Transforming growth factor- β 1 inhibits luteinization and promotes apoptosis in bovine granulosa cells

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Abstract

We have previously shown that TGF β 1 inhibits estradiol (E_2) and progesterone (P_4) biosynthesis in FSH-stimulated bovine granulosa cells by selective inhibition of steroidogenic enzymes. The objective of this study was to assess the effects of TGF β 1 on E_2 and P_4 steroidogenesis in bovine granulosa cells cultured in the absence of FSH and to measure the effects of TGF β 1 on cell proliferation and apoptosis in the presence and absence of FSH. Bovine granulosa cells from 2 to 5 mm follicles were cultured in serum-free medium for 2–6 days. In the absence of FSH, the secretion of P_4 increased with time in culture ($P < 0.05$). Addition of TGF β 1 for 6 days decreased P_4 secretion and mRNA levels of the P_4 synthesis-associated genes *STAR*, *CYP11A1*, *HSD3B1*, and *GSTA* ($P < 0.05$). In the absence of FSH, the secretion of E_2 decreased and addition of TGF β 1 for 6 days partially reversed this decline and stimulated E_2 biosynthesis, *CYP19A1* and *HSD17B1* mRNA levels and CYP19A1 activity ($P < 0.05$). Conversely, TGF β 1 did not affect *HSD17B7* expression and HSD17B-reducing activity. TGF β 1 decreased the proportion of cells in the G0/G1 and S + G2/M phases in FSH-stimulated and unstimulated granulosa cells ($P < 0.05$). Furthermore, in the presence or absence of FSH, TGF β 1 increased the proportion of cells in apoptosis measured by propidium iodide staining and flow cytometry and confirmed by increased levels of cleaved caspase-3 ($P < 0.05$). Our results therefore indicate that TGF β 1 inhibits luteinization in cultured bovine granulosa cells while maintaining an estrogenic phenotype, and this effect was associated with increased apoptosis.

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Introduction

In the ovary, the destiny of the majority of developing follicles is atresia, and only a limited number of follicles will develop to the ovulatory stage. Apoptosis of granulosa cells is a hallmark of follicular atresia (Tilly *et al.* 1991). Many signaling processes are involved in the initiation of apoptosis, one of which is the proteolytic activation of caspase-3, which is a caspase common to several different apoptosis pathways (Matikainen *et al.* 2001, Johnson & Bridgham 2002). Caspase-3 is actively involved in follicular atresia, and is present in the granulosa cells of atretic, but not healthy follicles (Boone & Tsang 1998, Nicholas *et al.* 2005). Research on the mechanisms that regulate cell cycle progression have identified specific phases in which cells are more susceptible to apoptosis (Quirk *et al.* 2004). During the first gap phase or G1, cells are quiescent but potentially receptive to mitogenic stimuli, which stimulate cells to embark into the proliferative phases of the cell cycle consisting of the DNA synthesis phase (S), the second gap phase (G2), and the mitotic phase (M). In granulosa cells of the developing follicle, the G1/S transition phase is a critical checkpoint, as progression

from the quiescent stage (G0/G1) to the S phase requires the action of cell proliferation and survival factors such as insulin-like growth factor-1 (IGF1) and estradiol (E_2 ; Hu *et al.* 2004, Quirk *et al.* 2004).

The secretion of E_2 and progesterone (P_4) and the autocrine or paracrine effects of these steroids are also important functions of granulosa cells during follicular development (Drummond 2006). Increased production of E_2 in granulosa cells requires transformation of androstenedione (A_4) to estrone (E_1) by cytochrome P450 aromatase (CYP19A1) activity and conversion of E_1 to E_2 by 17 β -hydroxysteroid dehydrogenase (HSD17B)-reducing enzyme activity (Mindnich *et al.* 2004, Zheng *et al.* 2008). In large antral and preovulatory follicles, granulosa cells develop the capacity to transform cholesterol into P_4 through the expression of P_4 -synthetic enzymes, including cytochrome P450 side-chain cleavage (CYP11A1), 3 β -hydroxysteroid dehydrogenase (HSD3B1), and α -glutathione S-transferase (GSTA; Bao & Garverick 1998, Rabahi *et al.* 1999, Raffalli-Mathieu *et al.* 2007).

To understand ovarian follicle development, studies have focused on the regulation of granulosa cell steroidogenesis, proliferation, and apoptosis. In the last

decade, many autocrine/paracrine growth factors, including transforming growth factor- β 1 (TGFB1), have been identified in the ovary and shown to regulate physiologic processes in granulosa cells. TGFB1 is expressed in the ovary of human, rodent, and sheep (Juengel & McNatty 2005). In earlier studies, TGFB1 knockout mice did not survive past weaning age, which prevented researchers from defining the ovarian role of TGFB1 *in vivo* (Shull *et al.* 1992, Kulkarni *et al.* 1993). More recently, female TGFB1 knockout mice on the immunocompromized *scid* background have provided evidence that follicular estrogen synthesis is less sensitive to TGFB1 deficiency than luteal cell P_4 synthesis, as TGFB1 null mutant mice had low serum P_4 levels, but estrogen was unaffected (Ingman *et al.* 2006). In agreement with this *in vivo* result, TGFB1 stimulated P_4 synthesis in cultured rodent granulosa cells (Dodson & Schomberg 1987); however, TGFB1 also stimulated E_2 production (Adashi *et al.* 1989). Conversely, TGFB1 inhibited granulosa cell secretion of E_2 and P_4 from pigs and ruminants (Chang *et al.* 1996, Wandji *et al.* 1996, Ford & Howard 1997, Juengel *et al.* 2004, Ouellette *et al.* 2005, Zheng *et al.* 2008). In the bovine species, TGFB1 expression was observed in granulosa cells of small but not of large antral follicles (Nilsson *et al.* 2003). In agreement with this finding, a previous study showed a negative relationship between follicular fluid TGFB1 and E_2 concentrations and between TGFB1 levels and follicle size (Ouellette *et al.* 2005). Concerning cell proliferation, TGFB1 inhibited EGF- or FSH-stimulated DNA synthesis and mitosis in cultured bovine, porcine, or ovine granulosa cells (Skinner *et al.* 1987, Gangrade & May 1990, Juengel *et al.* 2004), but it increased FSH-stimulated DNA synthesis and mitosis in rat granulosa cells (Dorrington *et al.* 1993). The conflicting results of TGFB1 function in ovarian physiology among animal species further emphasize the multifunctional roles of TGFB1, which have been reported in many other tissues (Herrmann *et al.* 2002). The effects of TGFB1 also appear to be dependent on the stage of differentiation and the state of stimulation of the tissues examined *in vitro*.

In bovine granulosa cells, it is well established that TGFB1 negatively affects secretion of E_2 and P_4 (Wandji *et al.* 1996, Ouellette *et al.* 2005, Zheng *et al.* 2008). Our previous study showed that TGFB1 inhibits E_2 and P_4 syntheses without totally shutting down steroidogenesis in FSH-stimulated granulosa cells. However, the effects of TGFB1 on steroidogenesis in granulosa cells cultured in basal conditions without FSH and its effects on cell proliferation and apoptosis in FSH-stimulated and -unstimulated granulosa cells remain unknown. Therefore, the objective of this study was to assess the effects of TGFB1 on steroidogenesis, cell proliferation, and apoptosis in cultured bovine granulosa cells.

Results

In granulosa cells cultured without FSH treatment, E_2 accumulation in culture medium decreased from day 2 to day 6 (Fig. 1A). P_4 secretion increased from day 2 to day 6 (Fig. 1B). In agreement with increased secretion of P_4 with time in culture, mRNA expression of the P_4 -related enzymes *STAR*, *CYP11A1*, *HSD3B1*, and *GSTA* also increased with time (Fig. 1D–G). A representative agarose gel from two replicates showing PCR products for each steroidogenic enzyme and housekeeping gene *1b15* is shown in Fig. 1C.

The addition of TGFB1 during the 6-day culture period caused a significant ($P < 0.05$) dose-dependent inhibition of P_4 , and increased E_2 secretion (Fig. 2A and B).

Figure 1 Effect of time in culture (2, 4, 6 days) on secretion of E_2 and P_4 with P_4 synthesis-associated gene expression in bovine granulosa cells cultured in serum-free medium in the absence of FSH. (A and B) The data represent the secretion of E_2 and P_4 during the previous 48 h of culture. (D–G) Transcript abundance of P_4 synthesis-associated genes *STAR*, *CYP11A1*, *HSD3B1*, and *GSTA* mRNA was measured by semi-quantitative RT-PCR and normalized to the housekeeping gene *1b15*. Data are means $\pm$ 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). (C) A representative agarose gel from two replicates showing PCR products for each steroidogenic enzyme and housekeeping gene *1b15*.

Figure 2 Effects of TGFB1 on (A) E₂ and (B) P₄ secretion in bovine granulosa cells cultured in the absence of FSH. Cells were cultured for 6 days without serum and treated with TGFB1 beginning at day 0. The medium was collected on day 6 and the data represent steroids produced during the final 48 h of culture. Data were corrected for total cell protein and represent means $\pm$ S.E.M. derived from four different pools of ovaries. The 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).

To determine whether the increase in E₂ secretion was due to an increase in CYP19A1 activity and HSD17B-reducing activity, conversion of the tritiated steroid hormone precursors of A₄, T, and E₁ to E₂ was measured. When granulosa cells were cultured with 1 ng/ml of TGFB1, there was a significant increase ($P < 0.05$) of combined CYP19A1 activity and HSD17B-reducing activity relative to untreated cells, as measured by the conversion of [³H]-A₄ to [³H]-E₂ (Fig. 3A). The intermediate product [³H]-E₁ was not detected. TGFB1 at a dose of 1 ng/ml caused a significant increase ($P < 0.05$) in CYP19A1 activity, as measured by the conversion of [³H]-T to [³H]-E₂ (Fig. 3B). TGFB1 did not alter HSD17B-reducing activity, as measured by the conversion of [³H]-E₁ to [³H]-E₂ (Fig. 3C). TGFB1 increased *CYP19A1* and *HSD17B1* mRNA levels ($P < 0.05$), but did not affect *HSD17B7* mRNA levels (Fig. 4A, C and D). Corresponding to the decreased secretion of P₄, TGFB1 caused a significant inhibition ($P < 0.05$) in mRNA expressions of *STAR*, *CYP11A1*, *HSD3B1*, and *GSTA* (Fig. 4E–H).

At day 0, the majority of granulosa cells were at G0/G1 phase (Fig. 5C and D). The percentage of cells in G0/G1 phase decreased with time in culture in the presence or absence of FSH, and TGFB1 caused a significant decrease in the percentage of cells in G0/G1 with or without FSH (Fig. 5C and D, $P < 0.05$). At day 0, the proportion of cells in the proliferative phase of the cell cycle (S+G2/M) was low ($\sim 10\%$). The proportion of S+G2/M cells did not change significantly with time in culture in FSH-stimulated cells, but TGFB1 treatment decreased the amount of proliferating cells at 144 h. In the absence of FSH, the proportion of S+G2/M cells decreased with time in culture and TGFB1 caused a marked decrease in the proportion of proliferating cells at 144 h (Fig. 5E and F, $P < 0.05$). The percentage of apoptotic granulosa cells (defined as $< 2n$) increased with time in culture in the presence or absence of FSH.

(Fig. 5A and B, $P < 0.05$). In FSH-stimulated granulosa cells, apoptosis increased by twofold compared with more than threefold increase in cells cultured without FSH. At 48, 96, and 144 h, TGFB1 significantly increased the percentage of apoptotic cells in the presence or absence of FSH, resulting in a proportion of more than 60% of apoptotic cells at 144 h (Fig. 5A and B, $P < 0.05$). The effect of TGFB1 on the proportion of cells in apoptosis measured by propidium iodide (PI) staining and flow cytometry was also confirmed by increased levels of cleaved caspase-3 ($P < 0.05$; Fig. 6A and B). In order to confirm the effects of TGFB1 on E₂ and P₄ concentration in FSH-stimulated and -unstimulated

Figure 3 Effect of TGFB1 on conversion of androgens to E₂ and HSD17B-reducing activity in bovine granulosa cells cultured in the absence of FSH. Cells were cultured for 6 days in serum-free medium with TGFB1 treatment beginning on day 0. The culture medium was replaced on day 6 with fresh medium containing labeled steroid precursor, and enzymatic activity assays were conducted as described in Materials and Methods. [³H]A₄ and [³H]T were incubated for 48 h and [³H]E₁ precursor was incubated with 10⁻⁶ M (1000-fold excess) unlabeled E₁ for 1.5 h. Data are means $\pm$ S.E.M. of three separate culture replicates. (A) Conversion of [³H]A₄ to [³H]E₂ (CYP19A1 + HSD17B-reducing activity) is shown. (B) Conversion of [³H]T to [³H]E₂ (CYP19A1 activity) is shown. (C) Conversion of [³H]E₁ to [³H]E₂ (HSD17B-reducing activity) is shown. The 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).

Figure 4 Effect of TGFB1 on mRNA expression of steroidogenic enzymes in bovine granulosa cells cultured in the absence of FSH. Cells were cultured in serum-free medium for 6 days in the presence of the stated doses of TGFB1 beginning on day 0. (A and C–H) Abundance of *CYP19A1*, *HSD17B1*, *HSD17B7*, *STAR*, *CYP11A1*, *HSD3B*, and *GSTA* mRNA was measured by semi-quantitative RT-PCR and normalized to the housekeeping gene *1b15*. Data are means $\pm$ S.E.M. of four separate culture replicates. Asterisk (*) indicates that mRNA content is significantly different from 0 dose control ($P < 0.05$, one-way ANOVA, with Dunnett's test). (B) Representative agarose gels from two replicates showing PCR products for each steroidogenic enzyme and the housekeeping gene *1b15* are shown.

granulosa cells, the concentration of these steroid hormones was measured at day 6 in the culture media of cells used for Figs 5 and 6. In FSH-stimulated granulosa cells, TGFB1 inhibited E_2 secretion (35.9 ± 5 in control versus 2.6 ± 1.6 ng/mg protein, $P < 0.05$, $n = 3$) and P_4 secretion (1151 ± 86 in control versus 125 ± 13 ng/mg protein, $P < 0.05$, $n = 3$). In the absence of FSH, TGFB1 stimulated E_2 secretion (3.5 ± 0.2 in control versus 5.7 ± 0.1 ng/mg protein, $P < 0.05$, $n = 3$) and inhibited P_4 secretion (1005 ± 153 in control versus 108 ± 14 ng/mg protein, $P < 0.05$, $n = 3$).

Discussion

In vitro, granulosa cells harvested from follicles always spontaneously luteinize (Meidan *et al.* 1990). However, in cultured bovine granulosa cells harvested from small bovine antral follicles, the addition of low dose of FSH maintains follicle characteristics by stimulating E_2 and *CYP19A1* for several days (Gutierrez *et al.* 1997, Silva &

Price 2002, Sahmi *et al.* 2004) and appears to counteract the process of luteinization by inhibiting *STAR* mRNA expression (Zheng *et al.* 2008). Our previous study showed that TGFB1 inhibits stimulation of E_2 and P_4 synthesis in bovine granulosa cells cultured in the presence of FSH (Zheng *et al.* 2008). In bovine granulosa cells cultured in the absence of FSH, the present study also shows that TGFB1 inhibits P_4 secretion and the expression of P_4 -related enzymes *STAR*, *CYP11A1*, *HSD3B1*, and *GSTA*, indicating an inhibition of luteinization. Similarly, inhibition of P_4 secretion by TGFB1 has also been observed in bovine luteal cells (Hou *et al.* 2008). Conversely, in the absence of FSH, this is the first study to show that TGFB1 increases E_2 secretion and the expression of E_2 -synthetic enzyme *CYP19A1* and its activity in bovine granulosa cells cultured for 6 days. However, the actual amount of E_2 stimulation by TGFB1 was sixfold lower compared with E_2 stimulation by FSH, and thus the relatively low level of E_2 stimulation by TGFB1 only prevents the decline in

Figure 5 Effects of TGFB1 treatment on apoptosis and cell cycle phase of granulosa cells in the presence and absence of FSH. Cells were treated with TGFB1 for 6 days in the (A, C, and E) presence or (B, D, and F) absence of FSH, stained with propidium iodide and analyzed by FACS. (A and B) The percentage of apoptotic cells relative to total cell counts, (C and D) the percentage of G0/G1 cells, and (E and F) the percentage of cells in the proliferative phase of the cell cycle (S+G2/M) are shown. Asterisk (**) indicates significant differences ($P < 0.05$) between granulosa cells cultured with or without TGFB1 at individual time points. Different letters indicate significant differences ($P < 0.05$) between different time points in the presence of TGFB1 (a, b, c and d) or in the absence of TGFB1 (x and y). Data are means $\pm$ S.E.M. of three separate culture replicates.

E₂ that was observed in the absence of FSH. Furthermore, in the present study, TGFB1 stimulated expression of *HSD17B1* mRNA but did not affect expression of *HSD17B7* mRNA and *HSD17B*-reducing activity. In our previous study, using FSH-stimulated bovine granulosa cells, TGFB1 inhibited expression of *HSD17B1* mRNA but similar to the present study, TGFB1 did not affect expression of *HSD17B7* and *HSD17B*-reducing activity (Zheng *et al.* 2008). These results confirmed that the expression of *HSD17B1* mRNA does not consistently correlate with overall cellular *HSD17B* activity. Other candidates such as *HSD17B7* may therefore contribute to *HSD17B*-reducing enzyme activity in granulosa cells. In summary, the effects of TGFB1 in steroidogenesis in the absence of FSH indicate that this regulating factor inhibits luteinization while preserving the estrogenic phenotype of granulosa cells.

In a previous *in vitro* study using cultured bovine granulosa cells, <15% of total cells were in the proliferation phase during the first day of culture with a minimal dose of FSH (Rouillier *et al.* 1998). At day 0, our study shows a similar percentage of cells in the proliferation phase (10%), with most of the cells (80%) being at the G0/G1 phase and the remainder (10%) being in apoptosis. In the present study, spontaneous apoptosis of GCs increased with time in culture and is in agreement with a previous study using cultured bovine

granulosa cells obtained from different sized follicles (Yang & Rajamahendran 2000). In the same study, FSH was also shown to prevent apoptosis of GCs (Yang & Rajamahendran 2000). Our results show that TGFB1 increased apoptosis in both FSH-treated and -untreated cells. TGFB1 not only sharply induced apoptosis of GCs during time in culture but also inhibited proliferation. However, the proportion of cells in the proliferative phase was very low to start with, which makes it appear likely that the previously reported decrease in protein and DNA content by TGFB1 in cultured ruminant granulosa cells (Juengel *et al.* 2004, Zheng *et al.* 2008) is due to the induction of apoptosis by TGFB1. We cannot exclude the possibility that contact inhibition may have limited cell proliferation in our culture system, and a more marked effect may have been obtained under conditions optimized for cell proliferation.

The data presented herein indicate that, in the presence or absence of FSH stimulation, TGFB1 promotes apoptosis and reduces entry into the proliferative phase of the cell cycle. Whether these effects of TGFB1 are somehow related to its effects on luteinization and steroidogenesis or whether they are wholly independent remains unclear. Recent work in bovine granulosa cells has demonstrated that granulosa cells are thought to become resistant to apoptosis through luteinization (Quirk *et al.* 2004). In this regard, the

Figure 6 Western blot analysis of cleaved caspase-3 in granulosa cells treated with TGFB1 in the presence or absence of FSH. The 17 and 19 kDa cleaved caspase-3 bands were normalized to the housekeeping protein β -actin. Asterisk (*) indicate that cleaved caspase-3 is significantly different from 0 dose treatment of TGFB1. Data are means $\pm$ S.E.M. of three separate culture replicates. ($P < 0.05$, one-way ANOVA, with Dunnett's test).

increased rate of apoptosis that we observed in TGFB1-treated cells may simply be a consequence of their less luteinized state. In addition, the G1/S transition phase seems to act as a critical checkpoint, at which granulosa cells may either progress through the cell cycle or undergo apoptosis, according to the relative abundance of growth and pro- or anti-apoptotic factors that may be present (Quirk *et al.* 2004). The presence of TGFB1 may commit a higher proportion of cells to apoptosis at the G1/S transition, resulting in a decrease in the proportion of cells at the G0/G1 phase and fewer cells in the S and G2/M phases of the cycle and therefore a lesser rate of proliferation. Finally, we cannot exclude the possibility that TGFB1 also affects cell proliferation more directly by negatively regulating cell cycle progression.

Although it is often difficult to relate observations in cultured granulosa cells to follicle development *in vivo*, our findings may provide some insight into mechanisms of follicle selection. The bovine ovarian cycle is characterized by the recruitment of waves of small follicles, which proceed to grow until a poorly understood selection process designates a dominant follicle to continue development, while the remaining recruited follicles undergo atresia (Ireland 1987, Fortune *et al.* 2001). TGFB1 is expressed in the granulosa cells of bovine small antral follicles (Nilsson *et al.* 2003). A recent study has suggested that TGFB1 increases atresia in primary and preantral follicles (Rosairo *et al.* 2008). Given the pro-apoptotic properties of TGFB1 that we have observed in cultured bovine granulosa cells, it seems reasonable to speculate that its relative abundance in a given small follicle may be a determinant if it will undergo atresia (i.e. apoptosis) or achieve dominant

status. In surviving follicles, the anti-luteinization properties of TGFB1 may serve to prevent the premature luteinization of follicular granulosa cells. For continued development of these surviving follicles, FSH would have to overcome TGFB1 inhibition of FSH-induced conversion of androgens to E_2 . One possible way to achieve this effect could be down-regulation of TGFB1 action. Indeed, some studies provide evidence that FSH treatment decreases *TGFB1* and *TGFB1R* (activin receptor-like kinase 5) mRNA expressions in cultured bovine granulosa cells (Nilsson *et al.* 2003, Jayawardana *et al.* 2006). Further studies, including experiments involving manipulation of TGFB1 activity *in vivo* and *in vitro*, will be required to explore these possibilities.

In conclusion, TGFB1 negatively regulates luteinization of bovine granulosa cells, as evidenced by the inhibition of P_4 secretion and P_4 -generating enzymes. In granulosa cells cultured in the absence of FSH, TGFB1 maintained an estrogenic phenotype. In addition, TGFB1 induced apoptosis in both FSH-stimulated and -unstimulated granulosa cells. By acting in this manner, TGFB1 may have a physiological role to control proliferation and steroidogenic differentiation of granulosa cell and may be involved in dominant follicle selection.

Materials and Methods

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 stage of the estrous cycle and transported to the laboratory at 37 °C in PBS containing penicillin (100 U/ml), streptomycin (100 μ g/ml), and fungizone (1 μ g/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.

Cells were seeded into 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 of α -MEM with L-glutamine containing sodium bicarbonate (10 mM), Hepes (20 mM), non-essential amino acid mix (1.1 mM), penicillin (100 IU/ml) and streptomycin (100 μ g/ml), protease-free BSA (0.1%), sodium selenite (4 ng/ml), transferrin (2.5 μ g/ml), A_4 (100 nM; all from Sigma), insulin (10 ng/ml; Invitrogen) and graded doses of recombinant active human TGFB1 (R&D Systems, Minneapolis, MN, USA), with or without ovine FSH (1 ng/ml; oFSH, AFP-5332B; NIDDKD, Torrance, CA, USA) starting on the first day of culture. Cultures were maintained at 37 °C in 5% CO_2 for 2–6 days, with 700 μ l medium being replaced every 2 days.

At the end of culture, medium was collected and frozen for subsequent steroid assays, and granulosa cells were collected for RNA or protein extraction. 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.

Steroid assays

Culture medium samples were assayed for E₂ as previously reported (Bélanger *et al.* 1990) but without C-18 column extraction. Cross-reaction of A₄ and E₁ with the E₂ assay was <0.1% (Bélanger *et al.* 1980). P₄ was measured in duplicate as described (Lafrance & Goff 1985). Intra- and inter-assay coefficients of variation were <15% for both assays. The sensitivity of the E₂ and P₄ assays were 5 and 18 pg per tube respectively. The steroid hormone concentrations were corrected for cell number by normalization to total cell protein.

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and treated with DNase (Qiagen). RT 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 (Zheng *et al.* 2008). 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* and *HSD3B1*) or 64 °C (*HSD17B1*, *HSD17B7*, and *GSTA*), or for 30 s at 62 °C (*STAR*, *1B15*, and *CYP19A1*); 3) elongation at 72 °C for 1 min; 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 27 cycles for *1b15*, 31 cycles for *STAR*, 35 cycles for *CYP11A1*, 34 cycles for *HSD3B1*, 25 cycles for *GSTA*, 38 cycles for *CYP19A1*, 36 cycles for *HSD17B1* and *HSD17B7*. 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 (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/ni-image/>). Specific gene mRNA abundance was normalized to *1b15* mRNA abundance.

Enzyme activity assays

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 A₄ but containing 6–11 nM (1.6 × 10⁶ DPM/ml medium) of either [³H]-E₁, [³H]-A₄, or [³H]-testosterone ([³H]-T) and 0, 0.5 or 1 ng/ml of TGFB1. Cells were incubated for 1.5 to 48 h at 37 °C. At the end of the incubation, medium was recovered and frozen at –20 °C until analysis of steroid metabolism by thin layer chromatography (TLC) as previously described (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, UK) in toluene:acetone (4:1). Each TLC plate contained [³H]-E₁, [³H]-E₂, [³H]-A₄, and [³H]-T 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, 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 radioactivity using PCS scintillation fluid (Amersham).

Activity assays were validated in bovine granulosa cells cultured without FSH by determining the conditions required to maintain excess substrate. In preliminary time course experiments without FSH treatment, 1.6 × 10⁶ DPM of [³H]-A₄ and [³H]-T (6–11 nM) were incubated with granulosa cells for 12, 24, and 48 h. In 12 and 24 h incubations, the conversion of [³H]-A₄ and [³H]-T precursors to [³H]-E₂ was too low to be detected. After 48-h incubation, the amount of conversion of [³H]-A₄ and [³H]-T precursors to [³H]-E₂ was 13.8 and 5.7% respectively, and thus an incubation time of 48 h was selected. For HSD17B-reducing activity, unlabeled E₁ (10^{–6} M) was added to the 8.8 pmol/ml of [³H]-E₁, and incubation time was 1.5 h. This resulted in 13.7% conversion of [³H]-E₁ to [³H]-E₂ and therefore 10^{–6} M unlabeled E₁ was added to all [³H]-E₁ incubations.

Cell cycle and apoptosis analysis by flow cytometry

Flow cytometry using a fluorescence-activated cell sorter (FACS) has been used to determine the proportion of granulosa cells in the different phases of the cycle, and is a recognized method to quantify the rate of apoptosis (Nicoletti *et al.* 1991, Rouillier *et al.* 1998). After removing the media, granulosa cells were detached by rinsing with PBS and were then centrifuged at 500 g for 5 min at 4 °C and washed a second time in PBS. Cells were fixed by drop-wise addition of 1 ml ice-cold 70% ethanol while vortexing. Ethanol-fixed cells were stored at 4 °C for at least 24 h before PI staining. Granulosa cells were centrifuged 500 g for 5 min at 4 °C, ethanol removed, and then treated with 0.5 ml of PI staining solution (1 mg/ml PI, 0.1% TritonX-100 and 0.2 mg/ml RNase A in 1 × PBS). Stained cells were held at room temperature in the dark for at least 1 h before FACS analysis. Immediately before analysis, cells were passed through a 70 µm nylon mesh sieve to obtain a single-cell suspension and to remove aggregated cells. Flow cytometry analysis was performed with a BD Bioscience FACS Vantage SE. Apoptotic cells or their isolated nuclei show a low DNA stainability resulting in a distinct, quantifiable region below the G0/G1 peak. The proportion of apoptotic cells and the proportion of cells in G0/G1, S, and G2/M phases of the cell cycle were calculated using Cell Quest Pro software (BD Bioscience, Oakville, ON, Canada).

Western blotting

Granulosa cells were lysed using Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific, Rockford, IL, USA) and 25 µg protein samples were separated electrophoretically on a 18% SDS-polyacrylamide gel, transferred to a PVDF membrane, blocked in 5% non-fat dried milk in Tris-buffered saline with Tween (TBST), and incubated overnight (4 °C) with the primary antibody, anti-cleaved caspase-3 (Asp175, Cell Signaling Technology, Danvers, MA, USA). After washing with TBST thrice, secondary antibody was added and incubated 1 h at room temperature and protein bands were visualized using ECL Plus (Amersham). The membranes were then stripped and re-probed with a mouse anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The molecular size of immunoreactive bands was determined by co-migration of a ladder of low-range rainbow molecular weight markers (Amersham) applied to a lane in each gel. Protein expression was calculated as the ratio of each specific band to corresponding β-actin signals. Experiments were repeated thrice.

Statistical analysis

All statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA). The data are presented as means ± S.E.M. of duplicate to triplicate measurements in at least three separate cultures. Data were transformed to logarithms if they were not normally distributed as verified using the Shapiro–Wilk test. One-way ANOVA was used to test the main effect of time and TGFB1 on E₂ secretion, P₄ secretion, steroidogenic enzyme activities, and protein expression of cleaved caspase-3. Differences between treatment dose of TGFB1 with the 0 dose control; or between 4 and 6 day cultures and the 2-day culture group were identified with Dunnett's test. Two sets of analyses were performed in flow cytometry experiments. First, the effect of time in culture on % apoptotic cells, % G0/G1, and % S+G2/M were analyzed from 0 to 144 h in the presence or absence of FSH and TGFB1. Differences at each time point were identified with Tukey HSD's all pairs comparison. Second, one-way ANOVA was used to test the effect of TGFB1 on the measured parameters of each time point. Differences between treatment doses of TGFB1 with the 0 dose control were identified with Dunnett's test. A probability of $P < 0.05$ was considered to be statistically significant.

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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