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Mesenchymal-Epithelial Interactions in the Ovarian Follicle Involve Keratinocyte and Hepatocyte Growth Factor Production by Thecal Cells and Their Action on Granulosa Cells*

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ABSTRACT

Mesenchymal-epithelial cell interactions between thecal and granulosa cells in bovine ovarian follicles were investigated. Experiments were designed to examine the local production and action of two mesenchymal (stromal)-derived growth factors, keratinocyte and hepatocyte growth factors (KGF and HGF). Using reverse transcription-polymerase chain reaction, gene expression for KGF and HGF was detected in the mesenchymal-derived thecal cells, but not in the epithelial granulosa cells. The bovine polymerase chain reaction products for KGF and HGF were sequenced and found to be similar to known mouse, rat, and human sequences. The bovine KGF sequence was found to have a high degree of identity (86–95%) with the other species, whereas bovine HGF has a lesser degree of identity (60–63%). Immunoprecipitation of radiolabeled thecal cell secreted proteins with a KGF antibody demonstrated production of the 28-kilodalton (kDa) KGF protein. An immunoblot of thecal cell secreted proteins with HGF antibodies detected the 87-kDa HGF as well as relevant 69- and 34-kDa subunits. Therefore, thecal cells were found to express the genes and secrete the proteins for KGF and HGF. Granulosa cells had no detectable KGF or HGF expression. Treatment with recombinant KGF or HGF stimulated the proliferation of granulosa cells, but not thecal cells. Therefore, the actions of KGF and HGF in the ovarian follicle appear to be restricted to granulosa cells. The combined results indicate that KGF and HGF are produced locally in the bovine ovarian follicle by thecal cells, and that both of these growth factors can act on granulosa cells to influence cell proliferation. These observations demonstrate that KGF and HGF can mediate mesenchymal-epithelial cell interactions between thecal and granulosa cells. The potential importance that the mesenchymal derived thecal cells may have in ovarian follicle development is discussed. (*Endocrinology* **135:** 569–575, 1994)

MESENCHYMAL-epithelial cell interactions are present in essentially every organ and are one of the most common cell-cell interactions between different cell types. It has long been recognized that differentiation of epithelial cells is directed by adjacent mesenchymal cells during embryonic development and optimally maintained by adjacent stroma in adult tissues (1, 2). Although the importance of mesenchymal-epithelial cell interactions has been established for most tissues (3), the molecular mechanisms involved in these cell-cell interactions remain to be elucidated.

The ovarian follicle provides a useful model system to investigate mesenchymal-epithelial cell interactions in a hormonally responsive tissue. During each reproductive cycle, the epithelial-derived granulosa cells support the oocyte and form the antrum of the follicle. The mesenchymal-derived thecal cells surround both the follicle and the outer layer of granulosa cells to provide structural integrity for the follicle. Theca cells have been shown to provide androgen to granulosa cells as a substrate for estrogen production (4). Thecal cells also produce transforming growth factor-α and -β, which can regulate the granulosa and thecal cell proliferation required for follicle development (5–8). Therefore, mesenchymal-epithelial interactions between thecal and granulosa cells appear to be important for ovarian physiology and follicle development.

Two recently identified mesenchymal-derived growth factors that mediate mesenchymal-epithelial interactions are keratinocyte and hepatocyte growth factors (KGF and HGF). KGF is a fibroblast growth factor (FGF-7)-related molecule that was originally isolated from keratinocyte-conditioned medium and found to stimulate epithelial cell proliferation (9). KGF is a 28-kilodalton (kDa) protein that appears to be produced primarily by mesenchymal cells. KGF acts mainly as an epithelial cell mitogen (10). The receptor to KGF is a FGF receptor splice variant of the FGFR2 isoform that specifically binds KGF (11–15). The KGF receptor appears to primarily be localized on epithelial cells (15). HGF is an 87-kDa protein composed of a 69-kDa α-subunit and a 34-kDa β-subunit that was originally isolated from human and rabbit plasma and rat platelets on the basis of its ability to stimulate mitogenesis of rat hepatocytes (16–21). HGF stimulates the proliferation of a number of epithelial cell types, including kidney epithelial cell lines (22–24). In addition, HGF promotes tubule formation and morphogenesis (20, 25). HGF is a unique protein containing a kringle structure, with identity to the protease domain of plasmin (26–29). Interestingly an alternately spliced low mol wt form of HGF acts as an antagonist of HGF action (29, 30). The receptor for HGF has been identified as the c-*met* protooncogene, which is a tyrosine kinase receptor (31–33). The HGF receptor (*met*) is

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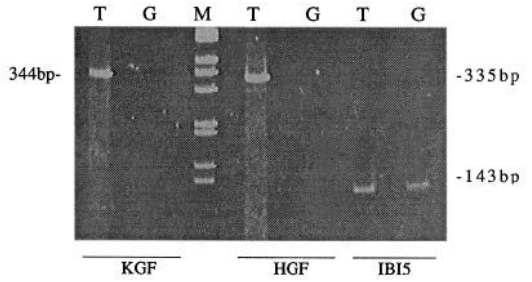

FIG. 1. Analysis of KGF and HGF gene expression. Reverse transcription-PCR analysis of KGF, HGF, and cyclophilin (IB15) messenger RNA in thecal cells (T) and granulosa cells (G). Basepair sizes are determined with respect to DNA mol wt standards (M). Data are representative of at least five experiments.

predominantly expressed in epithelial cells (34–36). Both KGF and HGF are useful candidates for mesenchymal growth factors that mediate mesenchymal-epithelial cell interactions.

The current study was designed to investigate the possible role that KGF and HGF may have in mediating theca-granulosa cell interactions in the ovarian follicle.

Materials and Methods

Tissue isolation and serum-free cell culture

Bovine ovaries were obtained from primarily young nonpregnant cycling heifers less than 10 min after slaughter. Ovaries were delivered fresh on ice by Golden Genes (Fresno, CA). Morphology was used to select follicular phase ovaries, and healthy developing follicles between 5–15 mm in diameter were isolated. Thecal and granulosa cells were isolated from fresh tissue and either used to prepare RNA or cultured as previously described (5, 8, 37, 38). Granulosa cells were shaken from the follicle, and the theca-interna shell was microdissected and cleaned of adhering granulosa and stromal cells. Cells were plated with an initial density of approximately 10^6 cells/2 cm^2 and maintained for 1–3 days at 37 C in a 5% CO_2 atmosphere in Ham's F-12 in the absence of serum (37, 38).

Immunoprecipitation and immunoblotting

Conditioned medium from thecal cell cultures was collected for 1–3 days and concentrated by ultrafiltration using a 3-kDa mol wt size exclusion membrane. For fluorography, cells were grown in methionine-free medium containing 5 μCi/ml [^{35}S]methionine. Before electrophoretic separation, the concentrated medium was treated with either a mouse monoclonal antibody directed against purified human KGF (generously provided by Dr. Jeff Rubin, NCI) or a polyclonal antibody raised in

(A) BOVINE KGF SEQUENCE

```
  1 TGACATGGAT CCTGCCAAGT TTGCTCTACA GATCATGCTT CCACATTATC TGTCTAGTGG
 61 GCACTATATC TTTAGCTTGC AATGACATGA CTCCAGAGCA AATGGCTACA AATGTGAACT
121 GTTCCAGCCC CGAGCGACAT ACAAGAAGTT ATGATTACAT GGAAGGAGGA GATATAAGAG
181 TGAGAAGACT CTTCTGTCGA ACACAGTGGT ATCTGAGGAT TGATAAACGC TAAGGCAAAG
241 TCAAAGGGAC TCAAGAGATG AAGAATAATT ACAACATCAT GGAAATCAGG ACCGTGGCAG
301 TTGGA
```

(B) SPECIES COMPARISON

```
BOVINE: TGACATGGATCCTGCCAAGTTTGCTCTACAGATCATGCTTCCACATTATCTGTCTAGTGG
HUMAN:  ------------------C--------------------T------------------
MOUSE:  -----C----------C-C----------------------C-CG-------------
RAT:    -----C----------G-C-CC----------C-G---------C-CG-------T----

BOVINE: GCACTATATCTTTAGCTTGCAATGACATGACTCCAGAGCAAATGGCTACAAATGTGAACT
HUMAN:  -T-------------------------------------------------------------
MOUSE:  ----------C------------------G---G-------C------G-G--------
RAT:    ----C----------------------G---------G-C---C--G-GC-------

BOVINE: GTTCCAGCCCCGAGCGACATACAAGAAGTTATGATTACATGGAAGGAGGAGATATAAGAG
HUMAN:  ----------T--------C-----------------------------G----------
MOUSE:  ------------------C--C----------C-------------G--------G-
RAT:    ----T-------------C--G----------C-------------G--------G-

BOVINE: TGAGAAGACTCTTCTGTCGAACACAGTGGTATCTGAGGATTGATAAACGCTAAGGCAAAG
HUMAN:  --------------------------------C--------C------A-...--------
MOUSE:  ----------G--------C--C--------C----------C------...--------
RAT:    ----G-----G--------C--C--------C----------C------...--------

BOVINE: TCAAAGGGACTCAAGAGATGAAGAATAATTACAACATCATGGAAATCAGGACCGTGGCAG
HUMAN:  -A--------C----------------------T----------------A--------
MOUSE:  -G--------C--G----------C-GC-----------------------------
RAT:    -G--------C--G-------G---C-GC------------------T---T-------

BOVINE: TTGGA
HUMAN:  -----
MOUSE:  -----
RAT:    -----
```

FIG. 2. DNA sequence of bovine KGF PCR product. Subcloned bovine KGF PCR product was sequenced from several primer sites within the vector, and a consensus of at least five sequences is shown. A, Partial DNA sequence of bovine KGF (305 bp). The *boxed nucleotides* indicate primer sequences and are not confirmed bovine sequence. B, Sequence comparison of bovine, human, mouse, and rat KGF sequence fragments. The bovine (*first*) sequence is shown, with the other sequences displayed as follows: *uppercase letters*, aligned nonidentical bases; —-, aligned identical bases; - - -, gap. Relative identities to the bovine sequence are: human, 95%; mouse, 90%; and rat, 86%.

(A) BOVINE HGF SEQUENCE

```
  1 ACAGCTTTTT GCCTTCGAGC TATCGGGGTA AAGACCTACA GGAAAACTAC TGTCGAAATC
 61 CTCGAGGGGA AGAAGGGGGA CCTTGGTGTT TCACAAGCAA TCCAGAGTTG AATGCATGAC
121 CTGCAATGGG GAAAGTTACC GAGGTCCCAT GGATCACACA GAAACAGGCA AGATTTGTCA
181 GCGCTGGGAT CATCAGACAC CACACCGGCA CAAATTCTTG CCAGAAAGAT ATCCCGACAA
241 GGGCTTTGAT GGGTTGATGC CGGTAAACTG TTGCNGCATA TCCCGACCAA GGGCTTTGAT
301 G
```

FIG. 3. DNA sequence of bovine HGF PCR product. Subcloned bovine HGF PCR product was sequenced from several primer sites within the vector, and a consensus of at least three sequences is shown. A, Partial DNA sequence of bovine HGF (301 bp). The *boxed nucleotides* indicate primer sequences and are not confirmed bovine sequence. B, Sequence comparison of human, bovine, mouse, and rat HGF sequence fragments. The human (*first*) sequence is shown, with the other sequences displayed as follows: *uppercase letters*, aligned nonidentical bases; *lowercase letters*, unaligned bases; —-, aligned identical bases; - - -, gap. Relative identities to the bovine sequence are: human, 63%; mouse, 60%; and rat, 63%.

(B) SPECIES COMPARISON

```
HUMAN: ACAGCTTTTTGCCTTCGAGCTATCGGGGTAAAGACCTACAGGAAAACTACTGTCGAAATC
BOVINE: ------------------------------------------------------------
MOUSE: ------------------------C-----------------------------------
  RAT: ------------------------C-----------------------------------

HUMAN: CTCGAGGGGAAGAAGGGGGACCCTGGTGTTTCACAAGCAATCCAGAGGTACGCTACGAAG
BOVINE: ---------------------T-----------------------T-.........---.
MOUSE: ------------------------------------------------------------
  RAT: ------------------------------------------------------------

HUMAN: TCTGTGACATTCCTCAGTG.....TTCAGAAGTTGAATGCATGAC.CTGCAA.TGGGGAG
BOVINE: -GCA----C-G-AATG-G-aaagt-A-C--G--CCC---G--C--a-A-A--cA--CA--
MOUSE: -------------------.....--------------------.-------.---T--A
  RAT: -------------------.....--------------------.-------.C--T--A

HUMAN: AGTTATCGAGGTCTCATGGATCAT.............ACAGAATCAGGC....AAGA
BOVINE: -T--G--.--..-G-TG-------cagacaccacaccggc---A-T--TT--caga----
MOUSE: --C--CA------C---------C.............------------....----
  RAT: --C--CA------C---------C.............------------....----

HUMAN: TTTGTC....AGCGC.....TGGGATCATCAGACACCACACCGGCACAAATTCTTGCCTG
BOVINE: -A-CC-gaca--G--tttga----T-G--.........G----..T---C-G----...
MOUSE: C-----....-----.....-----C--G--------------------G--------A-
  RAT: CA----....-----.....--------G----------------------------G-

HUMAN: AAAGATATCCCGA.CAAGGGCTTTGATG
BOVINE: .NGC---------c--------------
MOUSE: -------------.--------------
  RAT: -------------.--------------
```

rabbit, directed against human HGF (generously provided by Dr. Jeff Rubin, NCI). Immune complexes were then aggregated with 60 μl protein-A-Sepharose (Pharmacia, Piscataway, NJ) slurry in the presence of incubation NET buffer [NET buffer = 50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.25% gelatin, and 0.02% Na azide]. After incubation, the aggregated immune complexes were washed five times with NET buffer, the pellets were boiled in Laemmli buffer for 2–3 min, and the proteins were resolved by electrophoresis on a 10% polyacrylamide gel under reducing conditions. Gels were then fluorographed with diphenyloxazole in acetic acid, as previously described (39).

For immunoblotting, concentrated media were further purified on a 1-ml heparin-Sepharose column (Pharmacia). The retained proteins were eluted with a 1 M NaCl solution in 10 mM phosphate buffer, dialyzed overnight against 50 mM NH_4HCO_3, concentrated by drying in a Speed-Vac centrifuge (Savant Instruments, Hicksville, NY), resuspended in Laemmli buffer, and separated by electrophoresis on a 10% polyacrylamide gel under reducing conditions. The separated proteins were then transferred to a polyvinylidone difluoride membrane (Immobilon Millipore, South San Francisco, CA) by electrophoresis in Tris-glycine buffer containing 20% methanol. The blot was then blocked with 3% nonfat milk [dissolved in 50 mM Tris (pH 7.4), 150 mM NaCl, and 0.05% Nonidet P-40] for 1 h at room temperature and probed for 2 h with a polyclonal antibody directed against human HGF [1:500 dilution in 1% nonfat milk, 50 mM Tris (pH 7.4), 150 mM NaCl, and 0.05% Nonidet P-40]. After three washes (15 min each), the blot was hybridized with a secondary antibody (1:3000 dilution; directed against rabbit immunoglobulin G) conjugated to horseradish peroxidase for 1 h at room temperature. After five washes under the same conditions as described above, detection of the immune complexes was performed using the chemiluminescent ECL method (Amersham Corp., Arlington Heights, IL).

Polymerase chain reaction (PCR) of KGF and HGF

Total RNA was extracted from bovine thecal and granulosa cells using a guanidinium thiocyanate procedure and further purified by centrifugation through a cesium chloride gradient (15). Total RNA was reverse transcribed to obtain complementary DNA using avian myoblastosis virus reverse transcriptase (Promega, Madison, WI). The complementary DNA template was amplified by PCR,using specific primers whose sequences were derived from published KGF and HGF sequences. The primer sequences were designed as follows. KGF primers were a consensus of rat, mouse, and human sequences: primer 1, 5′-GGG TCG ACC TGC AGT CTA GAA AGC TTA TAC TGA CAT GGA TCC TGC CA-3′; and primer 2, 5′-CTC GGA TCC TCC AAC TGC CAC GGT CCT GAT-3′ (10, 37). HGF primers were a consensus of rat, mouse, and human sequences: primer 1, 5′-GGG TCG ACT CTA GAA AGC TTA CAG CTT TTT GCC TTC GAG CTA-3′; and primer 2, 5′-GGA GAT CTG GAT CCC ATC AAA GCC CTT GTC GGG ATA-3′ (21, 29). Primer sequences designed to amplify cyclophilin (IB15 gene) were used as a positive control: primer 1, 5′-GGA TCC CTG CAG ACA CGC CAT AAT GGC ACT GG-3′; and primer 2, 5′-GAA TTC ATT TGC CAT GGA CAA GAT GCC-3′. Amplification was performed under stringent conditions for 45 cycles, using *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT). The 344-basepair (bp; KGF), 335-bp (HGF), and 143-bp

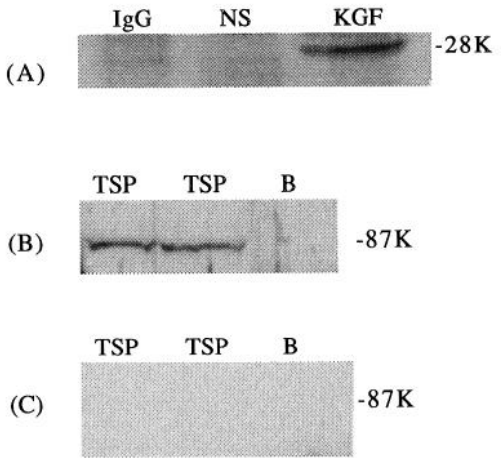

FIG. 4. KGF and HGF protein production by thecal cells. A, KGF immunoprecipitation of ^{35}S-labeled thecal cell secreted proteins with monoclonal KGF antibody (KGF), nonspecific mouse immunoglobulin G (IgG), or nonimmune serum (NS). Antibody complexes were precipitated, disrupted with sodium dodecyl sulfate sample buffer, electrophoresed under reducing conditions, and detected by fluorography. B, Immunoblot of thecal cell secreted proteins (TSP) with rabbit HGF antibodies (B indicates a blank lane). C, Immunoblot of thecal cell secreted proteins (TSP) with rabbit nonimmune serum (B indicates a blank lane). A representative of four different experiments is shown.

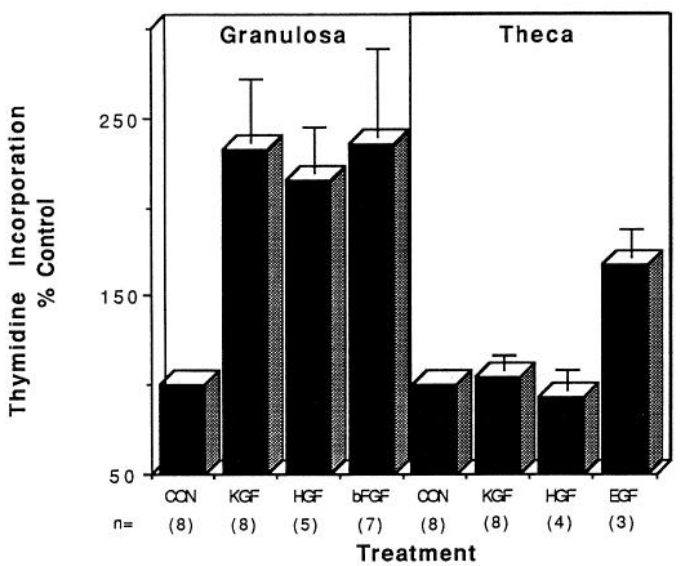

FIG. 5. Effects of KGF and HGF on proliferation of thecal and granulosa cells. [^{3}H]Thymidine incorporation into DNA. Data (mean ± SE) are presented as a percentage of the control (CON). The number of different experiments is indicated in *parentheses below each treatment.* Values for the control were generally greater than 2×10^3 cpm/μg DNA.

(cyclophilin, IB15) fragments were visualized by UV illumination on 5% polyacrylamide gels stained with 0.5 μg/ml ethidium bromide.

Cloning and DNA sequencing

KGF and HGF PCR products were ligated into Bluescript SK(-) at the *Sma*I site in the multicloning site. Blunt end ligations were performed using 5–10 U T4 DNA ligase (Boehringer Mannheim, Indianapolis, IN). All ligase reactions were initiated on wet ice, transferred to room temperature, and incubated for 30 min to overnight. DNA sequencing (40) was performed using the Sequenase version 2.0 chain termination DNA sequencing kit by incorporating [^{35}S]deoxy-ATP nucleotides (U.S. Biochemical, Cleveland, OH). Sequences were entered into GenBank to search for identities to KGF and HGF.

Growth assays

Cell growth was analyzed by quantitating [^{3}H]thymidine incorporation into newly synthesized DNA. Thecal and granulosa cells were plated (~1 million cells/cm^2) in 0.5 ml Dulbecco's Modified Eagle's Medium containing 0.1% calf serum. After 24 h, cells were treated with no growth factor (control), 10–100 ng/ml KGF, or 5–50 ng/ml HGF (generously provided by Dr. Jeff Rubin, NCI). Granulosa cells were also treated with 100 ng/ml basic FGF. Thecal cells were also treated with 50 ng/ml epidermal growth factor. Cells were plated for 24 h, then treated for 24 or 48 h. After treatment, 0.5 ml Dulbecco's Modified Eagle's Medium containing 2.5 μCi [^{3}H]thymidine was added to each well, and the cells were incubated for 4 h at 37 C and then sonicated. The quantity of [^{3}H]thymidine incorporated into DNA was determined, as previously described (41). Data were normalized to total DNA per well using an ethidium bromide procedure, described previously (38).

Results

Both thecal and granulosa cells were obtained from freshly isolated bovine ovarian follicles. Cells were used immediately for RNA preparation or placed in cell culture. The gene expression of KGF and HGF was examined with a reverse transcription-PCR procedure. Both KGF and HGF were found to be expressed in thecal cells, but not in granulosa cells (Fig. 1). A constitutively expressed protein, cyclophilin, was used to analyze the integrity of the RNA. With the appropriate primers a 143-bp cyclophilin, a 344-bp KGF, and a 335-bp HGF PCR product were observed from freshly isolated thecal cell RNA (Fig. 1). Negative controls with no template or each primer alone did not produce a PCR product (data not shown). Granulosa cells did not express detectable levels of steady state KGF or HGF transcripts, but did express cyclophilin (Fig. 1). Cultured granulosa cell RNA was also not positive for the growth factors. Both freshly isolated and cultured thecal cell RNA preparations were consistently positive for KGF and HGF. Therefore, thecal cells appear to express the KGF and HGF genes, but granulosa cells do not have any detectable KGF or HGF gene expression.

The bovine KGF and HGF PCR products were subcloned into the bacterial plasmid Bluescript by blunt end ligation (at the *Sma*I restriction site). Both the KGF and HGF DNA inserts were sequenced several times from different priming sites using the chain termination DNA-sequencing method (Figs. 2 and 3). These partial sequences are the only bovine KGF and HGF sequences published to date. A comparison of the bovine KGF sequence with the known rat (86% identity; 125–427 bp) (42), mouse (90% identity; 17–319 bp; GenBank accession no. Z22703), and human (95% identity; 462–764 bp) (10) sequences demonstrates a high degree of similarity in this region of the protein (Fig. 2). A similar comparison of the bovine HGF sequence with rat (63% identity; 624–915 bp) (21), mouse (60% identity; 482–773 bp; GenBank accession no. D10212), and human (63% identity; 555–846 bp) (29) sequences also demonstrates a significant yet reduced similarity. Rat, mouse, and human HGF nucleotide sequences in this region show 96% homology. Bovine HGF appears to

be less homologous in regard to nucleotide sequence in this region, due to several gaps and inserts (Fig. 3). The deduced amino acid sequence of bovine HGF, however, was found to have an 88–90% identity with the rat, mouse, and human HGF amino acid sequences in this region of the protein. In determining the amino acid sequence of bovine HGF from the nucleotide sequence, an apparent missing G residue at nucleotide residue 107 was identified that is probably due to a PCR artifact or a reproducible sequencing compression. Although the bovine HGF nucleotide sequence is less homologous, the deduced amino acid sequence is similar.

The ability of thecal cells to secrete KGF and HGF protein was examined to extend the above gene expression data. Thecal cells were cultured in the presence of [^{35}S]methionine, and then radiolabeled secreted proteins were collected from the conditioned medium. Radiolabeled secreted proteins were incubated with KGF monoclonal antibody and then precipitated, electrophoresed, and fluorographed. The KGF antibody specifically precipitated a 28-kDa protein from the radiolabeled thecal cell secreted proteins (Fig. 4). The HGF antibodies did not work well in immunoprecipitations and were used in an immunoblot of thecal cell secreted proteins. The HGF antibodies specifically detected an 87-kDa protein (Fig. 4) as well as relevant 69- and 34-kDa subunits (data not shown). The 87-kDa form of HGF detected is most likely the nonreduced or unprocessed form of HGF, lacking the proteolytic cleavage to generate the 69- and 34-kDa subunits. These proteins are the anticipated sizes for both KGF and HGF. Therefore, thecal cells have the capacity to synthesize and secrete both KGF and HGF proteins.

The actions of KGF and HGF in the ovarian follicle were examined through an analysis of the mitogenic activity of the growth factors on purified granulosa and thecal cells. After initial plating, freshly isolated granulosa and thecal cells were cultured in the absence or presence of recombinant KGF or HGF for 20 h and then in the presence of [^{3}H] thymidine for 4 h to assess the incorporation of [^{3}H]thymidine into DNA. Both KGF and HGF were found to stimulate the proliferation of bovine granulosa cells, as indicated by increases in thymidine incorporation (Fig. 5). Basic FGF (100 ng/ml) was used as a positive control to stimulate the growth of granulosa cells, and epidermal growth factor (50 ng/ml) was used to stimulate the growth of theca cells. The results indicate that both KGF and HGF can stimulate granulosa cell proliferation, but not that of thecal cells.

Discussion

Thecal cell-granulosa cell interactions are an example of an essential mesenchymal-epithelial cell interaction in the ovary. Mesenchymal cells are proposed to influence adjacent epithelial cells through the production of mesenchymal derived inducer proteins that act on the epithelium (1–3). Although the role that thecal cells have in regulating granulosa cell steroidogenesis is well established (4, 43–47), the role of thecal cells in regulating other granulosa cell functions remains to be elucidated. Previous observations have implied that thecal cells may influence granulosa cell proliferation through the local production of transforming growth factors (5–8, 38–42, 48). Two growth factors that appear to be restricted to mesenchymal-epithelial cell interactions are KGF and HGF. The current study was designed to identify the potential role KGF and HGF may have in mediating thecal-granulosa cell interactions.

The mesenchymal-derived thecal cells were found to express both the KGF and HGF genes as well as synthesize and secrete KGF and HGF proteins. In contrast, epithelial-derived granulosa cells had no detectable KGF or HGF messenger RNA expression. Therefore, the growth factors are produced by the mesenchymal cell population in the ovarian follicle, consistent with results from other tissues. The KGF and HGF receptors are primarily expressed by epithelial cells, such as the granulosa cells. Both KGF and HGF were found to stimulate granulosa cell proliferation, but not that of thecal cells. These observations imply that KGF and HGF have the capacity to mediate mesenchymal-epithelial cell interactions between thecal and granulosa cells. Further analysis of KGF, HGF, and their receptor expression during follicle development with *in situ* hybridization, immunocytochemistry, and direct measurements is needed. This analysis will assess whether KGF and HGF function during early and/or late follicle development. Analysis of the hormonal regulation of KGF and HGF expression will reveal the significance of this mesenchymal-epithelial cell interaction in the endocrine regulation of follicular growth.

Investigation of the actions of KGF and HGF demonstrated that granulosa cell proliferation is increased by these growth factors. Analysis of the actions of KGF and HGF on granulosa cell steroidogenesis is now needed to determine whether these growth factors also influence granulosa cell differentiation. The stage of follicle development may also influence the responsiveness of granulosa cells to KGF and HGF. Indeed, other growth factors known to influence granulosa cell growth and steroidogenesis have been shown to have altered actions during different stages of follicle development (37, 38). The actions of KGF and HGF on granulosa cell growth and differentiation (*i.e.* steroidogenesis) at various stages of follicle development are currently under investigation.

The current study demonstrates the local production and action of two mesenchymal-derived growth factors, KGF and HGF. These observations provide additional support for the importance of thecal cell-granulosa cell interactions in the regulation of follicle development. The previously identified concept that the mesenchymal cell population may have a significant role in the regulation of tissue growth is supported by the current observations. Mesenchymal controlled tissue growth may be a general phenomenon present in other tissues, but this requires more rigorous investigations. Although the current study suggests that KGF and HGF can mediate mesenchymal-epithelial cell interactions between thecal and granulosa cells, the *in vivo* expression, action, and physiological importance of this cell-cell interaction remain to be elucidated.

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References

1. **Grobstein C** 1967 Mechanisms of organogenetic tissue interaction. Natl Cancer Inst Monogr 26:279–299
2. **Kratochwil K** 1972 Tissue interaction during embryonic development. In: Tarin D (ed) General Properties of Tissue Interactions in Carcinogenesis. Academic Press, New York, pp 1–47
3. **Cunha GR, Chung LWK, Shannon JM, Taguchi O, Fujii H** 1983 Hormone-induced morphogenesis and growth: role of mesenchymal-epithelial interactions. Recent Prog Horm Res 39:559–598
4. **Dorrington JH, Moon YS, Armstrong DT** 1975 Estradiol-17β biosynthesis in cultured granulosa cells from hypophysectomized immature rats; stimulation by follicle-stimulating hormone. Endocrinology 97:1328–1331
5. **Skinner MK, Coffey Jr RJ** 1988 Regulation of ovarian cell growth through the local production of transforming growth factor-α by theca cells. Endocrinology 123:2632–2638
6. **Lobb DK, Kobrin MS, Kudlow JE, Dorrington JH** 1989 Tranforming growth factor-alpha in the adult bovine ovary: identification in growing ovarian follicles. Biol Reprod 40:1087–1093
7. **Kudlow J, Kobrin MS, Purchio AF, Twardzik DR, Hernandez R, Asa SL, Adashi EY** 1987 Ovarian transforming growth factor-α gene expression: immunohistochemical localization to the theca-interstitial cells. Endocrinology 121:1577–1579
8. **Skinner MK, Keski-Oja J, Osteen K, Moses HL** 1987 Ovarian thecal cells produce transforming growth factor-β which can regulate granulosa cell growth. Endocrinology 121:786–792
9. **Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA** 1989 Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc Natl Acad Sci USA 86:802–806
10. **Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA** 1989 Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. Science 245:752–755
11. **Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA** 1991 Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. Science 251:72–75
12. **Bottaro DP, Rubin JS, Ron D, Finch PW, Florio C, Aaronson SA** 1990 Characterization of the receptor for keratinocyte growth factor. Evidence for multiple fibroblast growth factor receptors. J Biol Chem 265:12767–12770
13. **Eisemann A, Ahn JA, Graziani G, Tronick SR, Ron D** 1991 Alternative splicing generates at least five different isoforms of the human basic-FGF receptor [published erratum appears in Oncogene 6:2379, 1991]. Oncogene 6:1195–1202
14. **Werner S, Duan DS, de Vries C, Peters KG, Johnson DE, Williams LT** 1992 Differential splicing in the extracellular region of fibroblast growth factor receptor 1 generates receptor variants with different ligand-binding specificities. Mol Cell Biol 12:82–88
15. **Miki T, Bottaro DP, Fleming TP, Smith CL, Burgess WH, Chan AM, Aaronson SA** 1992 Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. Proc Natl Acad Sci USA 89:246–250
16. **Gohda E, Tsubouchi H, Nakayama H, Hirono S, Sakiyama O, Takahashi K, Miyazaki H, Hashimoto S, Daikuhara Y** 1988 Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. J Clin Invest 81:414–419
17. **Zarnegar R, Michalopoulos G** 1989 Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. Cancer Res 49:3314–3320
18. **Nakamura T, Nawa K, Ichihara Kaise N, Nishino T** 1987 Purification and subunit structure of hepatocyte growth factor from rat platelets. FEBS Lett 224:311–316
19. **Miyazawa K, Kitamura A, Kitamura N** 1991 Structural organization and the transcription initiation site of the human hepatocyte growth factor gene. Biochemistry 30:9170–9176
20. **Montesano R, Matsumoto K, Nakamura T, Orci L** 1991 Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell 67:901–908
21. **Tashiro K, Hagiya M, Nishizawa T, Seki T, Shimonishi M, Shimizu S, Nakamura T** 1990 Deduced primary structure of rat hepatocyte growth factor and expression of the mRNA in rat tissues. Proc Natl Acad Sci USA 87:3200–3204
22. **Rubin JS, Chan AM-L, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW, Aaronson SA** 1991 A broad spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci USA 88:415–419
23. **Kan M, Zhang G, Zarnegar R, Michalopoulos G, Myoken Y, McKeehan WL, Stevens JL** 1991 Hepatocyte growth factor/hepatopoietin A stimulates the growth of rat kidney proximal tubule epithelial cells (RPTE), rat nonparenchymal liver cells, human melanoma cells, mouse keratinocytes and stimulates anchorage-independent growth of SV-40 transformed RPTE. Biochem Biophys Res Commun 174:331–337
24. **Igawa T, Kanda S, Kanetake H, Saitoh Y, Ichihara A, Tomita Y, Nakamura T** 1991 Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. Biochem Biophys Res Commun 174:831–838
25. **Tsarfaty I, Resau JH, Rulong S, Keydar I, Faletto DL, Vande Woude GF** 1992 The *met* proto-oncogene receptor and lumen formation. Science 257:1258–1261
26. **Petersen TE, Martzen MR, Ichinose A, Davie EW** 1990 Characterization of the gene for human plasminogen, a key proenzyme in the fibrinolytic system. J Biol Chem 265:6104–6111
27. **Degen SJF, Davie EW** 1987 Nucleotide sequence of the gene for human prothrombin. Biochem 26:6165–6177
28. **Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S** 1989 Molecular cloning and expression of human hepatocyte growth factor. Nature 342:440–443
29. **Miyazawa K, Kitamura A, Naka D, Kitamura N** 1991 An alternatively processed mRNA generated from human hepatocyte growth factor gene. Eur J Biochem 197:15–22
30. **Chan AM, Rubin JS, Bottaro DP, Hirschfield DW, Chedid M, Aaronson SA** 1991 Identification of a competitive HGF antagonist encoded by an alternative transcript. Science 254:1382–1385
31. **Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronson SA** 1991 Identification of the hepatocyte growth factor receptor as the c-*met* proto-oncogene product. Science 251:802–804
32. **Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Woude GV** 1987 Sequence of *MET* protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci USA 84:6379–6383
33. **Chan AM-L, King HWS, Deakin EA, Tempest PR, Hilkens J, Kroezen V, Edwards DR, Wills AJ, Brookes P, Cooper CS** 1988 Characterization of the mouse *met* proto-oncogene. Oncogene 2:593–599
34. **Park M, Dean M, Cooper CS, Schmidt M, O'Brien SJ, Blair DG, Vande Woude GF** 1986 Mechanism of met oncogene activation. Cell 45:895–904
35. **Di Renzo MF, Narsimhan RP, Olivero M, Bretti S, Giordano S, Medico E, Gaglia P, Zara P, Comoglio PM** 1991 Expression of the *Met*/HGF receptor in normal and neoplastic human tissues. Oncogene 6:1997–2003
36. **Lyer A, Kmiecik TE, Park M, Daar I, Blair D, Dunn KJ, Sutrave P, Ihle JN, Bodescot M, Vande Woude GF** 1990 Structure, tissue-specific expression, and transforming activity of the mouse *met* protooncogene. Cell Growth Differ 1:87–95
37. **Skinner MK, Osteen KG** 1988 Developmental and hormonal regulation of bovine granulosa cell function in the preovulatory follicle. Endocrinology 123:1668–1675
38. **Roberts AJ, Skinner MK** 1990 Hormonal regulation of thecal cell function during antral follicle development in bovine ovaries. Endocrinology 127:2907–2917
39. **Skinner MK, Griswold MD** 1983 Fluorographic detection of radioactivity in polyacrylamide gels with 2,5 diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem J 209:281–284
40. **Sanger F, Niklen S, Coulsen AR** 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
41. **Skinner MK, Fritz IB** 1986 Identification of a non-mitogenic par-

acrine factor involved in mesenchymal-epithelial cell interactions between testicular peritubular cells and Sertoli cells. Mol Cell Endocrinol 44:85–97
42. **Yan G, Nikolaropoulos S, Wang F, McKee W** 1992 Sequence of rat keratinocyte growth factor (heparin-binding growth factor type-7). In Vitro Cell Dev Biol 27:437–438
43. **Adashi EY, Resnick CE, Twardzik DR** 1987 Transforming growth factor-α attenuates the acquisition of aromatase activity by cultured rat granulosa cells. J Cell Biochem 33:1–13
44. **Knecht M, Feng P, Catt K** 1987 Bifunctional role of transforming growth factor-β during granulosa cell development. Endocrinology 120:1243–1249
45. **Dodsen WC, Schomberg DW** 1987 The effect of transforming growth factor-β on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology 120: 512–516
46. **Mondschein JS, Canning SF, Hammond JM** 1988 Effect of transforming growth factor-β on the production of immunoreactive insulin-like growth factor I and progesterone on ^{3}H-thymidine incorporation in porcine granulosa cell cultures. Endocrinology 123:1970–1976
47. **Skinner MK, Parrott JA** 1994 Growth factor mediated cell-cell interactions in the ovary. In: Findlay JK (ed) Cellular and Molecular Mechanisms in Female Reproduction. Academic Press, New York, pp 67–79
48. **Evans G, Dobias M, King GJ, Armstrong DT** 1981 Estrogen, androgen, and progesterone biosynthesis by theca and granulosa of preovulatory follicles in the pig. Biol Reprod 25:673–682