

# Developmental and Hormonal Regulation of Hepatocyte Growth Factor Expression and Action in the Bovine Ovarian Follicle<sup>1</sup>

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## ABSTRACT

Ovarian hormones (i.e., estrogen and LH) may promote folliculogenesis by regulating the local production of mesenchymal “inducer proteins” that mediate theca cell-granulosa cell interactions. Theca cells produce hepatocyte growth factor (HGF) that can stimulate granulosa cell growth. In order to investigate the physiological role of HGF in the ovarian follicle, the developmental and hormonal regulation of HGF was examined during follicular development in the bovine ovary. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to examine HGF expression in theca cells and the HGF receptor (HGFR or *c-met*) in granulosa cells. Both HGF and HGFR were detected throughout follicular development in small (< 5 mm)-, medium (5–10 mm)-, and large (> 10 mm)-sized follicles. Steady-state levels of HGF and HGFR mRNAs were determined using sensitive quantitative RT-PCR assays. Developmental regulation of HGF in theca cells and HGFR in granulosa cells was analyzed in freshly isolated small-, medium-, and large-sized follicles. Observations demonstrate that expression of HGF (in theca cells) and HGFR (in granulosa cells) was highest in large-sized follicles. Hormonal regulation of HGF was analyzed in hormone-treated theca cell cultures. Steady-state levels of HGF mRNA in theca cells were increased by treatment with hCG (an LH agonist), but estradiol had no effect. These results suggest that LH may promote ovarian follicular growth (i.e., granulosa cell proliferation) in part by stimulating the local production of HGF by theca cells. Effects of HGF on granulosa cell differentiated functions were examined. Treatment with HGF reduced basal and FSH-stimulated levels of aromatase activity in bovine and rat granulosa cells. In addition, HGF inhibited the ability of hCG to stimulate progesterone production by granulosa cells. The inhibition of granulosa cell steroid production by HGF is proposed to be the indirect effect of promoting cellular proliferation. Therefore, HGF directly stimulates granulosa cell proliferation and indirectly inhibits granulosa cell differentiated functions. The developmental and hormonal regulation of HGF and HGFR during folliculogenesis provides evidence that HGF may be important for hormone-induced granulosa cell proliferation. As a result, HGF may be essential for establishing the granulosa cell population and microenvironment required for oocyte maturation in the female.

## INTRODUCTION

The development and function of essentially every organ is influenced by paracrine interactions between mesenchymal cells and epithelial cells. It has long been recognized that growth and differentiation of epithelial cells is directed by adjacent mesenchymal cells during embryonic devel-

opment and optimally maintained by adjacent stroma in adult tissues [1–5]. Identification and characterization of inducer proteins that mediate these mesenchymal-epithelial cell interactions are necessary for understanding of the mechanisms by which cells communicate in an organ. The ovarian follicle is a useful model system for studying these cell-cell interactions.

Hepatocyte growth factor (HGF) is produced by mesenchymal-derived theca cells and stimulates epithelial granulosa cell proliferation during follicular development 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 [7]. Factors such as HGF that promote the growth of the granulosa cell population help establish and maintain 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 [8–12]. Therefore the effects of estrogen and LH on follicular growth in vivo are proposed to be indirectly mediated by locally produced growth factors such as HGF.

HGF is an 87-kDa protein composed of a 69-kDa  $\alpha$  subunit and a 34-kDa  $\beta$  subunit that is important for the organogenesis and morphogenesis of various tissues and organs [13–19]. HGF is primarily produced by mesenchymal-derived cells in many tissues and acts as an epithelial cell mitogen. The receptor to HGF (HGFR) is the product of the *c-met* protooncogene that is primarily localized to epithelial cells [20–24], but it can also be expressed by macrophages, neurons, endothelial cells, muscle cells, and cytotrophoblasts [25–28]. Two alternatively spliced forms of HGF, known as NK1 and NK2, that may act as HGF agonists or antagonists have been documented [29–33]. Previous studies have documented the ability of HGF to mediate mesenchymal-epithelial cell interactions in many tissues such as the hair follicle [34], bone [35], prostate [36, 37], stomach [38], pancreas [39], heart [40], tooth [41], blood cells [25], and muscle [42]. Expression of HGF has been shown to be regulated in an endocrine manner in many tissues including kidney, spleen, lung, and prostate [14, 36, 43–45]. These and other studies have established that HGF 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 HGF as a paracrine mediator of mesenchymal-epithelial cell interactions during ovarian follicular development. Experiments addressed the developmental and hormonal regulation of HGF in theca cells and the HGFR in granulosa cells.

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## MATERIALS AND METHODS

### *Tissue Isolation and 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 [46]. Theca interna layers were then microdissected away from the follicle wall and enzymatically dispersed with 2 mg/ml collagenase (Sigma, St. Louis, MO) in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -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%  $\text{CO}_2$  atmosphere. The indicated cells were treated with estradiol ( $10^{-7}$  M; Sigma), 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 as containing less than 3% contamination with endothelial cells. Rat granulosa cells were isolated as previously described [47] from 20-day estrogen-stimulated rats. Protocols for the use of rats were approved by the university animal care committee. The rat granulosa cells were cultured and maintained similarly to the bovine cell cultures [46].

### *Preparation of RNA and Polymerase Chain Reaction (PCR)*

Follicles were dissected from the bovine ovaries and separated into pools of small (< 5 mm)-, medium (5–10 mm)-, and large (> 10 mm)-sized follicles. Cells were pooled across ovaries from the follicular phase of the cycle. 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 [48]. Alternatively, total RNA was prepared using the RNA-Stat 60 kit (Tel-Test, Friendswood, TX). For qualitative analysis of gene expression, 10  $\mu\text{g}$  total RNA was reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 37°C for 1 h using oligo(dT)<sub>12–18</sub> primers (Gibco BRL). This cDNA template was amplified by PCR using specific primers for HGF, HGFR (*c-met*) or the constitutively expressed gene cyclophilin (1B15). The HGF primers were 5'-ACA GCT TTT TGC CTT CGA GCT ATC GGG GTA AAG ACC TAC AGG-3' (5' primer, 42 mer) and 5'-CAT CAA AGC CCT TGT CGG GAT A-3' (3' primer, 22 mer), which generated a specific 292-base pair (bp) HGF PCR product from bovine theca cells. The HGFR primers were 5'-GTA AGT GCC CGA AGT GTA AG-3' (5' primer, 20 mer) and 5'-GCC CTC TTC CTA TGA CTT C-3' (3' primer, 19 mer), which generated a specific 313-bp HGFR PCR product from bovine granulosa cells. The primers for cyclophilin (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  $\mu\text{M}$  each primer, 100  $\mu\text{M}$  dNTPs, 1.5 mM  $\text{Mg}^{2+}$ , 1.25 U *Taq* polymerase in 50  $\mu\text{l}$  total volume. Each PCR amplification consisted of an initial denaturing reaction (5 min, 95°C); 35 cycles of denaturing (30 sec, 95°C), an-

nealing (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 HGF, HGFR, and 1B15 PCR products were subcloned into the Bluescript plasmid (Stratagene, La Jolla, CA) at the *Sma* I site. All subclones were sequenced in both directions and confirmed to be bovine HGF, HGFR, and 1B15. These subclones were used as standard templates in the quantitative PCR procedure described below.

### *Quantitative RT-PCR Assay*

Steady-state levels of HGF, HGFR, and 1B15 mRNAs were measured using a specific quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay for each gene. The primers used in this quantitative analysis of HGF, HGFR, and 1B15 were the same as described above. 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  $\mu\text{g}$ ) was reverse transcribed for 1 h at 37°C using the following conditions: 1  $\mu\text{g}$  total RNA, 1  $\mu\text{M}$  specific 3'-primers of interest (up to 4 different primers including 1B15), 0.1 mM dNTPs, 10 mM dithiothreitol, 40 U RNase inhibitor (Promega, Madison, WI), and 200 U MMLV reverse transcriptase (Gibco BRL) in 40  $\mu\text{l}$  RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM  $\text{MgCl}_2$ ). After 1 h, samples were heated to 95°C for 5 min to inactivate the reverse transcriptase enzyme. Samples were immediately diluted 2.5-fold, and carrier DNA (Bluescript plasmid; Stratagene) was added to a final concentration of 10 ng/ $\mu\text{l}$ . This concentration of Bluescript carrier DNA (10 ng/ $\mu\text{l}$ ) was included in all subsequent dilutions of samples and standards. Immediately before amplification, each unknown sample was further diluted 1:10 in order to improve the fidelity of the PCR [49]. Plasmid DNAs containing bovine HGF, HGFR, or 1B15 subclones were used to generate standard curves from 1 ag/ $\mu\text{l}$  ( $10^{-15}$  g/ $\mu\text{l}$ ) to 10 pg/ $\mu\text{l}$  ( $10 \times 10^{-9}$  g/ $\mu\text{l}$ ) each containing 10 ng/ $\mu\text{l}$  Bluescript carrier DNA. Identical 10- $\mu\text{l}$  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  $\mu\text{M}$  each primer, 16  $\mu\text{M}$  dNTPs, and 1.25 U AmpliTaq polymerase in 50  $\mu\text{l}$  GeneAmp PCR buffer (containing 1.5 mM  $\text{MgCl}_2$ ; 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  $\mu\text{Ci}$  of  $^{32}\text{P}$ -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 PhosphoImager (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 to the appropriate standard curve. All HGF and HGFR data were normalized for 1B15.

Optimal cycle number for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity (i.e., logarithmic phase of PCR reactions). Both HGF and HGFR quantitative PCR products were amplified for 31 cycles, while the 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/ $\mu$ g total RNA. For each assay, all samples were simultaneously measured in duplicate, resulting in intraassay variabilities of 13.6% (HGF), 8.7% (HGFR), and 6.5% (1B15).

#### Aromatase Activity Assay

Aromatase activity was assessed by the release of  $^3\text{H}_2\text{O}$  from aromatization of [ $1\beta$ - $^3\text{H}$ ]androstenedione as previously described [50] with the following modifications. Approximately 1.5 million/cm<sup>2</sup> granulosa cells were plated in 24-well plates and immediately treated with no factor (control), HGF (50 ng/ml), FSH (100 ng/ml), or FSH+HGF. After 20 h, 0.5 ml medium containing 0.5 mCi [ $1\beta$ - $^3\text{H}$ ]androstenedione (New England Nuclear, Boston, MA) was added, and incubation was carried out for 6 h at 37°C in a CO<sub>2</sub> 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% (w:v) 70K dextran (Sigma), activated 2% (w:v) charcoal, 10 mM Tris, and 1 mM EDTA, pH 7.5, for 2 h at 4°C; it was then centrifuged at 13 000  $\times$  g for 15 min. Aliquots of the supernatant containing  $^3\text{H}_2\text{O}$  were then combined with 5 ml scintillation cocktail (ICN, Costa Mesa, CA) and counted. Blank values were established from identical incubations in the absence of cells, which contained less than 100 cpm. Aromatase activity, measured in counts per minute of  $^3\text{H}_2\text{O}$  released, was normalized per  $\mu$ g DNA using an ethidium bromide procedure, described previously [46].

#### Progesterone Assay

Progesterone production by granulosa cells was determined by quantitating progesterone accumulation in the culture medium. Approximately 1.5 million/cm<sup>2</sup> granulosa cells were plated in 24-well plates and immediately treated with no factor (control), HGF (50 ng/ml), hCG (100 ng/ml), or hCG+HGF. After 72 h the medium was collected and assayed for progesterone using the ImmunoChem <sup>125</sup>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 [46].

#### Statistical Analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute Inc., Cary, NC). Effects of hormones on HGF were examined by a one-way ANOVA. Significant differences between treated cells and control (untreated) cells were determined using Dunnett's test, which guards against the high alpha size (type I) error rate across the hypothesis tests [51]. Effects of follicle size on steady-state HGF or HGFR mRNA levels were examined by a one-way ANOVA as described above. Significant differences between small-, medium-, and large-sized follicles were determined using the Tukey-Kramer HSD (honestly significant difference) test, which protects the significance

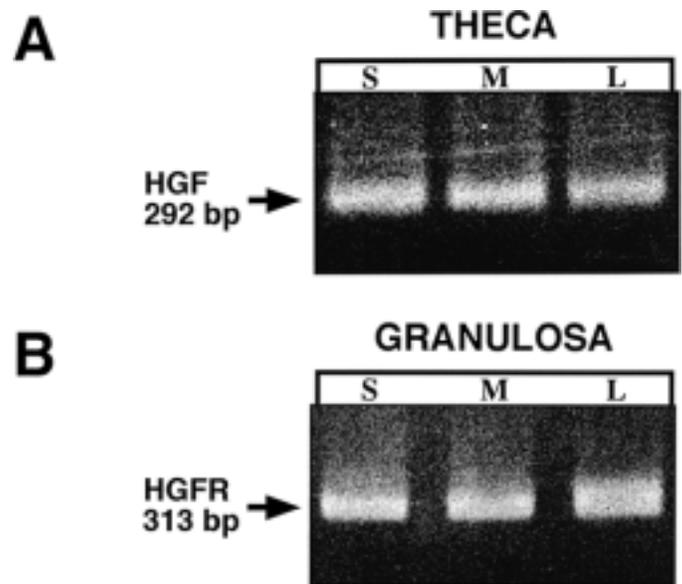


FIG. 1. Expression of HGF and HGFR mRNA in bovine ovarian follicles. RT-PCR analysis was performed with 10  $\mu$ g total RNA. Amplification was nonquantitative. **A**) Specific primers were designed to amplify HGF from bovine theca cDNA template; 292-bp HGF PCR products were detected in theca cells from small-, medium-, and large-sized follicles. **B**) Specific primers were designed to amplify HGFR from bovine granulosa cDNA template; 313-bp HGFR PCR products were detected in granulosa cells from small-, medium-, and large-sized 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.

tests of all combinations of pairs [52–54]. Effects of HGF, estradiol, and hCG on granulosa cell aromatase activity and progesterone production were analyzed by one-way ANOVA as described above; significant differences between control (untreated), HGF, FSH, and HGF+FSH (for aromatase activity) and between control (untreated), HGF, hCG, and HGF+hCG were determined using the Tukey-Kramer HSD test. These multiple comparisons tests are recommended for multiple comparisons with control (Dunnett's) or multiple comparisons of all pairs (Tukey-Kramer HSD) [55, 56].

#### RESULTS

Mesenchymal theca cells and epithelial granulosa cells were obtained from pools of small (< 5 mm diameter)-, medium (5–10 mm)-, and large (> 10 mm)-sized follicles from freshly isolated bovine ovaries. The gene expression of HGF and HGFR was determined by RT-PCR. Using appropriate primers, the 292-bp HGF PCR product was observed in theca cell RNA isolated from small-, medium-, and large-sized follicles (Fig. 1A). The 313-bp HGFR PCR product was observed in granulosa cell RNA from small-, medium-, and large-sized follicles (Fig. 1B). These PCR products were blunt-end subcloned into the Bluescript plasmid, sequenced, and confirmed to be bovine HGF and HGFR cDNA (data not shown). Previous studies have shown HGF mRNA expression in bovine theca cells [6] and HGFR mRNA expression in mouse granulosa cells [57]. The results in Figure 1 indicate that HGF and HGFR are expressed throughout follicular development, since HGF and HGFR mRNAs were observed from small-, medium-, and large-sized follicles.

In order to examine the potential developmental regu-

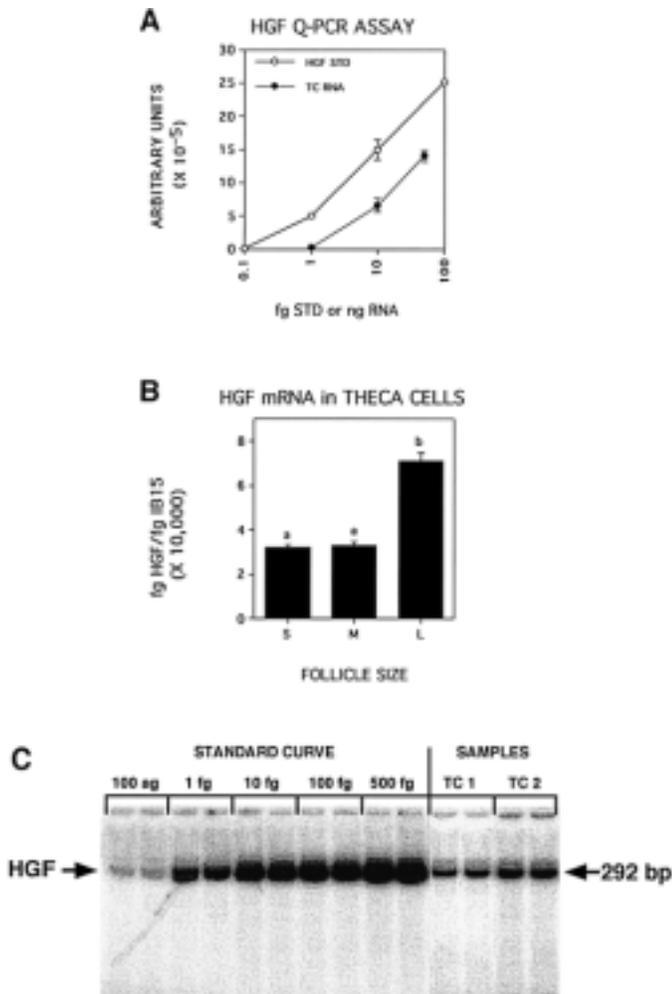


FIG. 2. Developmental regulation of HGF mRNA in bovine theca cells. Steady-state levels of HGF mRNA in theca cells from small, medium, and large follicles were determined using quantitative RT-PCR. **A)** Line graph of a typical experiment validating the assay. Parallel curves of a standard bovine HGF subclone (open circles) and a fresh theca cell total RNA sample (closed circles) are shown. Raw data are represented as arbitrary units  $\pm$  SEM and are directly proportional to actual counts per minute. **B)** Analysis of steady-state HGF mRNA levels in theca cells from small-, medium-, and large-sized follicles. Levels of HGF mRNA were determined and normalized to levels of cyclophilin (1B15) mRNA (fg HGF mRNA/fg 1B15 mRNA). Data are presented as the mean  $\pm$  SEM of duplicate determinations from at least 10 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 separation of HGF PCR products in a 4% polyacrylamide gel with five standards and two theca cell samples shown in duplicate.

lation of HGF and HGFR during follicular development, sensitive quantitative RT-PCR assays were developed for both genes. HGF steady-state mRNA levels in theca cells and HGFR steady-state mRNA levels in granulosa cells were determined in total RNA samples from small-, medium-, and large-sized follicles. These assays utilized the bovine HGF and HGFR subclones shown in Figure 1 as template to generate standard curves. Specific reverse transcription and amplification conditions were utilized as described in *Materials and Methods*. These quantitative assays for HGF and HGFR mRNAs are extremely sensitive ( $< 10^{-12}$  g/sample) and have intraassay variabilities of 13.6% and 8.7%, respectively. As shown in Figures 2 and 3, each assay is linear over several orders of magnitude

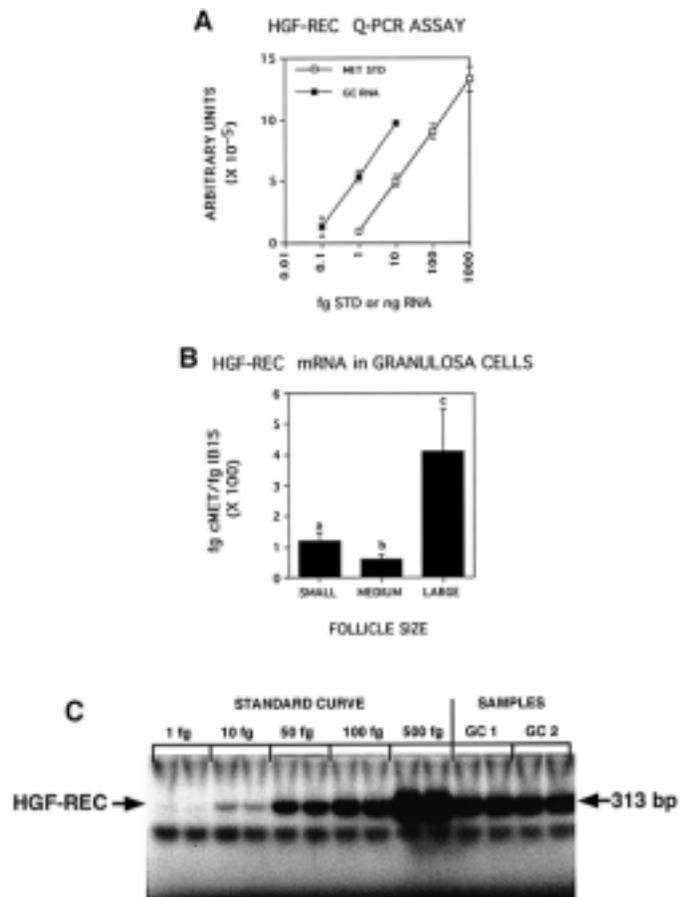


FIG. 3. Developmental regulation of HGFR mRNA in bovine granulosa cells. Steady-state levels of HGFR mRNA in granulosa cells from small, medium, and large follicles were determined using quantitative RT-PCR. **A)** Line graph of a typical experiment validating the assay. Parallel curves of a standard bovine HGFR subclone (open squares) and a fresh granulosa cell total RNA sample (solid squares) are shown. Raw data are represented as arbitrary units  $\pm$  SEM and are directly proportional to actual counts per minute. **B)** Analysis of steady-state HGFR mRNA levels in granulosa cells from small-, medium-, and large-sized follicles. Levels of HGFR mRNA were determined and normalized to levels of cyclophilin (1B15) mRNA (fg HGFR mRNA/fg 1B15 mRNA). Data are presented as the mean  $\pm$  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 separation of HGFR PCR products in a 4% polyacrylamide gel with five standards and two granulosa cell samples shown in duplicate.

(0.1–1000 fg/sample). Each assay was validated by demonstrating apparent parallel dilution curves between the appropriate RNA samples and standards (Figs. 2A and 3A). Separation of PCR products in 4% polyacrylamide gels demonstrated minimal background and precise duplicates. All samples were normalized for constitutively expressed cyclophilin mRNA (1B15) as determined by the same procedure. The expression of 1B15 was found to be constitutive; it did not vary with the developmental or hormonal status of the cells. Consequently, 1B15 expression corrected for changes in cell number (data not shown). This normalization also corrected for the amount and integrity of initial mRNA as well as small differences in the efficiency of reverse transcription between samples. The results are shown in Figures 2B and 3B. The steady-state levels of HGF mRNA in theca cells was highest in large-sized follicles and lower in small- and medium-sized follicles (Fig.

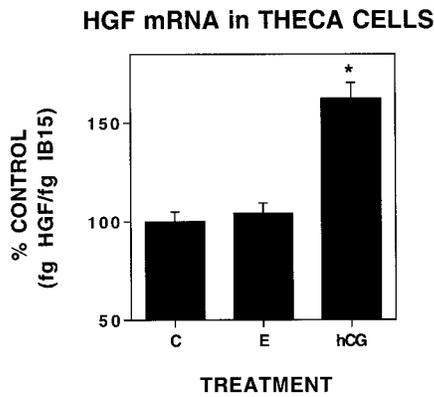


FIG. 4. Hormonal regulation of HGF mRNA in bovine theca cells. Steady-state levels of HGF mRNA in cultured theca cells were determined using quantitative RT-PCR. Theca cells were placed in serum-free culture and treated with no factor (control),  $10^{-7}$  M estradiol (E), or 100 ng/ml hCG. After 72 h in culture, cells were harvested and total RNA was prepared. Steady-state levels of HGF mRNA were determined as described for Figure 2. Levels of HGF mRNA were determined and normalized to levels of cyclophilin (1B15) mRNA (fg HGF mRNA/fg 1B15 mRNA). Data are presented as the mean  $\pm$  SEM of duplicate determinations from six 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 ( $p < 0.05$ ).

2B). Steady-state levels of HGFR were dramatically higher in granulosa cells from large-sized follicles than in small- or medium-sized follicles (Fig. 3B). In addition, steady-state levels of HGF mRNA in theca cells (0.0003–0.0005 fg HGF/fg 1B15) were two orders of magnitude lower than the levels of HGFR mRNA in granulosa cells (0.005–0.04 fg HGFR/fg 1B15). These results demonstrate that the HGF and HGFR genes are developmentally regulated during normal follicular development. Both HGF (in theca cells) and HGFR (in granulosa cells) were highest in large-sized follicles. Therefore, stimulation of HGF and HGFR expression may be important for granulosa cell proliferation in large-sized follicles.

It has been proposed that HGF expression is regulated by cytokines, steroids, and other extracellular signals through the activation of multiple putative *cis*-acting elements in the HGF promoter [23, 37, 58–61]. The hypothesis that estrogen and LH indirectly control follicular development by regulating the local production of HGF was tested. Fresh theca cells were placed in serum-free culture and treated with estradiol or hCG, an LH agonist. After 3 days, theca cell total RNA was isolated, and steady-state levels of HGF mRNA were measured using quantitative RT-PCR. The results are shown in Figure 4. Under these conditions, hCG stimulated HGF mRNA in bovine theca cells, but estradiol had no effect. These results are the first to demonstrate regulation of the HGF gene by a gonadotropin (e.g., hCG/LH). The potential regulation of ovarian HGF expression by other factors remains to be elucidated.

Granulosa cell steroidogenesis was examined to investigate the actions of HGF in the ovary. Granulosa cells were cultured in serum-free media and assayed for two functional markers [46]. The first functional parameter examined was the ability of granulosa cells to aromatize androstenedione to produce estradiol. Both bovine and rat granulosa cells were utilized to quantitate aromatase activity in the presence or absence of HGF. These results are shown in Figure 5. HGF significantly reduced basal levels of aromatase activity in bovine and rat granulosa cells. In addition, HGF reduced the ability of FSH to stimulate aroma-

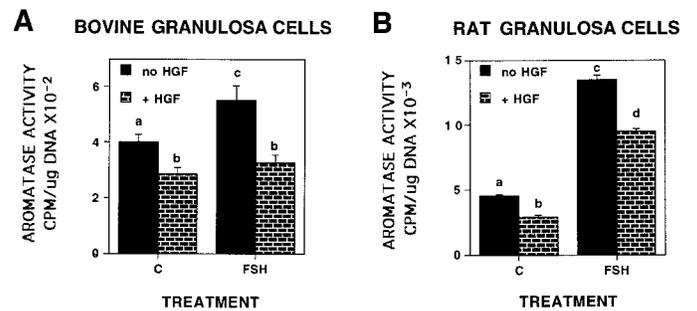


FIG. 5. HGF inhibits granulosa cell aromatase activity. Bovine (A) or rat (B) granulosa cells were cultured in the absence (solid bars) or presence (bricked bars) of HGF. Additionally, cells were treated with FSH as a positive control. Bovine granulosa cells can be isolated in large numbers, but rat granulosa cells express higher levels of aromatase in culture. Aromatase activity was determined on Day 1 of culture and normalized per  $\mu$ g DNA (cpm/ $\mu$ g DNA). Data are presented as mean  $\pm$  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 ( $p < 0.05$ ).

tase activity (Fig. 5). A similar effect has been observed with transforming growth factor alpha in the ovary [62]. The current results demonstrate that HGF can negatively regulate granulosa cell function.

The second functional parameter examined was progesterone production. Bovine granulosa cells were cultured in serum-free media for 72 h in the absence or presence of HGF. Cells were stimulated with hCG as a positive control. After 72 h, media were collected and progesterone accumulation was determined by RIA. The results for granulosa cell progesterone production are shown in Figure 6. HGF had no effect on basal levels of progesterone production but dramatically inhibited the ability of hCG to stimulate progesterone. Granulosa cells primarily express aromatase during the follicular phase or early stages of folliculogenesis. During the luteal phase or later stages of folliculogenesis, granulosa cells produce increasing amounts of progesterone. The observation that HGF influences both aromatase activity and progesterone production in granulosa

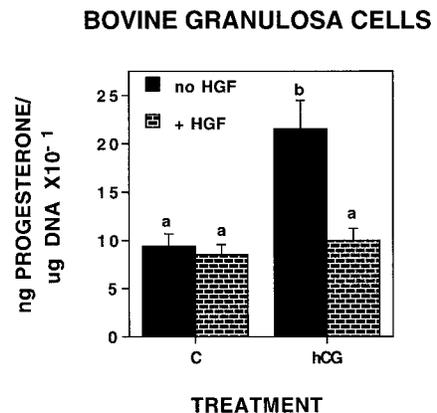


FIG. 6. HGF inhibits granulosa cell progesterone production. Bovine granulosa cells were cultured in the absence (solid bars) or presence (bricked bars) of HGF. Additionally, cells were treated with hCG as a positive control. Progesterone accumulation was determined on Day 3 of culture and normalized per  $\mu$ g DNA (ng progesterone/ $\mu$ g DNA). Data are presented as mean  $\pm$  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 ( $p < 0.05$ ).

cells suggests that HGF can act on granulosa cells during the follicular and luteal phases of follicle development.

## DISCUSSION

HGF is a candidate inducer protein that mediates mesenchymal-epithelial cell interactions in many tissues including the ovary [6, 57, 61, 63]. Endocrine hormones have been shown to act on tissues such as skin [59, 60, 64] and prostate [36, 37] by locally regulating the production and actions of growth factors such as HGF. In the ovary, HGF is expressed by theca cells and acts on granulosa cells [6]. The current study was designed to examine the developmental and hormonal regulation of HGF in theca cells and HGFR in granulosa cells.

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 [7]. The number of granulosa cells increases at least 10 000-fold during development from preantral to ovulatory follicles. The rate of proliferation is highest in large-sized follicles [65]. Theca cell-derived HGF is proposed to be important for this growth process. HGF and HGFR gene expression in theca cells and granulosa cells, respectively, was observed throughout follicular development (Fig. 1). Expression of HGF and HGFR was highest in large-sized follicles (Figs. 2 and 3). Large-sized ovarian follicles contain increased numbers of proliferating granulosa cells. The high level of expression of HGF and HGFR in large-sized follicles suggests that HGF actions may be involved in this cellular proliferation. These results demonstrate that expression levels of HGF and HGFR mRNA are developmentally regulated during follicular development. Additional studies are necessary to determine whether expression of HGF protein is also developmentally regulated in the ovarian follicle. It remains possible that mRNA levels of these genes vary during follicular development while protein levels may not change. The observation that HGF and HGFR gene expression is highest in large-sized follicles suggests that increased production of HGF may be important prior to ovulation.

Endocrine hormones (i.e., gonadotropins and steroids) such as estrogen and LH stimulate follicular growth *in vivo* [8–12]. The actions of such hormones in the ovary are necessary for follicular development and reproductive viability. Although these hormones increase follicular growth *in vivo*, no proliferative effect is apparent on purified cells *in vitro*. As a result, the hypothesis has developed that LH and other hormones indirectly stimulate follicular growth by influencing mesenchymal-epithelial cell interactions in the ovary [6, 61]. This hypothesis is supported by the results shown in Figure 4. Steady-state levels of HGF mRNA in purified theca cells were increased by treatment with hCG (an LH agonist). LH is known to stimulate theca cell functions through the cAMP pathway. In addition, cAMP analogues have been shown to stimulate HGF gene expression in other tissues [37, 58, 60]. As a result, LH stimulation of HGF expression may be mediated by activation of a cAMP pathway in theca cells. Previously, a number of regulatory agents have been shown to influence HGF expression. Phorbol esters (e.g., 12-*O*-tetradecanoylphorbol 13-acetate) [58, 59], serum, cAMP analogues (e.g., 8-bromo-cAMP and dibutyl cAMP) [37, 58, 60], and cAMP-elevating agents (e.g., forskolin, cholera toxin, and prostaglandin E<sub>2</sub>) [60] regulate HGF mRNA in cultured skin fibroblasts. In addition, two estrogen response element (ERE) sequences are present in the promoter and first intron of

the HGF gene that mediate estrogen-induced transcription of the HGF promoter in RL95–2 cells [61]. Results suggest that HGF in part mediates the actions of LH in the ovary. The ability of hCG (i.e., LH) to stimulate HGF expression in theca cells helps explain the mechanism of hormone-induced growth in the ovarian follicle. Endocrine hormones such as LH may stimulate follicular growth by stimulating the local production of growth factors such as HGF.

Estrogen did not directly stimulate HGF mRNA expression in theca cells. This result is surprising, since a single injection of estradiol resulted in a transient increase in HGF mRNA levels in the ovaries of immature mice [61]. The HGF promoter has been cloned, and it contains many recognizable regulatory elements that are important for transcription in mesenchymal cells [23, 66, 67]. Two ERE are present, including one in the first intron of the HGF gene (mouse positions –872 and +511) [61, 66]. Liu et al. [61] demonstrated that these ERE elements can confer estrogen action to homologous and heterologous promoters when transfected into endometrial carcinoma RL95–2 cells and mouse fibroblast NIH 3T3 cells. In addition, nuclear extracts from either estrogen receptor-transfected RL95–2 cells, or from mouse liver, bound to the ERE elements in gel-shift assays *in vitro*. The results in the current study suggest that the ERE sequences in the HGF promoter may not confer estrogen responsiveness in the ovarian follicle. The lack of estrogen action on theca cell HGF mRNA levels was not due to the inability of the cells to respond to estrogen. Additional analysis demonstrated that estrogen treatment increased keratinocyte growth factor mRNA levels in the same theca cell cultures [68]. The theca cells used in these experiments were pooled from several ovarian follicles. It is possible that estrogen may regulate theca cell HGF expression at specific stages of the estrous cycle. Such cycle-specific effects may not be observed in these pooled theca cell cultures. Analysis of estrous cycle-specific regulation of HGF in theca cells will require the quantitation of HGF mRNA in individually staged follicles. An additional possibility is that estrogen does not regulate HGF expression in theca cells.

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 [69]. Progression of the cell into the cell cycle results in the indirect effect of reducing the differentiated functions of the cell [70]. HGF has been shown to stimulate granulosa cell proliferation [6], but no information has previously been available concerning the effects of HGF on granulosa cell differentiated functions. The current results suggest that HGF inhibits granulosa cell differentiated functions. This effect was observed as the inhibition of basal or hormone-stimulated aromatase activity and progesterone production. Bovine granulosa cells can be isolated in large quantities but contain low levels of aromatase after 48 h in culture. Rat granulosa cells have more robust levels of aromatase and maintain aromatase activity for several days *in vitro*. Similar effects were observed on bovine and rat granulosa cells. The inhibitory effects of HGF on granulosa cell steroid production were similar to the actions of another growth factor produced in the ovary. Roberts and Skinner [62] demonstrated that transforming growth factor alpha also stimulates proliferation and inhibits steroid production by bovine granulosa cells. A general phenomenon may exist in which ovarian growth factors that stimulate cellular proliferation also inhibit differentiated functions.

Previous studies have described HGF as an important mediator of mesenchymal-epithelial cell interactions in many tissues. This study shows that HGF may be an important mediator of mesenchymal-epithelial cell interactions during ovarian follicular development. Higher levels of HGF and HGFR in large-sized follicles may be important for the rapid proliferation of granulosa cells at this stage of development. Results demonstrate that HGF in part mediates the actions of endocrine hormones such as LH but may not be a direct estromedin in the ovary. Other locally produced growth factors may also mediate the actions of LH 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. This study helps to establish that HGF in part mediates the stimulatory effects of LH on follicular growth and helps provide an understanding of factors that control mesenchymal-epithelial cell interactions in the ovary.

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