0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 1
Printed in U.S.A.

Developmental and Hormonal Regulation of Keratinocyte Growth Factor Expression and Action in the Ovarian Follicle*

JEFF A. PARROTT AND MICHAEL K. SKINNER

Reproductive Endocrinology Center, University of California, San Francisco, California 94143-0556; and Center for Reproductive Biology, Department of Genetics and Cell Biology, Washington State University, Pullman, Washington 99164-4231

ABSTRACT

The developing ovarian follicle is one of the most rapidly proliferating normal tissues *in vivo*. Mesenchymal-epithelial cell interactions between theca cells and granulosa cells are essential for this follicular expansion. Ovarian hormones (*i.e.* estrogen and LH) may promote follicular development by regulating the local production of mesenchymal inducer proteins that mediate theca cell-granulosa cell interactions. Recently, theca cells were shown to produce keratinocyte growth factor (KGF) that can act in a paracrine manner to stimulate granulosa cell growth. In this study, the developmental and hormonal regulation of KGF was examined during follicular development in the bovine ovary. Expression of KGF in theca cells and the KGF receptor (KGFR, or splice variant of the fibroblast growth factor family receptor family, FGFR-2) in granulosa cells was examined using RT-PCR. Both KGF and KGFR were detected throughout follicular development in small (<5 mm), medium (5–10 mm), and large (>10 mm) follicles. Quantitative RT-PCR assays were used to determine steady-state levels of KGF and KGFR messenger RNAs. Developmental regulation of KGF and KGFR was analyzed in freshly isolated theca cells and granulosa cells from small, medium, and large follicles. Observations demonstrated that expression of KGF (in theca cells) and KGFR (in granulosa cells) was highest in large follicles. These results suggest that KGF actions are important for the rapid proliferation of granulosa cells in large follicles. Estrogen and LH are the primary endocrine hormones that regulate theca cell function *in vivo*. Therefore, hormonal regulation of KGF was analyzed by treating serum-free theca cell cultures with estrogen and human CG (hCG, an LH agonist). Results showed that both estrogen and hCG stimulated KGF gene expression in theca cells. These results suggest that estrogen and LH may promote follicular growth (*i.e.* granulosa cell proliferation), in part, by stimulating the local production of KGF. Effects of KGF on granulosa cell differentiated functions were examined. Treatment with KGF reduced basal levels and FSH-stimulated levels of aromatase activity in bovine and rat granulosa cells. In addition, KGF inhibited the ability of hCG to stimulate progesterone production by granulosa cells. The inhibition of granulosa cell steroid production by KGF was likely the indirect effect of promoting cellular proliferation. Therefore, KGF directly stimulates granulosa cell proliferation and indirectly inhibits granulosa cell differentiated functions. Combined results suggest that theca cell production of KGF may be important for ovarian folliculogenesis. This is the first report of the regulation of KGF expression in the ovary. The developmental and hormonal regulation of KGF and KGFR during folliculogenesis provides evidence that KGF may be important for hormone-induced granulosa cell proliferation. As a result, KGF may be essential for establishing the microenvironment required for oocyte maturation in the ovary. (*Endocrinology* **139:** 228–235, 1998)

MESENCHYMAL-EPITHELIAL cell interactions occur in many organs and are one of the most common cell-cell interactions between different cell types. It has long been recognized that growth and differentiation of epithelial cells is directed by adjacent mesenchymal cells during embryonic development (1–5) and optimally maintained by adjacent stroma in adult tissues (6, 7). Identification and characterization of the inducer proteins that mediate mesenchymal-epithelial cell interactions are necessary to understand the mechanisms of how cells communicate in an organ. The ovarian follicle is a useful model system to study these cell-cell interactions.

Keratinocyte growth factor (KGF) is produced by mesenchymal-theca cells and stimulates epithelial granulosa cell proliferation during follicular development in the ovary (8). *In situ* hybridization has confirmed the localization of KGF gene expression in theca cells in the ovary (6). The ovarian follicle is one of the most rapidly proliferating normal tissues known *in vivo*, and granulosa cell growth accounts for the majority of this follicle expansion (9). Factors, such as KGF, that promote the growth of the granulosa cell population (8) help establish the microenvironment required for oocyte maturation and fertility in the female. Endocrine hormones, such as estrogen and LH, have been shown to stimulate follicle development and granulosa cell growth *in vivo*, but neither of these hormones acts as a granulosa cell mitogen *in vitro* (10–14). Therefore, the effects of estrogen and LH on follicular growth *in vivo* are proposed to be indirectly mediated by factors such as KGF.

KGF is a 28-kDa protein that is a member of the fibroblast growth factor family (FGF-7) (15, 16). KGF is primarily produced by mesenchymal-derived cells in many tissues and acts as an epithelial-cell-specific mitogen (17–19). The receptor to KGF (KGFR) is a splice variant of the FGF receptor family (FGFR-2) that is primarily localized on epithelial cells (17, 20–22). KGF mediates mesenchymal-epithelial cell interactions in many tissues, including the ovary (8), placenta

Received May 8, 1997.
Address all correspondence and requests for reprints to: Michael K. Skinner, Center for Reproductive Biology, Department of Genetics and Cell Biology, Washington State University, Pullman, Washington 99164-4231. E-mail: skinner@mail.wsu.edu.
* This research was supported by grants from the United States Department of Agriculture and the National Institutes of Health.

(23), endometrium (24), bladder (25, 26), hair follicle (27), stomach (28), lung (29, 30), ventral prostate (31), and seminal vesicle (32). Expression of KGF can be regulated by endocrine hormones such as androgen, progesterone, and glucocorticoids (24, 33, 34). These and other studies have established that KGF can mediate the actions of endocrine hormones and act as a paracrine mediator of mesenchymal-epithelial cell interactions in many tissues.

The current study was designed to investigate the role of KGF as a paracrine mediator of mesenchymal-epithelial cell interactions during ovarian follicular development. Experiments address the developmental and hormonal regulation of KGF in theca cells and the KGFR in granulosa cells. Estrogen and LH are proposed to stimulate granulosa cell proliferation *in vivo*, in part, by stimulating theca cell KGF production. Effects of KGF on granulosa cell steroid production are also examined.

Materials and Methods

Tissue isolation and serum-free cell culture

Bovine ovaries were obtained from young nonpregnant cycling heifers less than 10 min after death. Ovaries were delivered fresh on ice by Golden Genes (Fresno, CA). Granulosa cells were isolated by microdissection from fresh tissue, as previously described (35). Theca interna layers were then microdissected away from the follicle wall and enzymatically dispersed with 2 mg/ml (20 U) collagenase type I (Sigma, St. Louis, MO) in Ca^{++}/Mg^{++}-free buffer. For culture, cells were immediately plated in serum-free Ham's F-12 medium containing 0.1% BSA and maintained at 37 C in a 5% $C0_2$ atmosphere. The indicated cells were treated with estradiol (10^{-7} M; Sigma), human CG (hCG) (100 ng/ml; 4010 IU/mg, Calbiochem, La Jolla, CA), or FSH (100 ng/ml, National Pituitary Agency, Baltimore, MD). Cell preparations obtained by this procedure have been characterized cytochemically to contain less than 3% contamination with endothelial cells.

Preparation of RNA and PCR

Follicles were dissected from the bovine ovaries and separated into pools of small (<5 mm), medium (5–10 mm), and large (>10 mm) follicles. Granulosa and theca cell total RNA was extracted from each pool of samples using a guanidium thiocyanate procedure, followed by centrifugation through a cesium chloride gradient (36). Alternatively, total RNA was prepared using the RNA-Stat 60 kit (Tel-Test, Friendswood, TX). For qualitative analysis of gene expression, 10 ug total RNA was reverse transcribed with Moloney's murine leukemia virus RT (Gibco BRL, Gaithersburg, MD) at 37 C for 1 h using oligodeoxythymidine$_{12–18}$ primers (Gibco BRL). This complementary DNA (cDNA) template was amplified by PCR using specific primers for KGF, KGFR, or the constitutively expressed gene cyclophilin (1B15). The KGF primers were 5′-ATA CTG ACA TGG ATC CTG CCA AGT TTG CTC TAC AGA TCA TGC TTC-3′ (5′ primer, 45-mer) and 5′-TCC AAC TGC CAC GGT CCT GAT-3′ (3′ primer, 21-mer), which generated a specific 306-bp KGF PCR product from bovine theca cells. The KGFR primers were 5′-GAA GTG CTG GCT CTG TTC AAT GTG AC-3′ (5′ primer, 26-mer) and 5′-TTG GAG TTC ATG GAG GAG CTG GAC TC-3′ (3′ primer, 26-mer), which generated a specific 359-bp KGFR PCR product from bovine granulosa cells. The primers for 1B15 were: 5′-ACA CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC-3′ (5′ primer, 33-mer) and 5′-ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG-3′ (3′ primer, 33-mer), which generated a specific 105-bp product from all cell types, demonstrating the integrity of the RNA samples. Amplification was performed with AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) for 35 cycles using the following conditions: 0.8 μM of each primer, 100 μM deoxynucleotide triphosphates (dNTPs), 1.5 mM Mg^{++}, 1.25 U *Taq* polymerase in 50 μl total vol. Each PCR amplification consisted of an initial denaturing reaction (5 min, 95 C); 35 cycles of denaturing (30 sec, 95 C), annealing (2 min, 60 C), and elongation (3 min, 72 C) reactions; and a final elongation reaction (10 min, 72 C). PCR products were visualized by UV illumination (312 nm) of 2% agarose gels stained with ethidium bromide.

The KGF, KGFR, and 1B15 PCR products were subcloned into the Bluescript plasmid (Stratagene, La Jolla, CA) at the *Sma*I site. Each subclone was sequenced in both directions and confirmed to be bovine KGF, KGFR, and 1B15. These subclones were used as standard templates in the quantitative PCR procedure below.

Quantitative RT-PCR assays

Steady-state levels of KGF, KGFR, and 1B15 messenger RNAs (mRNAs) were measured using a specific quantitative RT-PCR assay for each gene. The primers used in this quantitative analysis of KGF, KGFR, and 1B15 were the same as described above. These were 3′ primers, which significantly increased the reproducibility of the reverse transcription and eliminated the variability normally found with oligodeoxythymidine. This eliminated the need for internal standards in the PCR. Before reverse transcription, tubes containing total RNA and specific 3′-primers were heated to 65 C for 10 min to facilitate denaturing and cooled to room temperature to facilitate annealing. Total RNA (1 μg) was reverse transcribed for 1 h at 37 C using the following conditions: 1 μg total RNA, 1 μM specific 3′-primers of interest (up to 4 different primers, including 1B15), 0.1 mM dNTPs, 10 mM dithiothreitol, 40 U ribonuclease inhibitor (Promega, Madison, WI), and 200 U M-MLV RT (Gibco BRL) in 40 μl RT buffer (50 mM Tris-HCl (ph 8.3), 75 mM KCI, 3 mM $MgCl_2$). After 1 h, samples were heated to 95 C for 5 min to inactivate the RT enzyme. Samples were immediately diluted 2.5-fold, and carrier DNA (Bluescript plasmid, Stratagene) was added to a final concentration of 10 ng/μl. This concentration of Bluescript carrier DNA (10 ng/μl) was included in all subsequent dilutions of samples and standards. Immediately before amplification, each unknown sample was further diluted 1:10 to improve the fidelity of the PCR reaction (37). Plasmid DNAs containing bovine KGF, KGFR, or 1B15 subclones were used to generate standard curves from 1 attogram/μl (10^{-15} g/μl) to 10 pg/μl (10×10^{-9} g/μl), each containing 10 ng/μl Bluescript carrier DNA. Identical 10-μl aliquots of each sample and standard were pipetted in duplicate into a 96-well reaction plate (Marsh Biomedical Products, Rochester, NY) and sealed with adhesive film (Marsh Biomedical Products) for PCR amplification. By this design, it was possible to simultaneously assay 5 known standard concentrations and 40 unknown samples for each gene. Amplification was performed in a Perkin Elmer 9600, equipped with a heated lid, using the following conditions: 0.4 μM each primer, 16 μM dNTPs, and 1.25 U AmpliTaq polymerase in 50 μl GeneAmp PCR buffer (containing 1.5 mM $MgCl_2$, Perkin Elmer). Each PCR amplification consisted of an initial denaturing reaction (5 min, 95 C); 25–31 cycles of denaturing (30 sec, 95 C), annealing (1 min, 60 C), and elongation (2 min, 72 C) reactions; and a final elongation reaction (10 min, 72 C). At least 0.25 uCi of ^{32}P-labeled dCTP (Redivue, Amersham Life Sciences, Arlington Heights, IL) was included in each sample during amplification for detection purposes. Specific PCR products were quantitated by electrophoresing all samples on 4–5% polyacrylamide gels, simultaneously exposing the gels to a phosphor screen for 8–24 h, followed by quantitating the specific bands on a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each gene was assayed in separate PCR reactions from the same RT samples. Equivalent steady-state mRNA levels for each gene were determined by comparing each sample with the appropriate standard curve. All KGF and KGFR data were normalized for 1B15. The IB15 PCR was performed on the same sample and reverse transcribed together with the appropriate 3′ primers. The expression of IB15 did not vary on a per-cell basis between cells and was similar, independent of source. The IB15 corrected for cell number and for amount and integrity of the RNA sample used.

Optimal cycle number for amplification was determined for each assay to achieve maximum sensitivity while maintaining linearity (*i.e.* logarithmic phase of PCR reactions). KGF quantitative PCR products were amplified for 28 cycles, KGFR PCR products were amplified for 31 cycles, and 1B15 PCR products were amplified for 25 cycles. The sensitivity of each quantitative PCR assay was below 1 fg, which corresponds to less than 125 fg target mRNA/μg total RNA. For each assay, all samples were simultaneously measured in duplicate, resulting in intraassay variabilities of 11.3% (KGF), 10.2% (KGFR), and 6.5% (1B15).

Assay for aromatase activity in granulosa cells

Aromatase activity was assessed by the release of ^3H_2O from aromatization of [1b-^{3}H]androstenedione, as previously described (38), with the following modifications: Approximately 1.5 million granulosa cells/cm^2 were plated in 24-well plates and immediately treated with no-factor (control), KGF (50 ng/ml) or FSH (100 ng/ml) in the presence or absence of KGF. After 20 h, 0.5 ml medium containing 0.5 mCi [1b-^{3}H]androstenedione (New England Nuclear, Boston, MA) was added and incubated for 6 h at 37 C in a CO_2 atmosphere. The culture was then sonicated, and an aliquot was removed for DNA analysis. The remaining sample was transferred to a tube and incubated with dextran-coated charcoal [0.2% (wt/vol) 70K dextran (Sigma), activated 2% (wt/vol) charcoal, 10 mM Tris, and 1 mM EDTA, pH 7.5] for 2 h at 4 C, then centrifuged at 13,000 $\times$ *g* for 15 min. Aliquots of the supernatant, containing ^3H_2O, were then combined with 5 ml scintillation cocktail (ICN, Costa Mesa, CA) and counted for 5 min in a scintillation counter. Blank values were established from identical incubations, in the absence of cells, and contained less than 100 cpm. Aromatase activity, measured in counts per min of ^3H_2O released, was normalized, per microgram DNA, using an ethidium bromide procedure described previously (35).

Assay for progesterone production

Progesterone production by granulosa cells was determined by quantitating progesterone accumulation in the culture medium. Approximately 1.5 million granulosa cells/cm^2 were plated in 24-well plates and immediately treated with no-factor (control), KGF (50 ng/ml), or hCG (100 ng/ml) in the presence or absence of KGF. After 72 h, the medium was collected and assayed for progesterone using the ImmunoChem ^{125}I progesterone kit (ICN). The sensitivity of the progesterone assay was 0.01 ng/ml. Progesterone data were normalized, per total DNA per well, as previously described (35).

Statistical analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute Inc., Cary, NC). Effects of hormones on KGF or KGFR mRNA levels and on granulosa cell aromatase activity and progesterone production were analyzed by a one-way ANOVA. Significant differences between treated cells and control (untreated) cells were determined using the Dunnett's test, which guards against the high α-size (Type I) error rate across the hypothesis tests (39). Effects of follicle size on steady-state KGF or KGFR mRNA levels were analyzed by a one-way ANOVA, as described above. Significant differences between small, medium, and large follicles were determined using the Tukey-Kramer HSD (honestly significant difference) test which protects the significance tests of all combinations of pairs (40–42). These multiple comparisons tests are recommended for multiple comparisons with control (Dunnett's) or multiple comparisons of all pairs (Tukey-Kramer HSD) (43, 44).

Results

Mesenchymal theca cells and epithelial granulosa cells were obtained from freshly isolated bovine ovaries. Cells were independently isolated from pools of small (<5-mm diameter), medium (5–10 mm) and large (>10 mm) follicles. Cells were used immediately for total RNA preparation or placed in cell culture. The gene expression of KGF and KGFR was determined by RT-PCR. Using appropriate primers, the 306-bp KGF PCR product was observed in theca cell RNA isolated from small, medium, and large follicles (Fig. 1A). The 359-bp KGFR PCR product was observed in granulosa cell RNA from small, medium, and large follicles (Fig. 1B). These PCR products were blunt-end subcloned into the Bluescript plasmid, sequenced, and confirmed to be bovine KGF and KGFR cDNA (data not shown). KGF and KGFR subclones were used below as standards for quantitative RT-PCR. Although KGF and KGFR are generally expressed in mesenchymal-derived cells and epithelial cells, respectively,

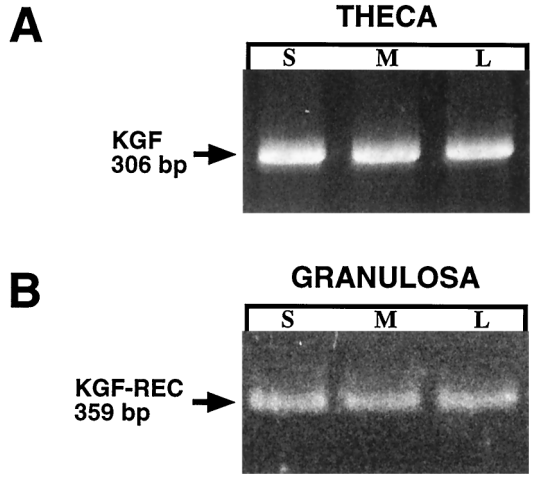

FIG. 1. Expression of KGF and KGFR mRNA in bovine ovarian follicles. RT-PCR analysis was performed with 10 μg total RNA. Amplification was nonquantitative. A, Specific primers were designed to amplify KGF from bovine theca cDNA template; 306-bp KGF PCR products were detected in theca cells from small, medium, and large follicles. B, Specific primers were designed to amplify KGFR from bovine granulosa cDNA template; 359-bp KGFR PCR products were detected in granulosa cells from small, medium, and large follicles. Standard DNA ladder (1 kB) was used for size determination (not shown). S, Small follicles; M, medium follicles; L, large follicles. Data are representative of at least four experiments.

their expression patterns have not been well characterized in the ovary. KGF mRNA expression has been shown in bovine theca cells (8), but KGFR expression has not been directly examined in the ovary. The results in Fig. 1 indicate that KGFR is expressed by epithelial granulosa cells in ovarian follicles. These results also demonstrate that KGF and KGFR are expressed throughout follicular development, because KGF and KGFR mRNAs were observed from small, medium, and large follicles.

To examine the potential developmental regulation of KGF and KGFR during follicular development, sensitive quantitative RT-PCRs were developed for both genes. KGF steady-state mRNA levels in theca cells and KGFR steady-state mRNA levels in granulosa cells were determined in total RNA samples from small, medium, and large follicles. Under specific amplification conditions, these assays used the bovine KGF and KGFR subclones from Fig. 1 as template, to generate standard curves. Samples, consisting of total RNA from freshly isolated theca cells or granulosa cells, were reverse transcribed using the specific 3′ primers of the gene(s) of interest. These unknown samples were simultaneously amplified by PCR, along with the known standards, to quantitate gene expression. Cycle number and annealing temperature were optimized for each gene to obtain maximum sensitivity and to maintain linearity. These quantitative assays for KGF and KGFR mRNAs are extremely sensitive ($<10^{-11}$ g/sample) and have intraassay variabilities of 11.3% and 10.2%, respectively. As is shown in Figs. 2 and 3, each assay is linear over several orders of magnitude (0.1–1000 fg/sample). Each assay was validated by demonstrating parallel curves between the appropriate RNA samples and standards (Figs. 2A and 3A). All samples were normalized for the constitutively expressed 1B15 mRNA, as determined by the

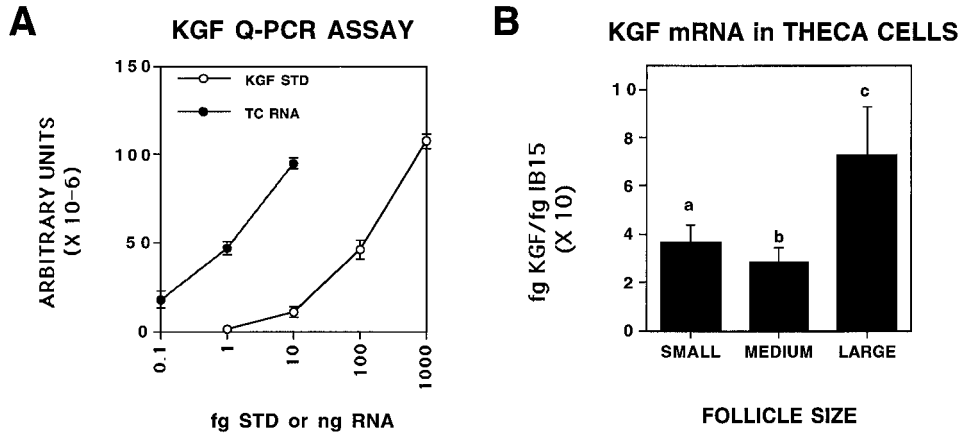

FIG. 2. Developmental regulation of KGF mRNA in bovine theca cells. Steady-state levels of KGF mRNA in theca cells from small, medium, and large follicles were determined using quantitative RT-PCR. A, Line graph of a typical experiment validating the assay. Parallel curves of a standard bovine KGF subclone (○) and a fresh theca cell total RNA sample (●) are shown. Raw data are represented as arbitrary units ± SEM (as read from the PhosphorImager) and are directly proportional to actual counts per minute. An inset of a representative gel is shown for both standard KGF subclone DNA (std) and sample RNA (RNA). B, Analysis of steady-state KGF mRNA levels in theca cells from small, medium, and large follicles. Levels of KGF mRNA were determined and normalized to levels of 1B15 mRNA (fg KGF mRNA/fg 1B15 mRNA). Data are presented as the mean ± SEM of duplicate determinations from seven different sets of theca cell RNA. An ANOVA was performed, and significant differences between follicle sizes were determined using the Tukey-Kramer HSD test. *Bars* with different *superscript letters* differ from each other ($P < 0.05$). C, Representative electrophorectic gel of standard and RNA sample (TC, theca cell) with the 306-bp PCR product indicated.

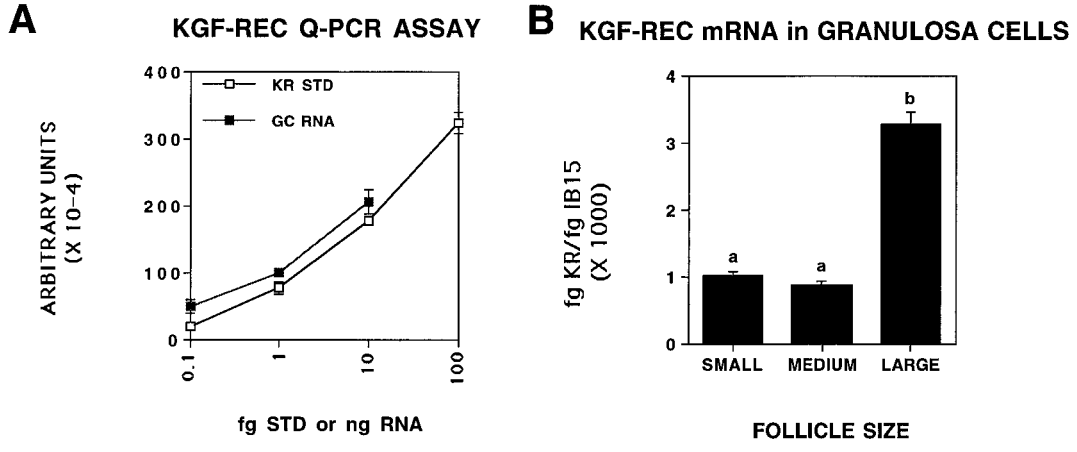

FIG. 3. Developmental regulation of KGFR mRNA in bovine granulosa cells. Steady-state levels of KGFR mRNA in granulosa cells from small, medium, and large follicles were determined using quantitative RT-PCR. A, Line graph of a typical experiment validating the assay. Parallel curves of a standard bovine KGFR subclone (□) and a fresh granulosa cell total RNA sample (■) are shown. Raw data are represented as arbitrary units ± SEM (as read from the PhosphorImager) and are directly proportional to actual counts per minute. An inset of a representative gel is shown for both standard KGFR subclone DNA (std) and sample RNA (RNA). B, Analysis of steady-state KGFR mRNA levels in granulosa cells from small, medium, and large follicles. Levels of KGFR mRNA were determined and normalized to levels of 1B15 mRNA (fg KGFR mRNA/fg 1B15 mRNA). Data are presented as the mean ± SEM of duplicate determinations from four different sets of granulosa cell RNA. An ANOVA was performed, and significant differences between follicle sizes were determined using the Tukey-Kramer HSD test. *Bars* with different *superscript letters* differ from each other ($P < 0.05$). C, Representative electrophoretic gel of standard and RNA samples (GC, granulosa cell) with the 359-bp PCR product indicated.

same procedure. This normalization corrects for the number of cells, amount and integrity of initial mRNA, and small differences in the efficiency of reverse transcription between samples. Inset representative gels are shown, to demonstrate that minimal background is detected and the same products are detected in the standard DNA and sample RNA. The results are shown in Figs. 2C and 3C. The steady-state levels of KGF mRNA in theca cells were highest in large follicles and lowest in medium follicles (Fig. 2B). A small (but significant) difference in KGF mRNA expression was observed between theca cells from small and medium follicles. Steady-state levels of KGFR were dramatically higher in granulosa cells from large follicles than in small or medium follicles (Fig. 3B). In addition, steady-state levels of KGF mRNA in theca cells (0.3–0.8 fg KGF/fg 1B15) were two orders of magnitude higher than the levels of KGFR mRNA in granulosa cells (0.001–0.003 fg KGFR/fg 1B15). These results demonstrate that the KGF and KGFR genes are developmentally regulated during normal follicular development. Both KGF (in theca cells) and KGFR (in granulosa cells) were

highest in large follicles. The IB15 normalization corrected for cell numbers and has previously been shown not to vary in expression levels between cells at different stages of development (data not shown). Because KGF has previously been shown to stimulate granulosa cell proliferation (8), expression of KGF and KGFR may be important for granulosa cell proliferation in large follicles.

Previous studies have shown that endocrine hormones can regulate KGF expression. Glucocorticoids regulate KGF mRNA and protein during wound healing (33), androgen stimulates KGF promoter activity in male accessory glands (34), and progesterone may directly stimulate KGF expression in endometrium (24). In the female, both estrogen and LH promote follicular development and increase granulosa cell proliferation *in vivo*. Neither hormone stimulates proliferation of purified granulosa cells. The hypothesis was tested that estrogen and LH indirectly promote follicular development by regulating the local production of KGF. Fresh theca cells were placed in serum-free culture and treated with estrogen and hCG (an LH agonist). After 3 days, total RNA was prepared and steady-state levels of KGF mRNA were measured using the KGF quantitative RT-PCR assay. The results are shown in Fig. 4. Both estrogen and hCG stimulated KGF mRNA in bovine theca cells. Similar results were obtained with 10^{-7} M and 10^{-6} M estradiol and 50 mg and 100 mg CG, indicating optimal stimulation (data not shown). These results are the first to demonstrate regulation of the KGF gene in the ovary. The regulation of KGF mRNA by estrogen and LH supports the hypothesis that these hormones promote follicular growth by stimulating the local production of KGF. The potential regulation of ovarian KGF expression by other factors remains to be elucidated.

To investigate the actions of KGF in the ovary, granulosa cell steroidogenic capacities in response to KGF were examined. Granulosa cells were cultured in serum-free media and assayed for two functional markers (35). The first functional parameter examined was the ability of granulosa cells to aromatize androstenedione to produce estradiol. Both bovine and rat granulosa cells were used to quantitate aromatase activity in the presence or absence of KGF. Bovine granulosa cells can be isolated in large quantities but contain low levels of aromatase after short-term culture. Rat granulosa cells have more robust levels of aromatase expression and maintain aromatase activity for several days *in vitro*. The analysis of bovine and rat granulosa cell aromatase activity is shown in Fig. 5. KGF significantly reduced basal levels of aromatase activity in bovine and rat granulosa cells. In addition, KGF reduced the ability of FSH to stimulate aromatase activity. Because KGF has previously been shown to stimulate granulosa cell proliferation (8), this negative effect on aromatase activity is probably the indirect effect of promoting cell cycle entry. A similar effect has been observed with transforming growth factor α (TGF-α) in the ovary (45). These results demonstrate that KGF negatively regulates granulosa cell function by decreasing aromatase activity.

The second functional parameter examined was progesterone production. Bovine granulosa cells were cultured in serum-free media for 72 h in the presence or absence of KGF. Cells were stimulated with hCG as a positive control. After 72 h, media were collected and progesterone accumulation was determined by RIA. The results of granulosa cell progesterone production are shown in Fig. 6. KGF had no effect on basal levels of progesterone production but dramatically inhibited the ability of hCG to stimulate progesterone. Therefore, KGF can inhibit both aromatase activity and progesterone production in granulosa cells. These results demonstrate that KGF has an inhibitory effect on the functional differentiation (*i.e.* aromatase activity and progesterone production) of granulosa cells. These differentiated functions are indirectly decreased because of the previously shown ability of KGF to stimulate granulosa cell.

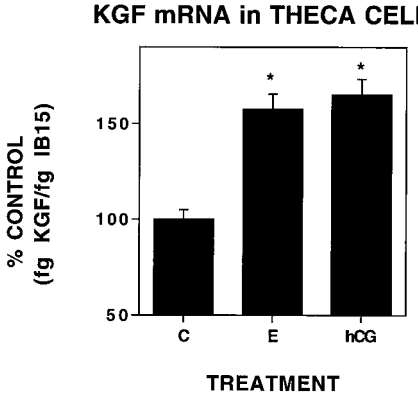

FIG. 4. Hormonal regulation of KGF mRNA in bovine theca cells. Steady-state levels of KGF mRNA in cultured theca cells were determined using quantitative RT-PCR. Theca cells were placed in serum-free culture and treated with no factor (control), 10^{-7} M estradiol (E), or 100 ng/ml hCG. After 72 h in culture, cells were harvested, and total RNA was prepared. Steady-state levels of KGF mRNA were determined as in Fig. 2. Levels of KGF mRNA were determined and normalized to levels of 1B15 mRNA (fg KGF mRNA/fg 1B15 mRNA). Data are presented as the mean ± SEM of duplicate determinations from five different sets of theca cell RNA. An ANOVA was performed and significant differences from control were determined using the Dunnett's test. *Bars* with *asterisks* differ from control ($P < 0.05$).

Discussion

Mesenchymal-epithelial cell interactions between theca cells and granulosa cells regulate follicular development in the ovary. KGF has been shown to mediate mesenchymal-epithelial cell interactions in many tissues (24, 31, 33, 34, 46), including the ovary (8). KGF is expressed by theca cells (6, 8) and acts on granulosa cells (8). The current study was designed to examine the developmental and hormonal regulation of KGF in theca cells, and KGFR in granulosa cells. By understanding the regulation and actions of KGF in the ovary, insight will be gained into mesenchymal-epithelial cell interactions that control follicular development.

The developing ovarian follicle is one of the most rapidly proliferating normal tissues known *in vivo*, and granulosa cell growth accounts for the majority of this follicle expansion (9). The number of granulosa cells increase at least 10,000-fold during development from preantral to ovulatory follicles, and the rate of proliferation is highest in large follicles (47). Locally produced KGF is proposed to be important for this growth process. Expression of KGF in theca cells, and KGFR in granulosa cells, was observed throughout follicular

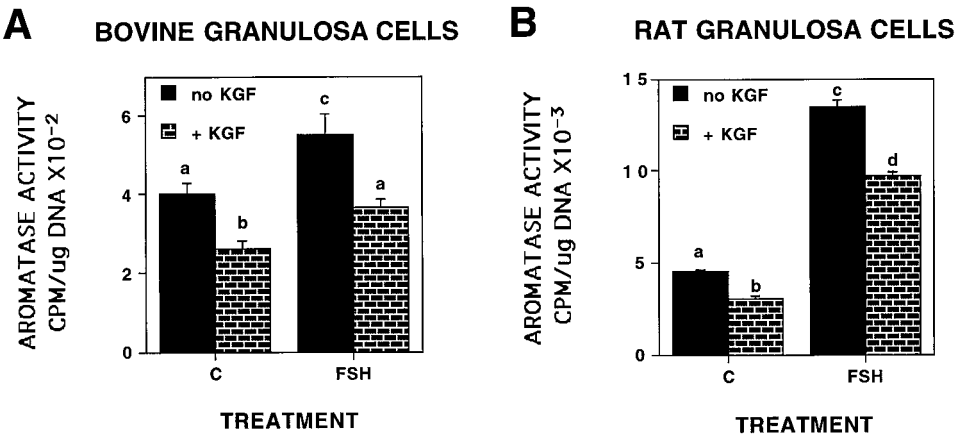

FIG. 5. KGF inhibits granulosa cell aromatase activity. Bovine (A) or rat (B) granulosa cells were cultured in the absence (■) or presence (▩) of KGF. Additionally, cells were treated with FSH as a positive control. Bovine granulosa cells can be isolated in large numbers, but rat granulosa cells express higher levels of aromatase in culture. Aromatase activity was determined on day 1 of culture and normalized per microgram DNA (cpm/μg DNA). Data are presented as mean ± SEM of triplicate determinations from at least three different experiments. An ANOVA was performed, and significant differences between treatments were determined using the Tukey-Kramer HSD test. *Bars* with different *superscript letters* differ from each other ($P < 0.05$).

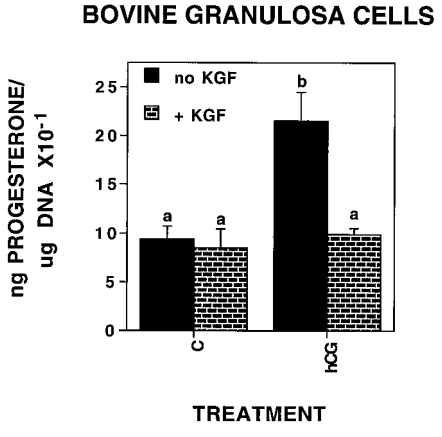

FIG. 6. KGF inhibits granulosa cell progesterone production. Bovine granulosa cells were cultured in the absence (■) or presence (▩) of KGF. Additionally, cells were treated with hCG as a positive control. Progesterone accumulation was determined on day 3 of culture and normalized per μg DNA (ng progesterone/μg DNA). Data are presented as mean ± SEM of triplicate determinations from three different experiments. An ANOVA was performed, and significant differences between treatments were determined using the Tukey-Kramer HSD test. *Bars* with different *superscript letters* differ from each other ($P < 0.05$).

development (Fig. 1). Levels of KGF and KGFR mRNAs were highest in large follicles (Figs. 2 and 3). Large follicles contain large numbers of proliferating granulosa cells. The high expression of KGF and KGFR in large follicles suggests that KGF actions may be involved in this cellular proliferation. KGF has previously been shown to stumunalte granulosa cell growth (8). These results demonstrate that expression levels of KGF and KGFR mRNA are developmentally regulated during follicular development. Additional studies are necessary to determine whether expression of KGF protein is also developmentally regulated in the ovarian follicle. It remains possible that mRNA levels of these genes vary during follicular development, whereas protein levels may not change. However, the observation that gene expression of both KGF and KGFR is highest in large follicles suggests that increased production of KGF is important in large follicles.

Endocrine hormones (*i.e.* gonadotropins and steroids), such as estrogen and LH, stimulate follicular growth (10–14). The actions of such hormones in the ovary are necessary for follicular development and reproductive viability. Although these hormones increase follicular growth *in vivo*, proliferative effects are not apparent on purified cells *in vitro*. These observations led to the hypothesis that estrogen and other hormones indirectly stimulate follicular growth by influencing mesenchymal-epithelial cell interactions in the ovary. This hypothesis is supported by the results in Fig. 5. Both estrogen and hCG (an LH agonist) treatment increased KGF mRNA levels in purified theca cells. These results suggest that KGF is an estromedin (*i.e.* local mediator of estrogen action) in the ovarian follicle. In addition, KGF may, in part, mediate the actions of LH in the ovary. The regulation of KGF expression by estrogen and LH helps explain the mechanism of hormone-induced growth in the ovary. Endocrine hormones may stimulate follicular growth by stimulating the local production of growth factors, such as KGF.

Factors that stimulate cellular proliferation generally inhibit differentiated functions. Many growth factors stimulate DNA synthesis in a particular cell by promoting entry of the cell into the cell cycle (48–52). Progression of the cell into the cell cycle results in the indirect effect of reducing the differentiated functions of the cell (53–58). KGF has been shown to stimulate granulosa cell proliferation (8), but no information has been available on the effects of KGF on granulosa cell differentiation. The current results suggest that KGF can indirectly inhibit granulosa cell differentiated functions. KGF reduced basal levels or hormone-stimulated levels of aromatase activity and progesterone production. Rat granulosa cells were included in these experiments because rat granulosa cells have a higher level of aromatase expression in culture than bovine granulosa cells. Similar effects were observed on bovine and rat granulosa cells. These inhibitory effects of KGF on steroid production were similar to the

inhibitory actions of TGF-α on granulosa cells (45). Roberts demonstrated that TGF-α also stimulates proliferation and inhibits steroid production by bovine granulosa cells.

This study shows that KGF may be an important mediator of mesenchymal-epithelial cell interactions during ovarian follicular development. Results demonstrate that KGF in theca cells, and KGFR in granulosa cells, are expressed throughout follicular development and are more highly expressed in large follicles. Estrogen and LH are known to directly act on theca cells and stimulate follicular growth *in vivo*. This study demonstrated that both estrogen and hCG (an LH agonist) increase KGF steady-state mRNA levels in purified theca cells. Therefore, KGF, in part, mediates the actions of endocrine hormones, such as estrogen and LH, in the ovary. Other locally produced growth factors may also mediate the actions of these hormones in the ovary. The rapid proliferation of granulosa cells is a critical aspect of follicular development and is proposed to be controlled through mesenchymal-epithelial cell interactions between theca cells and granulosa cells. Future studies to investigate the significance of KGF on follicle growth and development have been initiated and will examine inhibiting the actions of KGF in ovary organ cultures to assess effects on follicle development. The current study helps to establish that KGF, in part, mediates hormone-induced follicular growth and helps gain an understanding of factors that control mesenchymal-epithelial cell interactions in the ovary.

Acknowledgments

We thank Andrea Cupp, John Gordon, Linda Miyashiro, and Urvashi Patel for technical assistance with the ovary preparation and Steve Zippin for assistance with the progesterone RIA. We thank Naoki Itoh for providing continuous support and discussions concerning quantitative RT-PCR. We also thank Jaideep Chaudhary, Elena Levine, and B. Grant for special assistance.

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