0013-7227/87/1212-0786$02.00/0
Endocrinology

Vol. 121, No. 2
Printed in U.S.A.

# Ovarian Thecal Cells Produce Transforming Growth Factor-β Which Can Regulate Granulosa Cell Growth*

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**ABSTRACT.** Ovarian thecal cells in culture were found to synthesize and secrete transforming growth factor-β (TGFβ). A component in thecal cell-conditioned medium was immunologically similar to TGFβ, as assessed with a RIA, and inhibited specific binding of TGFβ to its cell surface receptors. Thecal cell-secreted proteins also contained TGFβ biological activity, which was determined by stimulation of soft agar colony formation by AKR-2B indicator cells. Specific TGFβ antibodies precipitated a 25 K protein from radiolabeled thecal cell-secreted protein that comigrated with purified platelet-derived TGFβ. Both bovine thecal cell and rat thecal/interstitial cell preparations produced TGFβ, which required acid treatment to obtain fully active samples. The physiological significance of TGFβ production by thecal cells was addressed through an analysis of the effects of TGFβ on bovine granulosa cell growth. TGFβ inhibited epidermal growth factor stimulation of granulosa cell growth, but alone it had no apparent influence. Observations indicate that ovarian thecal cells produce TGFβ, which can regulate granulosa cell growth and differentiation. Discussion of thecal cell-granulosa cell interactions and the possible functions of TGFβ in the ovary is presented. (*Endocrinology* **121:** 786–792, 1987)

A COMPLEX array of externally and locally derived regulatory agents is required for the maintenance of ovarian function (1). The somatic cell types in the ovarian follicle, thecal cells and granulosa cells, are the target cells for many of these regulatory agents. Cellular interactions between thecal cells and granulosa cells are postulated to have an integral role in the control of oocyte development. Granulosa cells provide the cytoarchitectural support for the oocyte, while thecal cells surround the follicle and are in contact with the outer layer of granulosa cells. A basement membrane is formed between the two cell types, which helps provide the structural integrity of the follicle. An additional cell-cell interaction between thecal and granulosa cells is mediated by steroids. Thecal cells produce testosterone, which is used by granulosa cells as a substrate for estrogen production (2). Therefore, thecal cell-granulosa cell interactions are important in the maintenance of ovarian function as well as the endocrine status of the animal.

One important aspect of ovarian function is the need for a rapid stimulation and inhibition of follicular growth. Granulosa cells are one of the most rapidly growing normal cell types known *in vivo*. The majority of developing follicles, however, undergo atresia, in which proliferation is stopped and the follicle degenerates. Therefore, the control of granulosa cell growth appears to be complex and requires factors that both stimulate and inhibit growth. Granulosa cells contain epidermal growth factor (EGF) receptors (3) and proliferate in response to EGF *in vitro* (4). Although a number of factors, including EGF, estrogen (5), fibroblast growth factor, and insulin-like growth factor (6), have been implicated in stimulating granulosa cell growth, inhibitors of granulosa cell growth have not been thoroughly investigated.

A protein that has both stimulatory and inhibitory effects on cell proliferation, as well as effects on cellular differentiation, is transforming growth factor-β (TGFβ) (7, 8). TGFβ generally inhibits the growth of epithelial cell types, particularly if they are responsive to EGF, and stimulates mesenchymal cell growth (7). This highly conserved protein is produced by many cell types (9) and acts via unique cell surface receptors (10, 11). Granulosa cells are an epithelial cell type that respond to EGF, therefore, it was postulated that TGFβ may influence granulosa cell growth. Recent observations that TGFβ can regulate granulosa cell steroidogenesis (12–14) support this hypothesis. The current study was designed to

Received February 12, 1987.
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* This work was supported by Mellon Foundation and NIH (HD-20922) grants (to M.K.S.) and a NCI grant (CA-42572 to H.L.M.).
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determine the possible local ovarian synthesis of TGFβ and investigate the action of TGFβ on granulosa cell growth.

## Materials and Methods

*Cell culture*

Immature female 20-day-old rats were treated daily for 4 days with 1 mg diethylstibestrol in 0.1 ml sesame oil. Rats were killed at 25 days of age. Granulosa cells were recovered from the ovaries by puncturing the follicles with a fine needle and cultured as previously described (15). Rat thecal interstitial cells were isolated from the remaining ovarian tissue after granulosa cell release. The tissue was chopped, and fragments were washed once and allowed to settle under gravity. The supernatant was discarded, and the remaining tissue was digested in a buffered salt solution containing 1 mg/ml collagenase and 5 μg/ml DNase (Sigma, St. Louis, MO) at 37 C for 45 min. Digested thecal sections were centrifuged at 50 × *g* for 5 min and further dispersed by passage through a Pasteur pipette. Remaining tissue sections were removed by sedimentation under gravity for 10 min. The supernatant containing small cell aggregates and single cells was centrifuged at 50 × *g* for 5 min, resuspended, plated in culture medium (Ham's F-12) containing 10% calf serum, and maintained at 37 C. After the cells had grown to confluence they were subcultured at 25% density and maintained in 10% calf serum. After confluent rat thecal/interstitial cell subcultures were obtained they were washed three times with serum-free medium over 8 h, whereupon fresh medium was added, and 48 h conditioned medium collections were commenced. Conditioned medium was centrifuged at 5000 × *g* for 20 min and frozen at −20 C. When designated, conditioned medium was concentrated by ultrafiltration.

Bovine ovaries were collected at a local abattoir from heifers less than 10 min after slaughter. Under sterile conditions the dominant follicle was removed from the ovary, cleaned of adhering interstitial tissue, and punctured by a hypodermic needle to drain out the follicular fluid. The follicle was flushed several times and cut into hemispheres, and gently scraped with a fine wire loop to remove the granulosa cells. The granulosa cells were centrifuged at 50 × *g* for 5 min, decanted, gently agitated with a Pasteur pipette, and resuspended in culture medium. After removal of the granulosa cells, layers of pure thecal cells were microdissected away from the remaining follicle. These thecal cell layers were then cleaned of adhering granulosa and interstitial cells, digested with collagenase and DNase for 1 h at 37 C, centrifuged, resuspended in medium, and plated in 10% calf serum. After the primary bovine thecal cell cultures had grown to confluence they were subcultured at 25% density and grown to confluence again, generally requiring 72 h. Serum was washed from the cells, and serum-free medium was collected every 48–72 h for 2 weeks. Medium was processed as described above.

*TGFβ RIA*

The RIA (Keski-Oja, J., R. M. Lyons, and H. L. Moses, manuscript submitted for publication) was carried out in 1.5-ml Eppendorf tubes in 1 ml PBS, pH 7.4, containing 0.1 mg/ml BSA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 100 U/ml aprotinin (Sigma), and 0.1% Triton X-100. The incubations were carried out for 2 h at room temperature, and the precipitated radioactivity was collected by adding 25 μl 50% (vol/vol; in PBS) protein A-Sepharose (Pharmacia, Upsala, Sweden) and incubating at room temperature with gentle rocking for 1 h. The Sepharose was washed four times with 1-ml aliquots of 10 mM Tris-HCl buffer, pH 7.5, containing 0.1% sodium deoxycholate, 0.1% Triton X-100, and 1% aprotinin and 1 mM PMSF as protease inhibitors. Titration of the anti-TGFβ antiserum (no. 278) indicated that a 1:2000 dilution was able to precipitate 50% of the immunoreactive radioiodinated TGFβ (curve not shown). Preimmune serum from the same animal gave no reactivity in the assays. This assay has a sensitivity of 0.1 ng TGFβ with a linear displacement up to 10 ng TGFβ. The inter- and intraassay coefficients of variation were 15% and 5%, respectively. The antibody raised against native porcine TGFβ did not cross-react with any other protein tested (16).

*TGFβ RRA*

TGFβ from human and porcine platelets was purified as previously described (17) with the addition of a final purification step consisting of reverse phase $C_{18}$ HPLC and used in a RRA previously described (7, 10). TGFβ was radioiodinated, and binding assays were carried out using dishes at a density of $1 \times 10^5$ to $2 \times 10^5$ AKR-2B cells/well. After 24 h, the cells were washed with binding buffer (Dulbecco's PBS, pH 7.4, containing 0.1% BSA and 5 mM $MgCl_2$). Then, 1 ml binding buffer containing 0.2 ng $^{125}$I-labeled TGFβ (15,000 cpm) with various levels of competitors was added to each well. Nonspecific binding was determined in the presence of 1 μg 50% pure unlabeled TGFβ. After 2 h of incubation at room temperature the cells were washed three times with binding buffer, and the bound radioactivity was extracted with PBS containing 0.5% Triton X-100.

*Immunoprecipitation, electrophoresis, and fluorography*

Cultures of rat thecal/interstitial cells or bovine thecal cells were radiolabeled for 48 h with 2 μCi/ml [$^{35}$S]cysteine (1000 Ci/mmol; New England Nuclear, Boston, MA) in cysteine-free medium. Radiolabeled conditioned medium was then collected, centrifuged at 5000 × *g* and frozen at −20 C. Aliquots of radiolabeled secreted proteins were incubated with 10 or 25 μl antisera to TGFβ or normal rabbit serum as a control. After an 18-h incubation at 4 C, 30 μl protein A-Sepharose (50%, vol/vol; in PBS) or 60 μl Pansorbin (Calbiochem, La Jolla, CA) beads were added. The samples were then incubated on a rotary shaker for 2 h. The precipitates were washed three times with 1-ml aliquots of 40 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, and 1 mM PMSF and once with PBS. The bound material was then dissolved in Laemmli's sample buffer. Electrophoresis was performed on 5–15% polyacrylamide gradient slab gels with the use of the Laemmli SDS buffer system (18). Samples were run nonreduced, and gels were fluorographed using diphenyloxazole in acetic acid, as previously described (19).

*Growth assays*

Soft agar assays were performed using nontumorigenic mouse AKR-2B indicator cells. Agar plates were prepared in 35-mm petri dishes (Falcon, Oxford, CA) by first applying a 1-ml layer of 0.8% agarose in McCoy's Medium 5a containing 10% fetal bovine serum. After the bottom layer had solidified, a top layer of 1 ml containing 0.4% agarose in medium with 10% fetal bovine serum was added along with $7.5 \times 10^3$ AKR-2B cells and the appropriate concentrations of TGFβ or thecal cell secreted proteins as described below. The plates were incubated at 37 C in a humidified atmosphere of 5% $CO_2$ for 7–14 days. Colonies were quantitated using a Bausch and Lomb Omnicon Feature Analysis Stem model II (Artec, NY). Colonies greater than 60 μm were scored as positive.

Bovine granulosa cells were plated at 25% confluence in 24-multiwell culture plates and incubated for 24 h in 1 ml culture medium containing 10% calf serum, followed by 72 h in culture medium containing 0.1% calf serum. Then, the cells were treated for 18 h with various concentrations of growth regulators. The medium was replaced with 0.5 ml culture medium containing 1 μCi [$^3$H]thymidine, and the cells were incubated for 4 h. The amount of [$^3$H]thymidine incorporated into DNA was then determined, as previously described (20).

## Results

A RIA for TGFβ had an effective displacement curve between 0.1 and 10 ng TGFβ (Fig. 1). Serum-free rat thecal/interstitial cell-conditioned medium was concentrated by ultrafiltration with a 10,000 mol wt exclusion limit membrane (~100-fold) and will be subsequently referred to as thecal/interstitial cell secreted proteins (TISP). Bovine thecal cell-conditioned medium was prepared in a similar manner and will be referred to as thecal cell secreted proteins (TSP). Rat TISP contained detectable amounts of TGFβ immunoreactivity and had a parallel displacement curve in the TGFβ RIA (Fig. 1). Bovine TSP gave similar results (data not shown).

The production of TGFβ by thecal cells was also determined with a TGFβ RRA. Rat TISP efficiently inhibited the binding of [$^{125}$I]TGFβ to its cell surface receptors (Fig. 2). Acid treatment of TISP at pH 1.5 for 1 h before neutrilization generated an increased amount of TGFβ competing activity in the RRA (Fig. 2) and RIA (data not shown). Bovine TSP had approximately an equivalent amount of activity in the RRA, with 54% displacement with 14 μg protein. As a control, concentrated bovine granulosa cell-conditioned medium was assayed and was found to have less reactivity in the TGFβ RRA (8% displacement with 11 μg protein).

The presence of TGFβ biological activity was analyzed with a soft agar growth assay. TGFβ stimulates colony formation of AKR-2B indicator cells in the soft agar assay, as shown in Fig. 3. Bovine TSP also significantly stimulated colony formation (Fig. 3). Acid treatment of TSP increased the ability of lower concentrations of TSP to stimulate colony formation. An acid enhancement of the soft agar growth with the higher concentration of

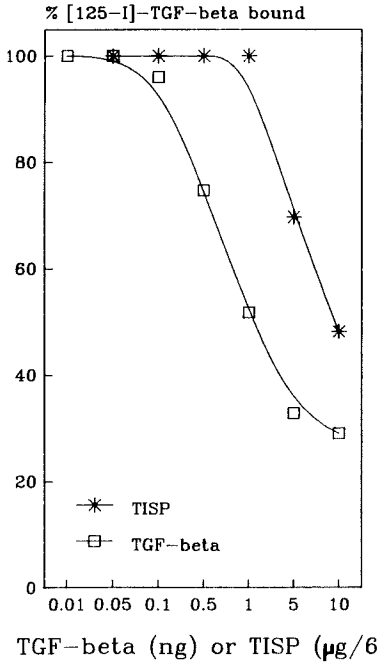


FIG. 1. Displacement curves of [$^{125}$I]TGFβ in the TGFβ RIA. Increasing concentrations of unlabeled TGFβ or rat TISP were used to displace [$^{125}$I]TGFβ in the RIA. Results are expressed as [$^{125}$I]TGFβ bound.

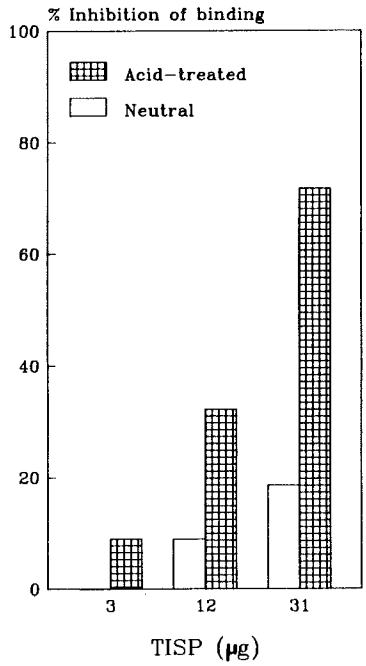


FIG. 2. TGFβ RRA of rat TISP. Increasing concentrations of TISP were used and were treated under neutral or acid conditions. The percent competition of [$^{125}$I]TGFβ binding to the cells was determined. Values are the mean of triplicate determinations from two separate experiments, with a coefficient of variation between 5–20% for each value.

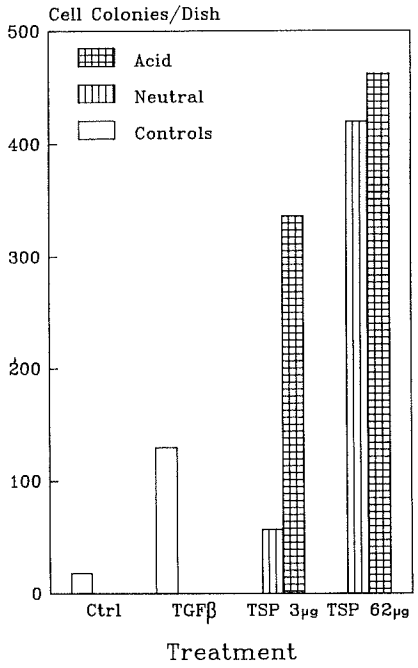


FIG. 3. Soft agar growth assay of bovine TSP and TGFβ. The number of cell colonies per dish was determined for the different treatments: untreated control (Ctrl); 1 ng TGFβ; and 3 or 62 μg TSP treated under neutral or acid conditions. Values are the mean of triplicate determinations, with a coefficient of variation between 10–15% for each value.

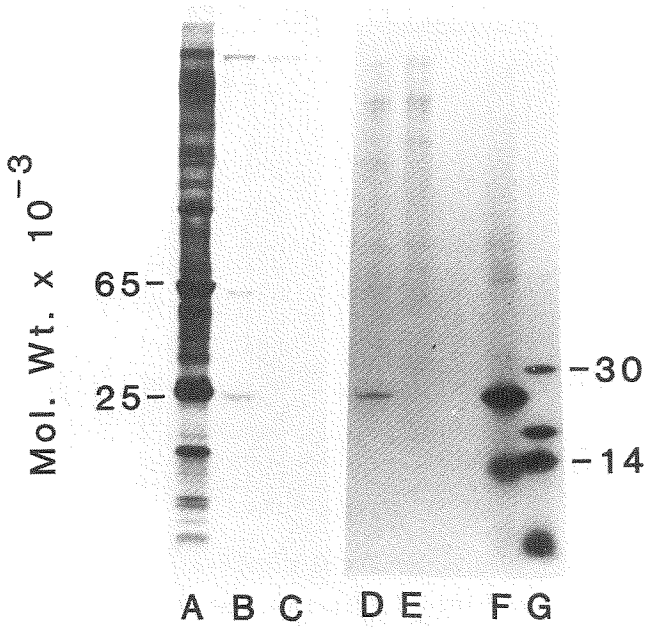


FIG. 4. Fluorograph of an electrophoretic profile of radiolabeled TSP and TGFβ immunoprecipitations. The lanes contain the following samples: A, bovine TSP; B, TGFβ immunoprecipitate of bovine TSP; C, nonimmune (rabbit) serum precipitate of bovine TSP; D, TGFβ immunoprecipitate of rat TISP; E, nonimmune (rabbit) serum precipitate of rat TISP; F, iodinated platelet-derived TGFβ (dimeric 25K and monomeric 12.5K forms); and G, $^{14}$C-labeled mol wt markers.

TSP was not as predominant, presumably due to the supraoptimal dose. The results obtained with the soft agar growth assay indicate that TSP contains TGFβ-like biological activity.

Several factors make an accurate determination of the amount of TGFβ in TSP difficult, including loss of material during the concentration procedure and variable levels of acid activation. A minimum estimate of 0.8 ng TGFβ/μg TSP is made with the data from the RIA, RRA, and colony formation assay. In contrast, a minimum of 0.2 ng TGFβ/μg granulosa cell secreted proteins is estimated from the RRA.

To determine active synthesis and secretion of TGFβ by thecal cells, radioactive [$^{35}$S]cysteine was incorporated into TSP and TISP. Immunoprecipitations were performed on radiolabeled TSP with immunoglobulin G purified from a TGFβ antiserum. Precipitation of rat TISP after acid treatment and neutralization revealed a single 25,000 mol wt protein that comigrated with authentic TGFβ (Fig. 4). The TGFβ antibody precipitated both 25,000 and 62,000 mol wt proteins from bovine TSP (Fig. 4). These observations indicate that both rat thecal/interstitial cells and bovine thecal cells synthesize and secrete TGFβ.

The physiological significance of TGFβ production by thecal cells was investigated through an analysis of the effects of TGFβ on bovine granulosa cell growth. The effect of growth regulators on [$^{3}$H]thymidine incorporation into granulosa cell DNA was determined. As previously demonstrated, EGF stimulated granulosa cell proliferation (Fig. 5). After an 18-h treatment, EGF stimulated thymidine incorporation into granulosa cell DNA 75% above control levels, which was statistically significant. This stimulation correlated to a corresponding increase in cell number after a 48-h treatment with EGF (data not shown). TGFβ alone had no influence on granulosa cell growth at various concentrations (5–25 ng/ml). However, TGFβ in combination with EGF inhibited the ability of EGF to stimulate [$^{3}$H]thymidine incorporation into granulosa cell DNA (Fig. 5). In Fig. 5 are shown the effects of an 18-h treatment with growth regulators on a synchronized bovine cell culture, followed by a 4-h incubation with [$^{3}$H]thymidine and a determination of counts per min of [$^{3}$H]thymidine per μg DNA. In experiments where [$^{3}$H]thymidine was coincubated with the growth regulators for 18 h an increase in [$^{3}$H]thymidine incorporation was observed which appears to be due to altered thymidine metabolism and not related to the growth of granulosa cells (data not shown).

## Discussion

Secreted proteins were prepared from both rat thecal/interstitial cell and bovine thecal cell preparations. Rat thecal/interstitial cell preparations contain thecal cells

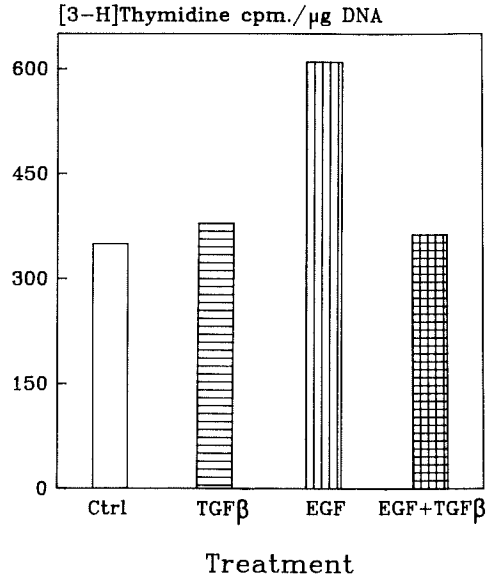


FIG. 5. Effects of TGFβ and EGF on bovine granulosa cell growth. The amount of [$^3$H]thymidine incorporated per μg granulosa cell DNA was determined after treatments with 50 ng/ml EGF, 5 or 25 ng/ml TGFβ, and a combination of EGF (50 ng/ml) and TGFβ (5 or 25 ng/ml) and compared to the control value (Ctrl). Values are the means of triplicate determinations from three experiments, with a coefficient of variation between 10–15% for each value.

and interstitial cells, such as fibroblasts and endothelial cells, that may contribute to the presence of TGFβ in TISP. Therefore, a highly purified population of bovine thecal cells was obtained to provide more direct evidence that thecal cells synthesize and secrete TGFβ. The use of TISP indicates local ovarian production of TGFβ, while the use of bovine TSP indicates production by thecal cells. Both rat TISP and bovine TSP preparations were positive with all methods used to detect TGFβ production.

Preparations of bovine TSP and rat TISP were found to contain TGFβ immunoreactivity. Parallel displacement curves for TSP, TISP, and TGFβ in the RIA demonstrate similar immunological reactivity. TSP was also found to compete in a TGFβ RRA. These observations imply that thecal cells secrete a protein that is immunologically similar to TGFβ and can bind to the TGFβ receptors. The presence of TGFβ biological activity was demonstrated with a soft agar growth assay. Bovine TSP significantly stimulated colony formation on soft agar. Previously it has been shown that acid treatment of TGFβ preparations converts an apparent precursor or inactive form of TGFβ to an active form (7). Acid treatment of TSP and TISP was also found to increase the amount of reactivity in all assays used. Therefore, activation of an inactive form of TGFβ appears to be required for the TGFβ produced by thecal cells. TGFβ can also be activated proteolytically by plasmin (Lyons, R. M., J. Keski-Oja, and H. L. Moses, manuscript submitted for publication). Therefore, plasminogen activator production by granulosa cells may play a role in activating the locally produced TGFβ.

Direct demonstration of the active synthesis and secretion of a protein by a given cell type requires radioactive amino acid incorporation into protein. Therefore, immunoprecipitation of radiolabeled secreted proteins with TGFβ antisera was performed. The TGFβ antibody specifically precipitated a 25K protein from rat TISP and 25K and 62K proteins from bovine TSP. The 25K proteins comigrated with authentic TGFβ. The 62K protein is frequently observed in immunoprecipitates using the anti-TGFβ antiserum (Keski-Oja, J., R. M. Lyons, and H. L. Moses, manuscript submitted), and its relationship to TGFβ remains to be established.

To further understand the possible physiological significance of TGFβ in the ovarian follicle, the production of TGFβ by granulosa cells was also analyzed. Both rat and bovine granulosa cell secreted protein preparations contained small amounts of TGFβ competing activity in the RRA. The levels produced were less than those produced by thecal cells. The molecular form(s) and the biological activity of the TGFβ produced by granulosa cells remain to be investigated. Further characterization of the TGFβ produced by granulosa cells will be required to determine the relative amounts produced. The production of TGFβ by granulosa cells will need to be considered in an examination of the physiological significance of TGFβ production in the ovary.

TGFβ is a versatile protein that has both growth stimulatory and inhibitory activities as well as an ability to influence the differentiation of cells (8). The granulosa cell within the ovarian follicle is a rapidly proliferating cell type that probably requires growth stimulators as well as growth inhibitors. Inhibition of granulosa cell growth will be required in primordial and atretic follicles. The effect of TGFβ on granulosa cell proliferation was examined to determine the possible function of TGFβ production by thecal cells. As found with most EGF-responsive epithelial cell types, TGFβ inhibited EGF-stimulated growth-promoting effects. TGFβ alone had no effect on granulosa cell growth. Therefore, one possible role for TGFβ in the follicle may be the inhibition of granulosa cell growth. The combined effects of a locally produced EGF-like activity and TGFβ may provide a mechanism to regulate granulosa cell growth. Analysis of the levels of TGFβ produced at different stages of follicle development will be informative.

In addition to the effects of TGFβ on granulosa cell growth, recent observations indicate an influence on granulosa cell function and differentiation. With the observation that inhibin, isolated from follicular fluid, has some homology with TGFβ (21), an analysis of the effects of TGFβ on granulosa cell function was initiated

(12). TGFβ has been shown to act synergistically with FSH to promote aromatase activity in granulosa cells (12, 13). TGFβ alone does not have an effect, but appears to augment the actions of FSH. In addition, TGFβ can regulate EGF receptor formation on granulosa cells (14) and induce LH receptor expression (22). Therefore, locally produced TGFβ may play a role in regulating both granulosa cell growth and differentiation. It is speculated that the growth inhibition induced by TGFβ may promote the expression of a steroidogenic (differentiated) state of the granulosa cell which allows the cell to be more responsive to regulators such as FSH. Further examination of the differential effects of TGFβ on granulosa cell growth and differentiation will provide insight into the physiological role of TGFβ in the ovary.

Production of TGFβ by thecal cells may result in a paracrine type of cellular interaction to regulate granulosa cell growth and differentiation. Steroid-mediated cellular interactions have previously been shown to act as paracrine factors between thecal and granulosa cells (1, 2). The possible regulation of granulosa cell growth by thecal cell products was previously postulated from the observation that thecal cell homogenates contain potential growth regulators for granulosa cells (23). Thecal-granulosa cell interactions provide an example of a mesenchymal-epithelial cell interaction. With the use of many different types of tissues it has been shown that mesenchymal cells appear to produce inducer substances that can modulate the function, growth, and differentiation of the adjacent epithelial cells (24). Therefore, TGFβ may be a possible paracrine factor involved in mesenchymal-epithelial cell interactions between thecal and granulosa cells. However, in addition to a paracrine role for TGFβ in the ovary, an autocrine role may be present. TGFβ produced by thecal cells may act as an autocrine factor to control thecal cell growth and differentiation. In addition, TGFβ produced by thecal cells may also influence interstitial cells. Although low levels of TGFβ were found to be produced by granulosa cells, the TGFβ produced may act as an autocrine factor for granulosa cells. Therefore, TGFβ production by both thecal and granulosa cells may provide a paracrine and/or autocrine factor to influence cellular growth and differentiation in the ovary. The current study demonstrates the local production of TGFβ by thecal cells and indicates that TGFβ can act as a growth inhibitor for granulosa cells. Further investigation into the relative levels of TGFβ produced by the different cell types and analysis of the production at different stages of development will elucidate the sites of action and physiological functions of locally produced TGFβ in the ovary.

## Acknowledgments

We thank Bryon Glenn, Lynn Black, and Mary Aakre for technical assistance; Mary Couey, Loretta Cheairs, and Trish Krabbendam for assistance in preparation of this manuscript; and Dr. R. M. Lyons for iodinated TGFβ. We also acknowledge the Vanderbilt University Reproductive Biology Research Center Tissue Culture Laboratory for the EGF, and Baltz Brothers Meat Packing Co. (Nashville, TN) for the bovine ovarian tissue.

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