). In our time-course experiments, in the presence of low dose of FSH, the production of P_4 increased as well as the expression of *STAR*, *CYP11A1*, *HSD3B*, and *GSTA*. These findings are in agreement with a previous study (Sahmi *et al.* 2004), which showed an increase with time in *HSD3B* and *CYP11A1*. Overall, these results indicate that P_4 increases spontaneously in cultured granulosa cells and that readily available stores of cholesterol are present in granulosa cells, which can be transformed to P_4 by *CYP11A1*, *HSD3B*, and *GSTA*. Similar to the effect of TGFB1 on E_2 synthesis, TGFB1 also inhibited the progestin synthetic pathway. TGFB1 inhibited P_4 secretion and abundance of mRNA encoding *STAR*, *CYP11A1*, *HSD3B*, and *GSTA*, which are required for sustained production of P_4 from cholesterol. These data are consistent with the effects of TGFB1 on *STAR*, *CYP11A1*, and *HSD3B* in thecal, adrenocortical, and adrenal tumor cells among different species (Rainey *et al.* 1991, Cherradi *et al.* 1995, Naaman-Reperant *et al.* 1996, Attia *et al.* 2000, Herrmann *et al.* 2002), and this is the first time that TGFB1 has been shown to inhibit mRNA encoding *GSTA*. Surprisingly, despite the reduced abundance of *HSD3B* and *GSTA* mRNA, TGFB1 did not affect total HSD3B activity. Similarly, TGFB1 did not significantly alter HSD3B activity in the rat fetal testis (Gautier *et al.* 1997). TGFB1 may have stabilized the corresponding enzyme proteins of *HSD3B* and *GSTA* or, alternatively, other unknown gene products with HSD3B activity may be involved.

In agreement with previous morphological studies (Gutierrez *et al.* 1997, Marsters *et al.* 2003), FSH increased cell number. In this model, it has been suggested that after dispersion, granulosa cells revert to

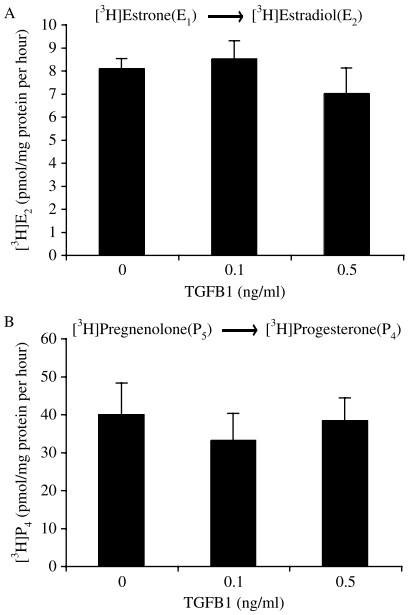

Figure 5 Effect of TGFB1 on (A) HSD17B reducing and (B) HSD3B dehydrogenase/isomerase activities in cultured bovine granulosa cells. The cells were cultured for 6 days in serum-free medium with TGFB1. Labeled steroid precursor ([^{3}H]E_1 or [^{3}H]P_5) was added on day 6 and enzymatic activity assays were conducted as described in Materials and Methods. The [^{3}H]E_1 precursor was incubated with 10^{-6} M (1000-fold excess) unlabeled E_1 for 1.5 h and [^{3}H]P_5 was incubated for 1.5 h without unlabeled P_5. Data are means ± S.E.M. of three separate culture replicates. Compared with the 0 dose control group, TGFB1 treatment did not significantly affect HSD17B reducing activity or HSD3B activity ($P > 0.05$, $n = 3$, one-way ANOVA, with Dunnett's test).

a less mature phenotype, re-establish cell–cell communications, and proliferate in the presence of FSH (Gutierrez *et al.* 1997, Marsters *et al.* 2003). The moderate increase in total cell protein observed in the presence of FSH could indicate that FSH stimulated proliferation or, alternatively, that FSH prevented apoptosis and increased cell survival. FSH and E_2 are known to act as survival factors to induce granulosa cell proliferation and prevent apoptosis (Gutierrez *et al.* 1997, Yang & Rajamahendran 2000, Jiang *et al.* 2003, Quirk *et al.* 2004). In the present study, TGFB1 caused visible differences in the morphology of granulosa cell clumps, which appeared smaller and more spherical than those in FSH-treated controls. TGFB1 at the highest dose also caused a slight decrease in total granulosa cell protein. It is unclear at the present time whether the TGF-induced reduction in steroidogenesis is a result or cause of the change in cell proliferation or survival. But, in the absence of FSH, TGFB1 did not significantly affect total granulosa cell protein. Similarly, TGFB1 alone had no effect on DNA synthesis in cultured bovine granulosa cells (Lerner *et al.* 1995). Therefore, the effects of TGFB1 on granulosa cells could be due to inhibition of the cell-surviving activity of FSH and/or mediated through a loss of E_2-stimulated cell survival (Yang & Rajamahendran 2000, Quirk *et al.* 2004). Ongoing experiments in our laboratory will test this hypothesis, by investigating the effects of TGFB1 on proliferation and apoptosis of bovine granulosa cells cultured with or without FSH.

We conclude that TGFB1 plays an inhibitory role in E_2 and P_4 steroidogenesis in granulosa cells cultured in conditions where E_2 and P_4 secretion is being stimulated, and that TGFB1 counteracts the stimulation of mRNA encoding steroidogenic enzymes. As TGFB1 inhibited *FSHR* mRNA levels and inhibited FSH-induced *CYP19A1* and *HSD17B1* but not *HSD17B7*, we propose that the inhibitory effects of TGFB1 on FSH-stimulated E_2 secretion may be due at least in part to the inhibition of *FSHR* (Fig. 8). These selective inhibitory effects suggest that TGFB1 may be acting in a physiological manner to limit the amount of E_2 and P_4 produced by the granulosa cells without totally shutting down the steroidogenic potential (i.e., HSD17B reducing activity and HSD3B activity are unaffected). By acting in this manner, the physiological role of TGFB1 may be to limit FSH-stimulated growth and differentiation of granulosa cells and play an active role in determining the fate of the developing follicle toward ovulation or atresia.

Materials and Methods

Experimental design

As it has not been established whether FSH stimulates all the steroidogenic enzyme genes in granulosa cells, we first determined the effect of FSH by culturing cells as described below with graded doses of FSH (0, 1, or 10 ng/ml) for 6 days. Based on these data, the development of gene expression during culture was assessed by culturing cells with 1 ng/ml FSH for 2, 4, or 6 days, and the cells were recovered at each time point for the extraction of RNA. Expression of the estrogenic enzyme genes was the highest on day 6; therefore, all subsequent experiments with TGFB1 were performed for 6 days in the presence of 1 ng/ml FSH.

Cell culture

Cell culture was performed essentially as described by Gutierrez *et al.* (1997). Briefly, ovaries were collected at a local abattoir from adult cows irrespective of the stage of the estrous cycle and transported to the laboratory at 37 °C in PBS containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and fungizone (1 μg/ml). Follicles of 2–5 mm in diameter were dissected from the ovaries, and granulosa cells were isolated mechanically by rinsing sections of follicle walls repeatedly through a disposable pipette. The granulosa cell suspension was filtered through a 150 mesh steel sieve (Sigma) to remove oocytes.

The cells were seeded onto 24-well tissue culture plates (Corning Glass Works, Corning, NY, USA) at a density of 10^6 viable cells (tested by Trypan blue exclusion) in 1 ml α-MEM with L-glutamine containing sodium bicarbonate (10 mM),

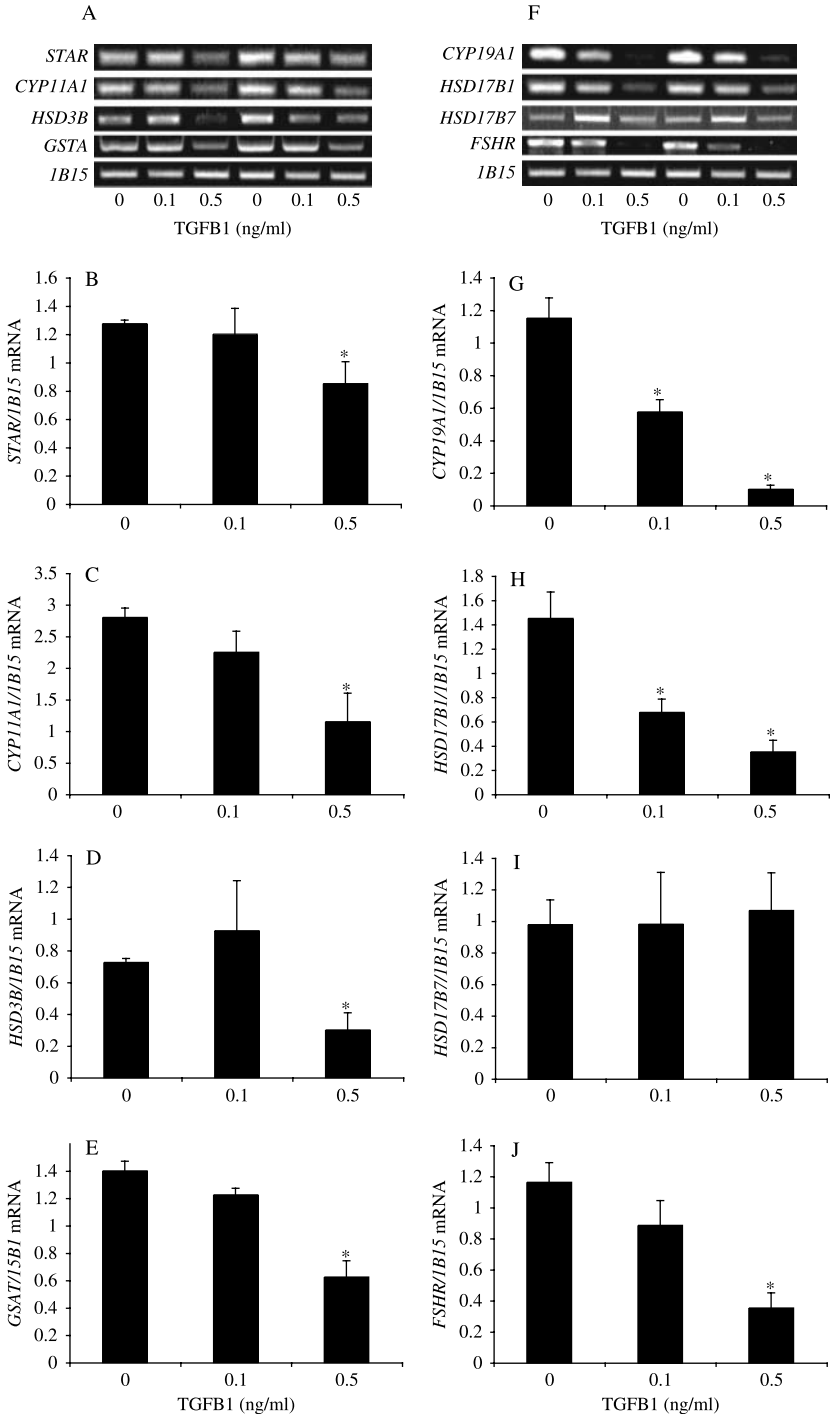

Figure 6 Effect of TGFB1 on mRNA expression of steroidogenic enzymes and *FSHR* in bovine granulosa cells. The cells were cultured in serum-free medium for 6 days in the presence of 1 ng/ml FSH with stated doses of TGFB1. (B–E and G–J) Abundance of *STAR*, *CYP11A1*, *HSD3B*, *GSTA*, *CYP19A1*, *HSD17B1*, *HSD17B7*, and *FSHR* mRNA was measured by semi-quantitative RT-PCR and normalized to the housekeeping gene *1B15*. Data are means ± S.E.M. of four separate culture replicates. Asterisk indicates that mRNA content is significantly different from the 0 dose control ($P<0.05$, one-way ANOVA, with Dunnett's test). (A and F) Representative agarose gels from two replicates showing PCR products for each steroidogenic enzyme, *FSHR* and constitutively expressed housekeeping gene *1B15*.

HEPES (20 mM), non-essential amino acid mix (1.1 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml), protease-free BSA (0.1%), sodium selenite (4 ng/ml), transferrin (2.5 µg/ml), A_4 (100 nM; all from Sigma), ovine FSH (1 ng/ml; oFSH, AFP-5332B; NIDDKD, Torrance, CA, USA), insulin (10 ng/ml; Invitrogen), and graded doses of recombinant active human TGFB1 (R&D Systems, Minneapolis, MN, USA) starting on the first day of culture. Cultures were maintained at 37 °C in 5% CO_2 for 6 days, with 700 µl medium being replaced every 2 days.

At the end of culture, the medium was collected and frozen for subsequent steroid assay and granulosa cells were collected for RNA extraction or lysed for total protein measurement. Total cell protein was extracted by the addition of 200 µl of 1 M NaOH to each well for 2 h at room temperature, followed by neutralization with 200 µl of 1 M HCl. Protein concentrations were measured using the Bio-Rad micro-assay (Bio-Rad). All experiments were performed with at least three independent cell cultures, and three to five wells per treatment were pooled for each assay.

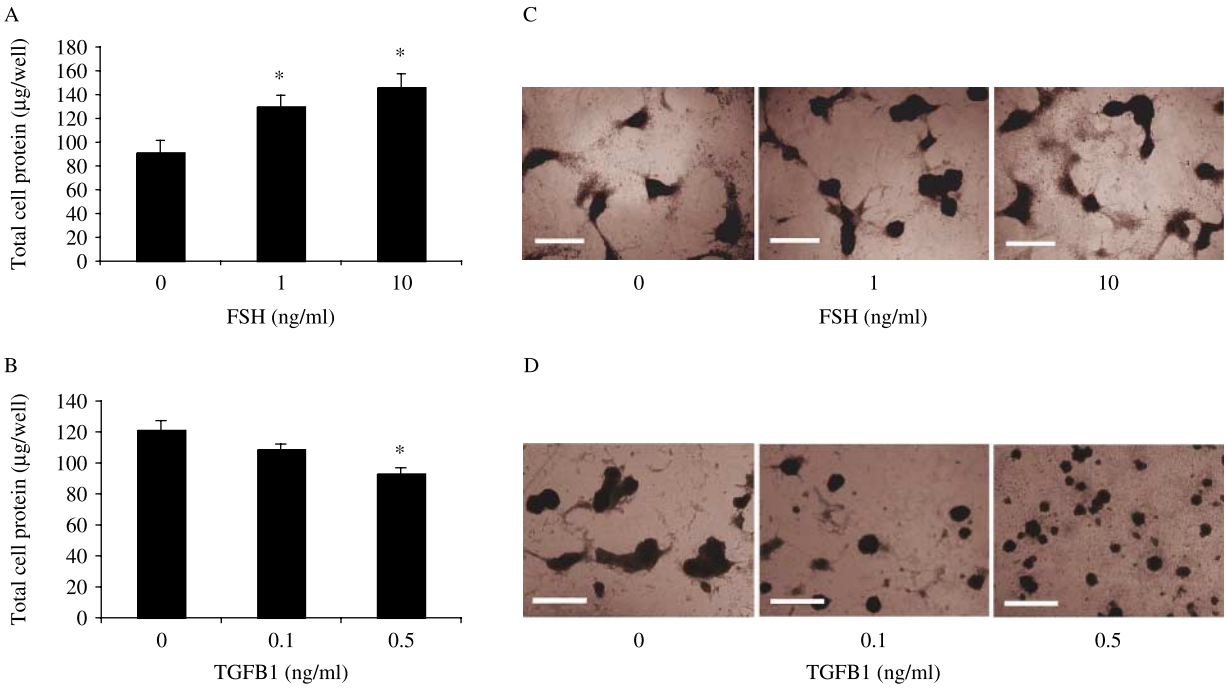

Figure 7 Effect of FSH and TGFB1 on total cell protein and morphology of granulosa cells. The cells were cultured for 6 days in serum-free medium with different doses of (A and C) FSH and (B and D) TGFB1 in the presence of 1 ng/ml FSH, as described in Materials and Methods. (A and B) Data for total protein content are means ± S.E.M. of four and six separate culture replicates respectively. Asterisk indicates significant differences compared with the 0 dose control ($P<0.05$, one-way ANOVA, with Dunnett's test). Scale bar, 500 µm.

Steroid assays

Culture medium samples were assayed for E_2 as reported previously (Bélanger *et al.* 1990), but without C-18 column extraction. Cross-reaction of A_4 and E_1 with the E_2 assay was less than 0.1% (Bélanger *et al.* 1980). P_4 was measured in duplicate as described (Lafrance & Goff 1985). Intra- and inter-assay coefficients of variation were less than 15% for both assays. The sensitivity of the E_2 and P_4 assays was 8 and 32 pg per tube respectively. The steroid hormone concentrations were corrected for cell number by normalization to total cell protein per well.

RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen), according to the manufacturer's instructions, and treated with DNase (Qiagen). The RT reaction was performed on 1 µg total RNA with Omniscript enzyme (Qiagen). Gene expression was measured by semi-quantitative PCR. The primers used were those described previously for *cyclophilin* (*1B15*; Bettegowda *et al.* 2006), *CYP11A1* (Vanselow *et al.* 2004), *HSD3B* (Vanselow *et al.* 2004), *CYP19A1* (Sahmi *et al.* 2004), and *FSHR* (Ndiaye *et al.* 2005). Sense (5′-TTGTGCGAGAGTCTGGCGATTCT-3′) and

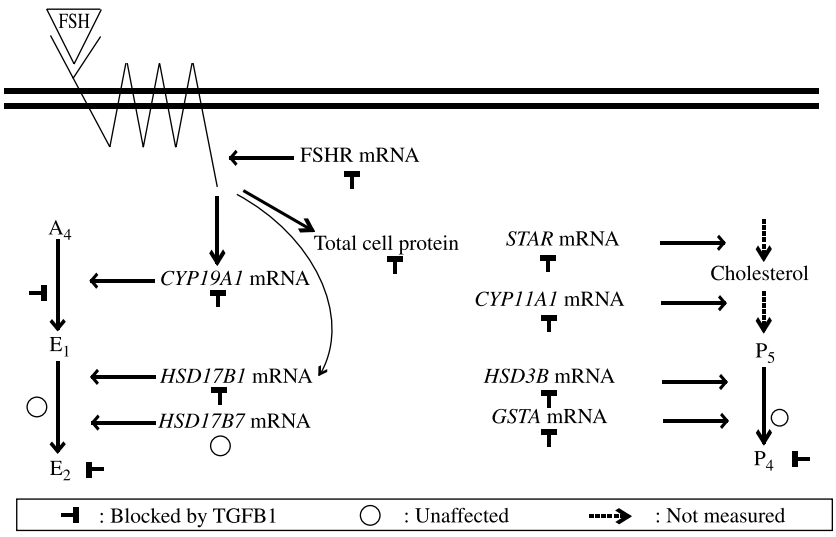

Figure 8 Schematic showing the effect of TGFB1 in the presence of FSH (1 ng/ml) on mRNA expression of steroidogenic enzymes and *FSHR*, total cell protein, and steroidogenic enzyme activity in cultured bovine granulosa cells. The meaning of the signs is shown in the rectangle underneath.

antisense (5′-AGGAATCGCTCGGTGGTGAAGTA-3′) primers for *HSD17B1* were designed based on the bovine sequence (NM_001102365) with a product size of 287 bp. Sense (5′-CGGTTGCTGGAAGAAGATGATG-3′) and antisense (5′-TCACCAGAGGATTGAGAGACTCG-3′) primers for *HSD17B7* were designed based on the bovine sequence (XM_581467) with a product size of 789 bp. Sense (5′-GACACGGTCATCACTCACGAGTT-3′) and antisense (5′-ATGCTGAGCAGCCAGGTGAGTT-3′) primers for *STAR* were designed based on the bovine sequence (XM_001250261) with a product size of 248 bp. Sense (5′-GAATGGAGTGCATTCGGTGGCTC-3′) and antisense (5′-GACTGCTGACTCTGGCTTTTAGG-3′) primers for *GSTA* were designed based on the bovine sequence (BTU49179) with a product size of 528 bp. Forward and reverse primers used in the PCRs were located in different exons to avoid amplification of any residual genomic DNA. The PCR products for each gene were sequenced to confirm the identity of the gene. PCR was performed under the following conditions: (1) initial denaturation at 94 °C for 3 min; (2) amplification cycles of denaturation at 94 °C for 45 s, annealing for 45 s at 60 °C (*CYP11A1*, *HSD3B*, and *FSHR*) or 64 °C (*HSD17B1*, *HSD17B7*, *GSTA*), or for 30 s at 62 °C (*STAR*, *1B15* and *CYP19A1*); (3) elongation at 72 °C for 1 min; and (4) final elongation at 72 °C for 5 min. Optimal cycle number for amplification during the exponential phase was determined for each gene. The reactions were performed for 31 cycles for *STAR*, 35 cycles for *CYP11A1*, 34 cycles for *HSD3B*, 25 cycles for *GSTA*, 29 cycles for *CYP19A1*, 36 cycles for *HSD17B1* and *HSD17B7*, 37 cycles for *FSHR*, and 27 cycles for *1B15*. The PCR products were separated on 2% agarose gels containing 0.001% ethidium bromide and visualized under u.v. light. Quantification of band intensity was performed with NIH Image J software (http://rsb.info.nih.gov/nih-image/). Target gene mRNA abundance was expressed relative to *1B15* mRNA abundance.

Enzyme activity assays

Short-term incubation of tritiated steroid hormone precursors was conducted at the end of day 6 of culture to measure specific enzyme activities. On day 6, all the medium of each well was removed and replaced with fresh medium without A_4 but containing 6–11 nM (corresponding to 1.6×10^6 disintegrations per minute (DPM)/ml medium) of either [^{3}H]E_1, [^{3}H]A_4, [^{3}H]T, or [^{3}H]P_5 and 0, 0.1, or 0.5 ng/ml TGFB1. Cells were incubated for 1.5 or 3 h at 37 °C. At the end of the incubation, the medium was recovered and frozen at −20 °C until analysis of steroid metabolism by thin layer chromatography (TLC) as described previously (Godin *et al.* 1999). Briefly, steroids were extracted from the medium using diethyl ether and resolved on DC-Alufolien neutral (type E) paper plates (Whatman, Maidstone, Kent, England) in toluene:acetone (4:1). Each TLC plate contained [^{3}H]E_1, [^{3}H]E_2, [^{3}H]A_4, [^{3}H]T, and [^{3}H]P_5 as standards. A culture medium control was performed by incubating tracer in culture medium without cells, and was used to obtain background radioactivity that was subtracted from product counts. After migration of samples, the TLC plates were exposed to phosphor screens designed for tritium detection, and tritiated steroid metabolites were localized with a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). The rate of production of specific steroid metabolites was quantified by scraping the corresponding sample and background spots from the TLC plate and counting the radioactivity using PCS scintillation fluid (Amersham).

Activity assays were validated by determining the conditions required to maintain excess substrate. In preliminary time-course experiments, 1.6×10^6 DPM of [^{3}H]precursor (6–11 nM) were incubated with granulosa cells for 3 and 6 h. The amount of conversion of [^{3}H]A_4 and [^{3}H]T precursors to [^{3}H]E_2 was similar with 29 and 58% conversion after 3 and 6 h respectively, and thus an incubation time of 3 h was selected to measure the rate of conversion of [^{3}H]A_4 and [^{3}H]T to [^{3}H]E_2. For HSD17B reducing activity, the amount of conversion of [^{3}H]E_1 precursor to [^{3}H]E_2 was 92 and 98% after 3 and 6 h respectively. Therefore, to obtain experimental conditions of excess precursor, increasing concentrations of unlabeled E_1 (10^{-7}, 10^{-6}, 10^{-5} M) were added to the 8.8 pmol/ml of [^{3}H]E_1, and incubation time was reduced to 1.5 h. This resulted in 6, 20, and 30% conversion of [^{3}H]E_1 to [^{3}H]E_2 respectively, and therefore 10^{-6} M unlabeled E_1 was added to all [^{3}H]E_1 incubations to quantify HSD17B reducing activity. For HSD3B activity, the amount of conversion of [^{3}H]P_5 precursor to [^{3}H]P_4 was 37, 69, 79, and 90% conversion after 1.5, 3, 4.5, and 6 h respectively, and thus an incubation time of 1.5 h was selected to measure the rate of conversion of [^{3}H]P_5 to [^{3}H]P_4.

Statistical analysis

Statistical analysis was performed using the JMP software (SAS Institute, Cary, NC, USA). Data are presented as means ± S.E.M. The data were normally distributed as verified using the Shapiro–Wilk test. One-way ANOVA was used to test the main effect of FSH, time, and TGFB1 on the measured parameters. Differences between treatment dose of FSH or TGFB1 with the 0 dose control or between 4- and 6-day cultures with the 2-day culture group were identified with Dunnett's test.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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