Transforming Growth Factor-α and -β Differentially Regulate Growth and Steroidogenesis of Bovine Thecal Cells during Antral Follicle Development

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ABSTRACT. The actions and interactions of transforming growth factor-α (TGFα) and TGFβ on growth and differentiation of bovine thecal cells were investigated. Bovine thecal interna cells were isolated from small (<5 mm), medium (5–10 mm), and large (>10 mm) antral follicles and cultured in the presence or absence of TGFα and/or TGFβ. Both [3H]thymidine incorporation and changes in cell number (i.e. DNA levels) were evaluated to determine effects on thecal cell growth. Short term treatment of cells with TGFα (18–24 h) stimulated thymidine incorporation and, longer term treatments (4 days) increased cell number. TGFβ suppressed thymidine incorporation below that observed in untreated cultures, but had no effect on cell number. When combined with TGFα, TGFβ suppressed the ability of TGFα to stimulate thymidine incorporation and increase cell number. The response to these growth factors was similar for cells isolated from the different stages of antral follicle development. The effects of TGFα and TGFβ on thecal cell differentiation were evaluated by quantitating changes in androstenedione and progesterone accumulation in cultures treated with TGFs in the absence (basal) or presence of hCG, estradiol (E2), or a combination of hCG and E2. E2 and hCG were included in this study because previous research has demonstrated that these hormones alter thecal cell steroidogenesis. Treatment with TGFα resulted in a suppression of basal and hormonally stimulated accumulation of androstenedione during days 0–3 of culture, whereas TGFβ did not significantly alter androstenedione accumulation. TGFα also suppressed progesterone accumulation during days 0–3 of culture in the absence or presence of hormones. In contrast, TGFβ stimulated accumulation of progesterone in cultures that did not contain E2, which suppressed progesterone during this period. Therefore, during days 0–3 of culture, TGFα appears to have suppressive effects on androstenedione and progesterone production, whereas TGFβ can stimulate progesterone production in the absence of E2. During days 3–6 of culture, thecal cell differentiation changes, and the capacity to produce androstenedione dramatically declines, while the capacity to produce progesterone increases. During this period, either TGFα or TGFβ slightly increased basal progesterone accumulation and partially suppressed the ability of hCG to stimulate progesterone. The effects of TGFs on thecal cell steroidogenesis were similar with cells isolated from the different stages of antral follicle development. Results from these studies provide evidence that TGFα and TGFβ can modulate thecal cell growth and differentiation (i.e. steroidogenesis). The inverse actions of these growth factors on thecal cell proliferation provide an efficient mechanism by which the growth of follicles may be regulated. The effects of TGFα and TGFβ on steroidogenesis may be associated with these growth effects. The ability of TGFα to stimulate cell growth may decrease cell differentiation, which correlates with the generally suppressive effects of TGFα on steroidogenesis observed. In contrast, TGFβ inhibits growth, which may promote cell differentiation, and this correlates with the stimulatory effects of TGFβ on steroidogenesis observed. The ability of thecal cells to produce and respond to these growth factors provides evidence that TGFα and TGFβ may act as important autocrine factors to influence the growth and differentiation of thecal cells during follicle development. (Endocrinology 129: 2041–2048, 1991)

O VARIAN function depends on the continual process of follicular growth and development to ensure ovulation during each estrous cycle. Follicle development is regulated by endocrine, paracrine, and autocrine processes. Gonadotropin secretion from the pituitary is important for the process of growth and differentiation (i.e. steroidogenesis) of ovarian follicles. In vivo administration of gonadotropins stimulates the proliferation of follicular cells (1, 2). When administered in vitro, however, gonadotropins fail to promote the growth of thecal or granulosa cells, providing evidence that gonadotropin-stimulated growth of follicles may be mediated indirectly via other factors. Therefore, the local production of growth factors may be important in regulating normal follicular development.

Previous research has demonstrated that growth-stimulating factors are present in conditioned medium obtained from cultures of rat (3) and bovine (4) thecal cells as well as in porcine follicular fluid (5). One of these growth-stimulating substance was found to be an epider-
mal growth factor (EGF)-like substance (5, 6). This EGF-like substance was found to be transforming growth factor-α (TGFα) (7, 8), which is structurally similar to EGF (9, 10). Both TGFα and EGF bind to a common receptor, which generally results in a mitogenic effect in most cell types shown to respond to these factors (9, 10). Expression and production of TGFα have been demonstrated in thecal cells, but appears to be absent in granulosa cells (7, 8, 11).

In addition to the growth-stimulating factors isolated in thecal cell-conditioned medium, factors that suppress the growth of bovine granulosa cells have been identified in rat thecal-interstitial cell-conditioned medium (6). The growth-suppressive factor in conditioned medium was determined to be TGFβ (12). TGFβ is a member of a supergene family which includes inhibin, activin, and Mullerian inhibiting substance. To date, five different genes for TGFβ have been identified. Although thorough studies concerning intrafollicular expression and production of the different forms of TGFβ have not been conducted, the available results indicate that expression and production of TGFβ occur primarily in the thecal cell component of the follicle (12), with some production observed in bovine (12) and porcine (13) granulosa cells. Recent evidence demonstrates that expression of TGFβ2 occurs in granulosa cells (14), whereas production of TGFβ occurs in thecal cells (15). Since TGFα and TGFβ are expressed and secreted within ovarian follicles, these growth-modulating agents may have an active role in regulating follicle development.

The ability of TGFα and TGFβ to alter the growth and differentiation of cells within the follicle has been demonstrated in several different species. The majority of the research has been conducted on cultures of granulosa cells. Treatment of granulosa cells with either EGF or TGFα results in a mitogenic effect in several species, including the bovine (7, 16), porcine (17–19), and human (17), but appears not to be mitogenic in rats (17, 20). In contrast, treatment with TGFβ appears to suppress the growth of porcine (18, 19) and bovine granulosa cells (12), but stimulates the growth of rat granulosa cells (21).

In addition to the effects that these growth factors have on growth, numerous studies demonstrate that granulosa cell differentiation is altered by treatment with EGF, TGFα, or TGFβ. Treatment of granulosa cells with EGF or TGFα suppressed the ability of FSH to stimulate aromatase activity in cells from human (22) and rat follicles (23, 24). Progesterone production in response to treatment with EGF or TGFα is suppressed in bovine granulosa cells (25), suppressed or stimulated in rat granulosa cells (24, 26, 27), and increased in human granulosa-luteal cells (28). Treatment of granulosa cells with TGFβ generally appears to have opposite effects from those observed with TGFα. TGFβ augments the actions of FSH in promoting estrogen production (21, 29–31) and the induction of receptors for LH in rat granulosa cells (32, 33). Treatment of porcine granulosa cells with TGFβ suppresses the ability of FSH to stimulate progesterone production (19). Collectively, these results demonstrate that the responses to these growth factors differ among the various species. Additional research will be required to fully understand the regulatory roles that these growth factors have in follicular development.

In contrast to the numerous studies that have investigated the regulatory roles that TGFα and TGFβ have in growth and differentiation of granulosa cells, limited data are available on the effects that these growth factors have on thecal cell proliferation and differentiation. The ability of TGFα to stimulate thecal cell growth has been reported in cells isolated from bovine follicles (7), and the effects of EGF on steroidogenesis have been studied in porcine thecal cells (34) and rat thecal-interstitial cells (30, 35). Studies concerning the effects of TGFβ on thecal cell differentiation have also been limited and have been conducted primarily on cells from rats (30, 36, 37) and pigs (34). Since limited information is available on the effects that TGFα and TGFβ have on regulation of thecal cell proliferation, a major objective of the present study was to investigate the effects that these growth factors have on the proliferation of bovine thecal cells during antral follicle development. Another objective of the present study was to investigate the effects that TGFα and TGFβ have on bovine thecal cell steroidogenesis, which is a monovulatory species, as opposed to previous research in rats and pigs, which are polyovulatory.

**Materials and Methods**

**Cell preparation and culture conditions**

Follicles ranging in diameter from approximately 2–20 mm were dissected from bovine ovaries that had been collected on ice at an abattoir. Healthy nonatretic follicles were selected based on previously defined criteria (38, 39). Follicular fluid was aspirated, and follicles were bisected. Granulosa cells were removed by scraping the follicle wall with a fine plastic loop and flushing the wall back and forth in a small beaker containing Ham's F-12. Thecal interna layers were then microdissected away from the follicle wall and enzymatically dispersed (40). Dispersed thecal cells were centrifuged for 4 min at 50 × g, resuspended in culture medium, and plated in either 24- or 48-well culture plates containing 1 or 0.5 ml Ham's F-12, respectively. All cell cultures were maintained at 37 °C in a 5% CO₂ atmosphere. In experiments in which androstenedione and progesterone production was evaluated, cells were plated and maintained in serum-free medium that contained no treatment (control), hCG (1 μg/ml), and/or estradiol (E₂; 1 μM) in the
in cultures treated with growth factor to that in cultures not treated with growth factor. Separate analyses were performed for each growth factor within each hormone treatment (i.e., control, hCG, E2, and hCG plus E2). For the sake of clarity and conciseness, the least squares means and SEM of the steroid accumulation data have been presented.

Results

The ability of TGFα and TGFβ to alter the growth of thecal cells isolated from small, medium, and large follicles was evaluated by analyzing [3H]thymidine incorporation into DNA and DNA content per culture well. Treatment of thecal cells with TGFα stimulated DNA synthesis and cell division, as indicated by increases in the quantity of DNA per culture well. In contrast, the stimulatory effect that TGFα had on growth, treatment with TGFβ resulted in a suppression of thymidine incorporation compared to that in nontreated controls (Fig. 1). This suppressive effect of TGFβ on thymidine incorporation was not paralleled by a decrease in DNA content below that observed in nontreated controls when treatment of cultures with TGFβ was extended to 4 days (Fig. 2). Treatment of thecal cells with a combination of TGFα and TGFβ revealed that TGFβ suppressed the ability of TGFα to stimulate growth. Thymidine incorporation in cells treated with TGFα and TGFβ was similar to that in cells treated with TGFβ alone (Fig. 1), demonstrating that TGFβ com-

Growth assays

Growth was analyzed by quantitating [3H]thymidine incorporation into newly synthesized DNA and changes in DNA content of individual cultures. In experiments in which thymidine incorporation was evaluated, thecal cells were plated in Ham's F-12 containing 0.5% CS, and 18–24 h after plating, cells were treated with no growth factor (control), 2.5 ng/ml TGFα, 2.5 ng/ml TGFβ, or a combination of 2.5 ng/ml TGFα and 2.5 ng/ml TGFβ. Approximately 40 h after plating, the culture medium was replaced with DMEM containing 0.1 μCi [3H]thymidine. Cells were incubated for 4 h, and then medium was removed, and the quantity of [3H]thymidine incorporated into DNA was determined, as previously described (41). Experiments designed to evaluate changes in DNA were conducted by plating cells in Ham's F-12 containing 10% CS for 18–24 h, and then medium was changed to 0.1% CS. Either 24 or 48 h after plating, cells were treated with no treatment (control), TGFα, and/or TGFβ for a duration of 4 days. Medium was then removed, and the DNA content per culture well was determined using an ethidium bromide procedure, described previously (40).

Steroid assays

In experiments in which steroid production was analyzed, medium was removed from cells at the end of the culture period and centrifuged to remove cellular debris. Medium was then stored frozen until concentrations of androstenedione and progesterone were determined by RIAs (40, 42). These assays are specific and show no detectable cross-reactivity with estrogen (40, 42). All steroid concentrations were normalized to the quantity of DNA per culture well at the time of medium collection.

Statistical analysis

All data were analyzed by a SAS program, as described by Freund and Littell (43). Effects of growth factor treatment on thymidine incorporation and DNA were analyzed by an analysis of variance procedure for a block design, using experimental replicate as the blocking factor. Pairwise comparisons were made using a least significant difference procedure when treatment effect was significant (P < 0.05) in the analysis of variance procedure. The effects of follicle size on thecal cell response to TGFα and TGFβ were analyzed by a general linear model procedure, which included experimental replicate as a blocking factor. A paired t test was used to compare steroid accumulation in cultures treated with growth factor to that in cultures not treated with growth factor. Separate analyses were performed for each growth factor within each hormone treatment (i.e., control, hCG, E2, and hCG plus E2). For the sake of clarity and conciseness, the least squares means and SEM of the steroid accumulation data have been presented.
FIG. 2. Effects of TGFα and TGFβ on cell proliferation of thecal cells from small, medium, and large antral bovine follicles. Cells were cultured at approximately 3–5 μg DNA/ml well in the absence of growth factors for 48 h, then treated for 4 days, followed by an analysis of micrograms of DNA per well. Data are presented as the mean ± SEM from five different experiments, each run in replicate, and are expressed as fold increase in DNA content over that in control nontreated cells. Different superscript letters denote a statistical difference from control nontreated cells and each other (P < 0.05).

Completely suppressed the stimulatory effects of TGFα on thymidine incorporation. However, longer treatment with the combination of TGFα and TGFβ resulted in DNA levels that were intermediate between those in cultures treated with TGFα or TGFβ alone (Fig. 2). Similar results were obtained with cell proliferation assays (alterations in DNA levels) when cultures were terminated earlier on day 3 or 4 of culture; however, the magnitude of the TGFα response was less. Comparison of the responses of thecal cells isolated from follicles at different stages of development to TGFα or TGFβ revealed no major differences in either thymidine incorporation (Fig. 1) or DNA quantity (Fig. 2), providing evidence that thecal cells from antral follicles of different sizes respond similarly. These findings support a mitogenic role for TGFα in the bovine follicle and demonstrate that TGFβ may suppress the mitogenic actions of TGFα.

To further evaluate the potential roles that TGFα and TGFβ may have in modulating follicular development, the effects of these growth factors on thecal cell steroidogenesis were investigated. Since previous studies have demonstrated that thecal cell steroidogenesis can be altered by treatment with hCG and/or E2 (44), it was also of interest to investigate the interactions that TGFα and TGFβ have with these hormones. Initial experiments revealed that androstenedione production in response to TGFα and TGFβ was similar in thecal cells isolated from the different sized follicles. Therefore, thecal cells from all sizes of antral follicles were combined and used in these studies. Treatment of thecal cells with TGFα during the first 3 days of culture resulted in a small suppression of androstenedione (Fig. 3) and progesterone (Fig. 4) accumulation in culture medium. Treatment of thecal

FIG. 3. Effects of TGFα and TGFβ on thecal cell androstenedione accumulation when cells were cultured in the absence (control) or presence of hCG (1 μg/ml), E2 (1 μM), and hCG plus E2 from days 0–3 of culture. Cells were cultured at approximately 1–5 μg DNA/ml well, and steroid production in control cultures was between 1–2 ng androstenedione/μg DNA. Data are presented as the least squares mean ± SEM from five to seven different experiments, each performed in replicate, and are expressed as fold increase over values in control cultures after calculation of nanograms of androstenedione per μg thecal cell DNA. The star denotes a statistical difference from cells not treated with TGF (□) within a treatment group (P < 0.05).

FIG. 4. Effects of TGFα and TGFβ on thecal cell progesterone accumulation when cells were cultured in the absence (control) or presence of hCG (1 μg/ml), E2 (1 μM), and hCG plus E2 from days 0–3 of culture. Cells were cultured at approximately 1–5 μg DNA/ml well, and steroid production in control cultures was between 3–4 ng progesterone/μg DNA. Data are presented as the least squares mean ± SEM from five to seven different experiments, each performed in replicate, and are expressed as fold increase over values in control cultures after calculation of nanograms of progesterone per μg thecal cell DNA. The star denotes a statistical difference from cells not treated with TGF (□) within a treatment group (P < 0.05).
cells with TGFα also decreased androstenedione accumulation in cultures stimulated with hCG, E2, or a combination of the two hormones (Fig. 3). Although treatment of thecal cells with hCG alone did not alter progesterone accumulation during the first 3 days of culture, treatment with TGFα in the presence of hCG resulted in suppression of progesterone accumulation similar in magnitude to that observed in cultures treated with TGFα alone (Fig. 4). As in previous studies (44), E2 suppressed progesterone accumulation. No additional suppression of progesterone accumulation was observed in cells treated with both E2 and TGFα compared to that in cultures treated with E2 alone (Fig. 4).

The effects of TGFβ on steroidogenesis were different from those of TGFα. Neither basal nor hormonally stimulated accumulation of androstenedione during days 0–3 of culture was altered by treatment with TGFβ (Fig. 3). Accumulation of progesterone was increased in thecal cell cultures treated with TGFβ in the absence of other hormone treatments, and no significant effect of TGFβ was observed in cultures in which progesterone production was suppressed by treatment with E2 (Fig. 4).

In addition to evaluating the effects of TGFα and TGFβ on progesterone production during the initial 3 days of culture, as reported above, evaluations were also carried out on longer term cultures, since previous studies demonstrated that thecal cells undergo a change in differentiation when cultured longer than 3 days (40, 44). The change in differentiation is characterized by a dramatic decline in androgen production and thecal cell production of progesterone becomes responsive to stimulation by gonadotropin treatment. Treatment with TGFα or TGFβ for a 6-day period caused a slight increase in the quantity of progesterone accumulated during days 3–6 of culture, but was not statistically significant (Fig. 5). Similar results were observed in cultures in which E2 was present (Fig. 5). In the presence of hCG or a combination of hCG and E2, both TGFα and TGFβ significantly reduced the ability of hCG to increase progesterone accumulation during days 3–6 of culture (Fig. 5). These results indicate that thecal cell responsiveness to TGFα and TGFβ changed with time in culture.

Discussion

Results from this study provide evidence that TGFα and TGFβ have the potential to regulate follicle development by altering thecal cell proliferation and steroidogenesis. Findings from the thymidine incorporation and DNA studies demonstrate that TGFα stimulates thecal cell proliferation, and TGFβ suppresses this stimulatory effect. Although previous research has demonstrated that TGFα stimulates bovine thecal cell growth (7), the present study is the first to examine the inter-actions that TGFα and TGFβ have on thecal cell proliferation. The ability of TGFβ to suppress EGF-stimulated growth of bovine granulosa cells has also been reported (6). Since TGFα and EGF both mediate their actions through a common receptor (9), the results in bovine granulosa cells appear to be very similar to those in bovine thecal cells. The inverse actions observed for TGFα and TGFβ provide a mechanism by which follicle growth may be regulated.

It is not clear from the present study whether TGFβ suppresses thecal cell proliferation or if TGFβ acts only to suppress the ability of TGFα to stimulate proliferation. Short term treatment with TGFβ suppressed thymidine incorporation below that in nontreated cultures, but after longer treatment periods DNA levels were similar in untreated and TGFβ-treated cultures. In both types of assays, TGFβ suppressed TGFα stimulation; however, in the cell proliferation assay (alterations in DNA levels), TGFβ did not completely suppress TGFα stimulation. The conditions used for the two assays were different with thymidine studies terminated on day 3 and DNA assays on day 6. The alteration in the apparent thecal cell differentiation during this culture period, previously noted (40, 44), could be a variable to consider, but similar data were obtained when DNA assays were terminated on day 3 or 4 of culture. Another possible explanation for the inability of TGFβ to completely suppress TGFα actions on day 6 may be due to the ability of TGFβ to alter extracellular matrix formation, which enhances thecal cell survival during longer cultures. Support for this hypothesis is provided by previous research which demonstrated that TGFβ stimulates formation and prevents degradation of extracellular matrix in other
cell types (45, 46). Additional research will be required to determine the extent that TGFβ is capable of suppressing thecal cell proliferation or if TGFβ only modulates the ability of TGFα to stimulate proliferation.

The observation that thecal cells isolated from different sized follicles responded similarly to treatment with TGFα and TGFβ indicates that the mechanisms involved in regulating the expression and production of these factors may be more important than regulation of cellular receptors for these factors. However, it has been shown by immunohistochemical staining that TGFα is first detected in bovine preantral follicles after interstitial cells differentiate into thecal cells, and staining persists in the thecal cell component of follicles throughout follicle development (8). These results provide evidence that TGFα is available and can act as a mitogenic factor throughout follicle development. Since expression and production of TGFα have not been studied in atretic follicles, one hypothesis is that discontinued production of this protein may be important in bringing about the demise of follicles which undergo the process of atresia. An alternative hypothesis is that TGFα is expressed and produced throughout follicle development in the bovine, but that expression and production of TGFβ are differentially regulated, such that TGFβ could inhibit TGFα stimulation of granulosa and thecal cell proliferation at different times during follicular development, which would suppress follicular development or result in follicular atresia. It has been reported that FSH suppresses the expression and production of TGFβ2 in rat granulosa cells (14). Thus, one possible mechanism by which FSH may stimulate follicle growth may be by decreasing intracellular production of TGFβ, which would allow granulosa and thecal cells to proliferate in response to TGFα. Since the thecal cell component of the follicle appears to be a primary source for TGFβ (12, 37) and TGFα (7, 8, 11), interactions between granulosa and thecal cells will undoubtedly be important in the regulation of follicle growth, just as the interactions between these cells have been shown to be important for steroidogenesis (44, 47, 48). Current research directed at evaluating the regulation of expression and production of these and other growth factors will add new insight into the mechanisms involved in regulating follicular development.

In addition to the effects that TGFα and TGFβ had on thecal cell proliferation, these growth factors also altered thecal cell steroidogenesis. TGFα suppressed basal and hormonally stimulated androstenedione and progesterone during days 0–3 of culture. Treatment of bovine granulosa cells with EGF has also been reported to suppress progesterone production (25). In the pig, EGF appears not to affect thecal cell production of androgens or progesterone, but does reduce basal and hCG-stimulated estrogen production (34). Treatment of rat thecal-interstitial cells with EGF reduces gonadotropin stimulation of androgen production (35). In contrast to the inhibitory effects observed for TGFα in the present study, TGFβ did not significantly alter androgen production by bovine thecal cells, but increased basal progesterone production during days 0–3 of culture. In rat thecal-interstitial cells, TGFβ reduced the ability of LH to stimulate androstenedione and androstenedione, but increased the quantity of progesterone and testosterone produced in response to LH (36, 37). Treatment of porcine thecal cells with TGFβ reduced androgen and progesterone production, but stimulated estrogen production (34).

Collectively, these studies demonstrate that thecal cell steroidogenesis is altered by TGFα and TGFβ. The effects of these growth factors on steroidogenesis are generally opposite each another and differ for the different species.

Culture of thecal cells for an additional 3-day period resulted in a change in the steroidogenic responses to TGFα and TGFβ. Instead of the opposite effects observed for TGFα and TGFβ on basal progesterone during days 0–3 of culture, both TGFα and TGFβ appeared to have stimulatory effects on basal progesterone production, but these growth factors reduced the quantity of progesterone produced in response to hCG stimulation during days 3–6 of culture. This change in thecal cell response to TGFα and TGFβ which occurs sometime after day 3 of culture is paralleled by other changes in thecal cell function. Thecal cell production of androgens dramatically declines after day 3 of culture, and thecal cell production of progesterone changes from relatively nonresponsive to hCG stimulation before day 3 to very responsive after day 3 of culture (40, 49). It is proposed that these changes are similar to those that occur during the process of luteinization (40, 49). Therefore, it is possible that the changes in the effects that TGFα and TGFβ had on thecal cell steroidogenesis after day 3 of culture may be indicative of those that would occur during luteinization.

Although data concerning changes in growth factor effects on thecal cells isolated from different stages of follicle development were not presented for the steroidogenic studies, no dramatic changes in thecal cell responsiveness to TGFα or TGFβ were observed in cells collected from small, medium, or large antral follicles. This observation is in agreement with the data obtained in the growth studies and provides additional evidence that thecal cell responsiveness to TGFα and TGFβ may remain relatively constant throughout antral follicle development. Collectively, the data from these studies demonstrate that TGFα may stimulate thecal cell proliferation, but androgen and progesterone may be inhibited during this process. In contrast, TGFβ may suppress the
ability of TGFα to increase thecal cell proliferation and at the same time promote progesterone production. Therefore, the effects of TGF on thecal cell differentiation (i.e. steroidogenesis) may be indirectly associated with effects on cell growth. The ability of TGFα to stimulate cell proliferation and put the cell into the growth cycle may reduce the differentiated state of the cell. This correlates with the suppressive effects TGFα has on steroidogenesis of thecal and granulosa cells in the bovine. TGFβ, however, inhibits growth and may promote cell differentiation, which correlates with the observation that TGFβ has stimulatory effects on thecal and granulosa cell steroidogenesis.

Observations indicate that thecal cells can produce and respond to TGFα and TGFβ. TGFα and TGFβ may act as autocrine factors for thecal cells and paracrine factors for granulosa cells. Therefore, the local production and action of these growth factors are postulated to play an important role in the regulation of ovarian follicle growth and differentiation. However, caution must be taken in the direct extrapolation of these in vitro data to in vivo follicle development.

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