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Regulation of Ovarian Cell Growth through the Local Production of Transforming Growth Factor-α by Theca Cells*

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ABSTRACT. The rapid proliferation of a tissue often requires the local production of a specific growth factor. The ovarian follicle is a rapidly growing tissue which contains two primary somatic cell types, granulosa cells and theca cells. Theca cells and granulosa cells were isolated from bovine ovaries and cultured to assess the possible local production of a growth factor within the ovarian follicle. Serum-free conditioned medium from theca cells, but not from granulosa cells, was found to contain a component that specifically bound to the epidermal growth factor (EGF) receptor. Therefore, theca cells appear to produce an EGF-like substance as a potential regulator of follicle cell growth. This result provides physiological significance to the previous observation that granulosa cells contain EGF receptors and respond to EGF to increase cell proliferation. Transforming growth factor-α (TGFα) is a protein that is structurally and functionally related to EGF and binds to the EGF receptor. Using a molecular probe to TGFα, theca cells were found to express the TGFα gene, which is consistent with the presence of an EGF-like substance in conditioned medium, but granulosa cells had no detectable TGFα gene expression. Similar analysis with a molecular probe to EGF demonstrated the apparent lack of EGF gene expression in theca cells or granulosa cells. As previously demonstrated with granulosa cells, the data presented indicate that theca cells also contain high affinity EGF receptors. TGFα was found to stimulate the growth of both granulosa and theca cells. These observations imply that within the ovarian follicle TGFα is produced by theca cells, which can subsequently have both a paracrine and an autocrine role in regulating follicle cell proliferation. Results presented demonstrate production of TGFα by a normal adult mesenchymal tissue and provide an example of a growth factor-mediated mesenchymal-epithelial cell interaction between theca cells and granulosa cells. (*Endocrinology* **123:** 2632–2638, 1988)

THE OVARIAN follicle is one of the most rapidly growing normal tissues known and requires a number of complex interactions with different growth factors and hormones (1, 2). Granulosa cells are the epithelial cells that make up the follicle and support the developing oocyte. The proliferation of granulosa cells is responsible for the majority of follicle cell expansion. Theca cells are the mesenchymal cells (*i.e.* stromal) that surround the follicle and contribute to the expansion of the follicle. Research dealing with growth factors and the ovary have primarily focused on factors that influence granulosa cell proliferation *in vitro*, including fibroblast growth factor (3), insulin-like growth factor (4), and epidermal growth factor (EGF) (3). High affinity EGF receptors have been shown to be present on granulosa cells (5). EGF can promote granulosa cell growth *in vitro* (3) and alter the hormonal regulation of steroidogenesis (1, 6). These observations have led to the proposal that EGF may have an important role in regulating ovarian cell growth and function (1). Because the circulating levels of EGF are negligible (7), studies have been designed to investigate the potential for the local production of an EGF-like substance in the follicle. Previously, a rat ovarian theca/interstitial cell preparation was found to produce an EGF-like substance with biochemical properties distinct from those of EGF (8).

Transforming growth factor-α (TGFα) is a protein that has structural homology with EGF, binds to the EGF receptor (9), and has similar, although not identical, biological activities as EGF (10). TGFα is a unique gene product that is produced as a precursor integral membrane protein which is processed into a soluble extracellular protein (11). TGFα was initially isolated from the conditioned medium of virally transformed fibroblasts (12) and has subsequently been shown to be produced by a large number of neoplastic cells (13). Cells of embryonic origin have also been shown to produce TGFα (14). These observations have led investigators to propose that TGFα may function during transformation as an autocrine

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growth factor (10). Recent reports have demonstrated that TGFα is produced by normal adult epithelial cell types, including bovine pituitary cells (15) and human keratinocytes (16). These results imply that TGFα may also be a growth regulator in normal adult tissue. Therefore, the current study examines the possibility that TGFα is produced locally in the ovarian follicle to participate in the regulation of cell proliferation.

Materials and Methods

Cell preparation and culture

Bovine ovaries were obtained from young nonpregnant cycling heifers less than 10 min after slaughter (Bio-Resources, Inc., Irving, TX). Ovaries are delivered fresh on ice in a buffered salt solution to the laboratory in less than 3 h after collection. The stage of the estrous cycle was determined morphologically, as previously described by Ireland *et al.* (17). Under sterile conditions the follicles were removed from the ovary, cleaned of adhering interstitial tissue, and punctured by a hypodermic needle to drain out the follicular fluid. Follicle diameter was determined, and 5- to 10-mm follicles were used. Healthy developing follicles are identified with the criteria previously established by Metcalf (18) for a vascularized pink theca externa and clear amber follicular fluid with no debris. The follicle was flushed several times, cut into hemispheres, and gently scraped with a fine plastic 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. Cells were plated in Ham's F-12 (Gibco, Grand Island, NY) in the absence of serum and maintained at 37 C in a 5% CO_2 atmosphere. Cells were plated at approximately 10^6 cells/2 cm^2, and medium was changed every 24–48 h. After removal of the granulosa cells, layers of pure theca cells were microdissected away from the remaining follicle. These theca 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 theca cell cultures had grown to confluence they were subcultured at a 25% confluent cell 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. Conditioned medium was centrifuged at 5000 × *g* for 20 min and frozen at −20 C. Conditioned medium was concentrated by ultrafiltration with a 3000 mol wt exclusion limit membrane (Amicon, Lexington, MA) approximately 100-fold.

EGF RRA and Scatchard analysis

An EGF RRA was established with a human fibroblast cell line, as previously described (19). Samples were incubated at 4 C for 4 h on human fibroblasts and [^{125}I]EGF binding was assessed. All data were obtained with both preincubation and coincubation conditions of sample with [^{125}I]EGF. Therefore, cells were either preincubated with sample before incubation with [^{125}I]EGF or coincubated with sample and [^{125}I]EGF to eliminate problems associated with possible proteolytic degradation of radiolabeled tracer.

The possible sequence of EGF receptors on cells was investigated with a Scatchard analysis using [^{125}I]EGF, as previously described (19, 20). Confluent cultures were incubated under serum-free conditions with increasing amounts of murine [^{125}I]EGF in the absence or presence of 200 ng nonradiolabeled EGF for 4 h at 4 C. The amount of [^{125}I]EGF specifically bound was determined and presented as a ratio of (bound/free) [^{125}I]EGF used in the incubation *vs.* the concentration of [^{125}I]EGF specifically bound (moles/liter).

Data obtained with both the RRA and Scatchard analysis were performed with the assistance of the Vanderbilt University Reproductive Biology Research Center, Tissue Culture Core Laboratory, directed by Dr. G. Carpenter.

RNA isolation and Northern analysis

Polyadenylated RNA was obtained from granulosa cells and theca cells. Cells were extracted with 5 M guanidine isothiocyanate, 10 mM EDTA, 50 mM Tris (pH 7.5), and 8% (vol/vol) β-mercaptoethanol, and then RNA was precipitated with 1 M LiCl for 18 h at −20 C. The RNA pellet was reconstituted in 1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.1 M NaCl, and 10 mM Tris-HCl and homogenized. Samples were then digested with proteinase-K and applied to an oligodeoxythymidine affinity column. Polyadenylated RNA was separated electrophoretically on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and analyzed with a Northern blot procedure previously described (21). The human TGFα cRNA probe was obtained from a cDNA that contained the complete coding sequence (22). The insert was subcloned into the plasmid SP65 in the antisense orientation with regard to the transcriptional direction of the SP6 promotor. The cRNA probe was labeled with [^{32}P]UTP as previously described (23). A human EGF cDNA probe (24) was labeled by a random primer extension method (25). A β-actin cDNA probe (26) in pBR322 was labeled by nick translation. A 700-basepair insert of plB15 (27), a rat cDNA that encodes cyclophilin, was subcloned into the plasmid SP65 promotor to produce a cRNA probe. β-Actin and 1B15, two genes that appear to be constitutively expressed, were used as control probes to demonstrate intact RNA. RNA was hybridized to the various probes at 65 C for 20 h (SP6 probes) (16, 28) or at 43 C for 40 h (nick-translated and primer-extended probes). Hybridization conditions were 50% deionized formamide, 0.75 M NaCl, 75 mM sodium citrate, 50 mM sodium phosphate (pH 6.5), 0.2% BSA, 0.2% Ficoll, 0.1% SDS, 0.2% polyvinylpyrrolidone, and 50 μg/ml sonicated denatured herring sperm DNA. Posthybridization washes [three 20-min washes in 0.1× standard saline citrate (0.15 M sodium chloride-0.015 M sodium citrate, pH 7.4)-0.1% SDS-1 mM EDTA] were performed at the same temperature as hybridization.

Cell growth assays

Granulosa and theca cells were plated at a 25% confluent cell density in 24-multiwell culture plates and incubated for 24 h in 1 ml culture medium containing 1% calf serum, followed by 48-h culture in medium containing 0.1% calf serum. 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 determined, as previously described (29). Alternatively, cells were cultured for 72 h in the presence of growth regulators, followed by a DNA assay to determine alterations in cell number.

DNA and protein assays

DNA was measured fluorometrically with ethidium bromide (30), as previously described (31). At the end of the culture period, the media were removed, ethidium bromide buffer (EBB, 20 mM sodium chloride, 5 mM EDTA, and 10 mM Tris, pH 7.5; Sigma, St. Louis, MO) was added to the wells, and the cells were sonicated. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 nM ethidium bromide and 100 U/ml heparin in EBB), then diluted 1:2 with EBB buffer and allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm was monitored with 350 nm excitation. A standard curve with calf thymus DNA was used to quantitate DNA levels in the culture wells. This assay has a sensitivity of approximately 0.1 μg DNA and is linear up to 2.5 μg DNA. Total protein concentration was measured according to the method of Bradford (32).

Results

Theca and granulosa cells were obtained from freshly isolated bovine ovaries from young cycling heifers and individual cell types were isolated by microdissection and cultured as previously described (29). Due to the isolation procedure and from a morphological analysis the cell preparations appeared homogeneous and did not contain any major cell contaminants. The theca cell preparation produced androgens and progestins in response to gonadotropin and the level of steroidogenesis was similar for primary cultures and subcultured cells that had been maintained for 2 weeks in culture (M. K. Skinner, unpublished observation and manuscript in preparation). In addition, the radiolabeled proteins secreted by freshly isolated theca cells were similar to those obtained from subcultured theca cells. Observations imply that the theca cell preparation is a hormone-responsive steroidogenic cell population that does not contain any major cell contaminants (M. K. Skinner, manuscript in preparation). Serum-free conditioned medium was obtained from theca cell and granulosa cell cultures and concentrated approximately 100-fold by ultrafiltration with a 3000 mol wt exclusion limit membrane (24, 26). The amount of protein secreted per cell was similar for theca and granulosa cells, while the protein concentrations of concentrated conditioned medium were 1.5 and 0.5 mg/ml, respectively. Concentrated conditioned medium contained cellular secreted proteins and was assayed for the presence of an EGF-like substance. An EGF RRA was established with a human fibroblast cell line, as previously described (19). Increasing concentrations of theca cell secreted proteins were found to specifically displace radioiodinated EGF from its receptor (Fig. 1). The displacement curve for murine EGF was parallel that of theca cell secreted proteins (data not shown). With the limitations of this RRA procedure considered the concentration of EGF-binding activity in theca cell secreted proteins was approximately 1 ng EGF-binding activity/10 μg theca cell secreted protein. Granulosa cell secreted proteins did not have the ability to displace radioiodinated EGF from the EGF receptor (Fig. 1). These results indicate that theca cell secreted proteins contain a component that can bind to the EGF receptor and is an EGF-like substance. Granulosa cell secreted proteins, however, did not contain a component that could be detected in the EGF RRA.

The possibility that the EGF-like substance produced by theca cells may be TGFα was investigated by examining TGFα gene expression in theca and granulosa cells. Polyadenylated RNA was obtained from freshly isolated and purified theca and granulosa cell populations and analyzed by Northern blot analysis (20). A human ^{32}P-labeled TGFα cRNA probe was used that was derived from a cDNA that contains the complete coding sequence for mature TGFα (16). The theca cell sample contained a 4.5- to 4.8-kilobase TGFα mRNA species (Fig. 2). The size of this transcript is similar to a TGFα mRNA species previously shown in a transformed colon cell line, SW620 (28). The granulosa sample, however, did not contain any detectable TGFα mRNA. RNA isolated from cultures of theca cells contained a more intense TGFα signal (Fig. 2). Whether the culture of theca cells or the culture

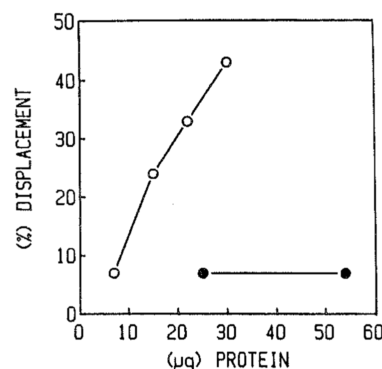

FIG. 1. EGF RRA of theca and granulosa cell secreted protein preparations. The ability of increasing concentrations of theca (○) and granulosa (●) cell secreted proteins to displace [^{125}I]EGF from an EGF receptor preparation is shown. Samples were incubated at 4 C for 4 h on human fibroblasts with [^{125}I]EGF in the absence or presence of excess of unlabeled EGF (100 ng). Specific binding was determined, and the percent inhibition of [^{125}I]EGF binding was assessed. Similar results were obtained when samples were either preincubated or coincubated with [^{125}I]EGF. Results presented are the mean from three experiments done in duplicate on three different preparations of secreted proteins.

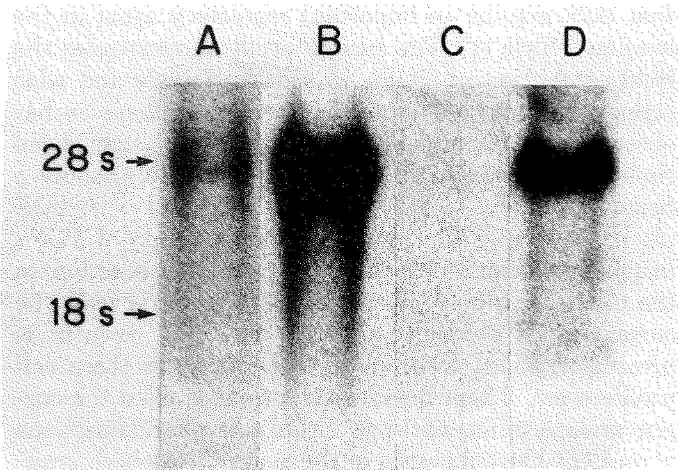

FIG. 2. Examination of TGFα gene expression in theca and granulosa cells. Polyadenylated RNA was prepared from freshly isolated theca cells (A), cultured theca cells (B), freshly isolated granulosa cells (C), and a colin cell line, SW620 (D). Similar amounts of RNA (2 μg) were applied to each lane, and the integrity of the RNA was examined with an ethidium bromide stain of the agarose gel after electrophoresis. An autoradiograph of hybridized ^{32}P-radiolabeled TGFα cRNA is shown. Data presented are representative of similar results obtained with three separate preparations of RNA analyzed in three different experiments.

conditions used were responsible for this increase in levels of TGFα message remains to be investigated. Hybridization with control β-actin (26) and 1B15 (27) probes demonstrated that the RNA from all samples was intact (data not shown).

A human EGF cDNA probe (24) was used in a Northern blot analysis to determine the possible presence of EGF gene expression. No hybridization was detected with an analysis of up to 10 μg polyadenylated RNA from theca or granulosa cells. A 5.2-kilobase RNA species was detected with the polyadenylated RNA from rat kidney (5 μg) as previously described (24) (data not shown). These observations indicate the apparent absence of EGF gene expression in theca or granulosa cells. Further investigation of the nature of the EGF-like activity in theca cell-conditioned medium used an immunoblot procedure previously described (33). An antisera to rat TGFα (Peninsula Laboratories, CA) was found to detect an apparent 6K protein in theca cell secreted proteins that had been electrophoretically separated, blotted to nitrocellulose, and detected with a lactoperoxidase-conjugated second antibody (data not shown). This observation implies that the EGF receptor-binding activity in theca cell secreted proteins is TGFα and helps confirm the expression of the TGFα gene in theca cells.

The observation that theca cells produce TGFα initiated an investigation of the potential sites of action of an EGF-like substance in the ovarian follicle. Previously, granulosa cells have been shown to contain high affinity EGF receptors and provide a site of EGF action in the

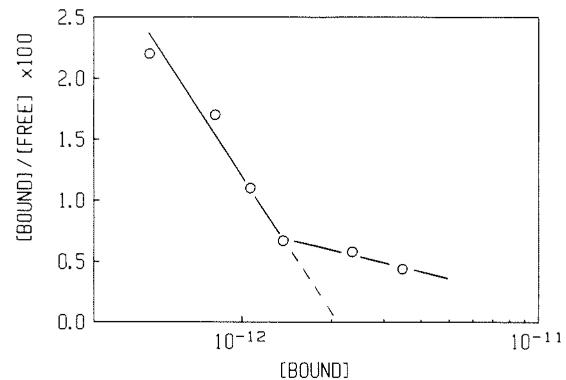

FIG. 3. Identification of high affinity EGF receptors on theca cells. Confluent cultures of theca cells were incubated with increasing amounts of murine [^{125}I]EGF in the absence or presence of 200 ng nonradiolabeled EGF for 4 h at 4 C. The amount of [^{125}I]EGF specifically bound was determined and presented as a ratio of bound/free [^{125}I]EGF used in the incubation *vs.* the concentration of [^{125}I]EGF specifically bound (moles/liter). Data presented are representative of similar results obtained with different theca cell and [^{125}I]EGF preparations in three different experiments.

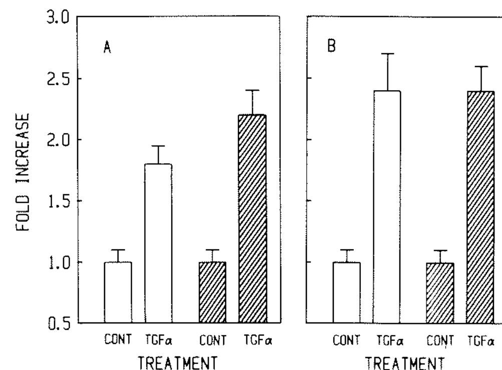

FIG. 4. Effect of TGFα on granulosa (□) and theca (▨) cell growth. Cultured cells were incubated in the absence of growth factors for 48 h, then in the absence (CONT) or presence of 10 ng/ml TGFα for 18 h. After a 4-h incubation with [^{3}H]thymidine the amount of [^{3}H] thymidine per μg DNA was determined (A). In separate experiments cell cultures were treated with TGFα for 72 h and then analyzed for DNA content to determine effects on cell proliferation (B). Data are presented as fold increase above control values and are represented as the mean ± SEM from three different experiments done in triplicate.

ovary (3). The possible presence of EGF receptors on theca cells was investigated with a Scatchard analysis using [^{125}I]EGF, as previously described (19, 28). Theca cells contained high affinity EGF receptors with an apparent K_d (±SEM) of $4.5 \times 10^{-11} \pm 0.8 \times 10^{-11}$ M and approximately 10,000 ± 1,500 binding sites/cell (Fig. 3). This observation demonstrates the presence of high affinity EGF receptors on theca cells, but the values calculated for binding affinity and binding sites per cell are approximate values due to a number of variables associated with this type of analysis, as previously discussed (28). Therefore, EGF receptors are present on both granulosa and theca cells, and both cell types provide poten-

tial sites of action for the TGFα produced by theca cells.

To confirm these potential sites of action, the effect of TGFα on both granulosa and theca cell growth was investigated. Granulosa and theca cells were cultured in the absence of serum for 48 h and then treated with TGFα (10 ng/ml) for an additional 24 h. Cultures were incubated in the presence of [^{3}H]thymidine for 4 h, followed by determination of the amount of [^{3}H]thymidine incorporated into DNA as previously described (18, 24). TGFα significantly stimulated DNA synthesis in both granulosa and theca cells (Fig. 4A). When cells were treated for 72 h with TGFα, a corresponding increase in cell number and DNA content in the cultures confirmed the proliferation of both cell types in response to TGFα (Fig. 4B). Experiments with an automated cell counter established that the increase in DNA correlated to an increase in cell number. The ability of exogenously added TGFα to stimulate theca cell growth implies that the level of TGFα production *in vitro* by theca cells is not sufficient to maintain optimal cell growth.

Discussion

The present study indicates that TGFα mRNA is expressed in ovarian theca cells and is supported by the observation that an EGF-like substance is present in theca cell secreted protein preparations. Previous observations have demonstrated the production of an EGF-like substance by rat theca/interstitial cells that had biochemical characteristics distinct from those of EGF (8). The rat theca/interstitial cell-derived EGF-like substance was also found to have no cross-reactivity with mouse, rat, or human antisera to EGF (8). Preliminary results indicate that TGFα can be detected immunologically in bovine theca cell secreted protein preparations with a Western immunoblot procedure and a TGFα antisera. No EGF gene expression was detected in theca or granulosa cells using a human EGF cDNA probe that recognized an EGF transcript in rat kidney. Further analysis with a more specific EGF molecular probe may be necessary to eliminate variations between species and confirm the absence of EGF gene expression in the ovarian follicle. Current data support the proposal that theca cells synthesize and secrete TGFα as the primary EGF-like activity in the follicle. Although theca cells are capable of producing TGFα, granulosa cells did not contain TGFα mRNA, nor was any EGF-like material detected in granulosa cell secreted protein preparations. Therefore, theca cells appear to be the principal site in the ovarian follicle for production of an EGF-like activity that is now identified as TGFα.

Previous studies have demonstrated the ability of EGF to alter granulosa cell growth (3) and hormonal regulation (1, 6). These observations have led to the suggestion that EGF may be an important regulatory agent in the ovarian follicle (1). The current study demonstrates the local production of TGFα within the follicle and adds support and physiological significance to these earlier observations. Granulosa cells have previously been shown to contain high affinity EGF receptors using a Scatchard analysis (5). As previously shown with EGF (3), the current study demonstrates the ability of TGFα to promote granulosa cell proliferation. In addition to the results previously obtained with granulosa cells, the current study demonstrates that the EGF receptor is present on theca cells, and TGFα promotes theca cell proliferation. These novel observations expand the current understanding of the potential actions and functions of an EGF-like substance in the ovary. Since both granulosa and theca cell proliferation is required for follicle growth, the presence of the EGF receptor and actions of TGFα on both cell types support the proposal that an EGF-like substance such as TGFα will be an important growth regulator in the ovarian follicle. In addition to growth regulation, TGFα production in the ovary may have a role in modulating hormone actions on granulosa cells (34).

The production of TGFα by theca cells provides a possible autocrine role for TGFα in the regulation of theca cell growth and a paracrine role in the regulation of granulosa cell growth. This is supported by the observation that preparations of bovine theca cell secreted protein can stimulate bovine granulosa cell growth (35). The ability of two different cell types to interact in a paracrine manner to control cell growth has been shown for several growth factors, but is a novel observation for TGFα (10). Growth factor-mediated paracrine interactions within a tissue provide an efficient mechanism for different cell types to form a functional unit to control cell proliferation. The evolution of this process indicates the importance of cell-cell interactions between different cell types within a tissue and implies that few cell types will be autonomous in the regulation of growth and differentiation. Theca-granulosa cell interactions provide an example of mesenchymal-epithelial cell interactions. The importance of mesenchymal-epithelial cell interactions has been demonstrated during development (36), but remains to be elucidated in adult tissues. Mesenchymal cells are postulated to produce inducer substances that can influence the differentiation of adjacent epithelial cells (36). The significance of the production of growth factors by mesenchymal cells that regulate the proliferation of adjacent epithelial cells remains to be elucidated. This type of growth factor-mediated cellular interaction may be potentially important for embryonic development as well as cell proliferation in adult tissues. The production of TGFα by theca cells and subsequent actions on granulosa cells are an example of a growth

factor-mediated mesenchymal-epithelial cell interaction in an adult tissue. Analysis of the generality of this type of interaction will provide insight into the importance of mesenchymal-epithelial interactions in the control of cell growth in embryonic and adult tissues.

The synthesis of TGFα by theca cells provides an example of TGFα production by a normal adult mesenchymal tissue. Previous studies have implicated TGFα as an important growth regulator during transformation (4) and embryonic development (14). Studies have also demonstrated that TGFα can be produced by normal pituitary epithelial cells (15) and keratinocytes (16). The present study provides the demonstration of TGFα production by a normal mesenchymal cell type. Therefore, the current observations support the suggestion that TGFα may also have an important role as a growth regulator in normal adult tissues (16). A number of rapidly growing tissues have been shown to contain EGF receptors, including uterus, intestine, skin, and mammary gland (20, 37). The local production of TGFα as a potential growth regulator for these tissues will be an interesting possibility to investigate.

Although the ovarian follicle is a rapidly growing tissue, the majority of follicles at various stages of development undergo atresia in which growth is inhibited. Therefore, growth regulation in the follicle would appear to require both a growth stimulator and a growth inhibitor. TGFβ is a unique gene product that, after activation, has the ability to inhibit the growth of most epithelial cell types (33). In particular, biologically active TGFβ inhibits the ability of EGF to promote growth of EGF-sensitive cell types (38). Recently, ovarian theca cells have been shown to produce TGFβ, which can inhibit the ability of EGF to promote granulosa cell growth (29). Therefore, current data indicate that theca cells produce both TGFα and TGFβ, which may function as a growth stimulator and growth inhibitor, respectively, in the ovarian follicle. The speculation is made that the production of TGFα by theca cells would be predominant during the growth phase of the follicle, and TGFβ production would be predominant when growth is inhibited. These inverse actions of TGFα and TGFβ provide an efficient mechanism to control the growth of a tissue that requires both rapid growth stimulation and inhibition. It is suggested that this type of growth control may exist for a number of tissues that undergo a rapid stimulation and inhibition of cell proliferation.

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