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Keratinocyte Growth Factor Acts as a Mesenchymal Factor That Promotes Ovarian Primordial to Primary Follicle Transition

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ABSTRACT

An important but poorly understood process in ovarian biology is the transition of the developmentally arrested primordial follicle to the developing primary follicle. Interactions between the epithelial and mesenchymal cells of the follicle are critical for the coordination of ovarian follicle development. The mesenchymal growth factor keratinocyte growth factor (KGF) (i.e., fibroblast growth factor-7) and the epithelial growth factor kit ligand (KITL) are known to interact to coordinate the growth of later-stage antral follicles. The hypothesis tested in the current study is that KGF acts as a mesenchymal factor to promote the primordial to primary follicle transition. A postnatal 4-day-old rat ovary organ culture system was used to investigate the actions of KGF. KGF treatment promoted 65% of follicles to undergo the primordial to primary follicle transition, but only 45% underwent development in control ovaries. Neutralizing antibody for KGF was found to attenuate the stimulatory action of KITL, but neutralizing antibody for KITL was not able to attenuate the stimulatory action of KGF. Further analysis demonstrated that KGF was found to stimulate the expression of KITL (i.e., mRNA levels) by granulosa cells. KITL in turn was found to stimulate the expression of KGF to create a positive feedback loop. Interestingly, KGF expression was localized to selected mesenchymal cells (i.e., precursor theca cells) surrounding the developing primordial follicle. Observations suggest that developing granulosa cells of the primordial follicles produce KITL, which helps recruit precursor theca cells to the follicle; the thecal cells then produce KGF, which acts on the granulosa to amplify KITL expression and support primordial follicle development. KGF appears to be a mesenchymal factor that promotes the primordial to primary follicle transitions.

follicle, granulosa cells, oocyte development, ovary, theca cells

INTRODUCTION

A critical process in ovarian biology is the transition of the developmentally arrested primordial follicle to the developing primary follicle. The primordial follicle consists of an oocyte arrested in meiosis I and surrounded by flattened epithelium termed the pregranulosa [1]. In most placental mammals, all of the primordial follicles are in place at or immediately following birth [1]. Although a recent study suggested that new primordial follicles might be formed from germ-line stem cells during adult life [2], the majority of previous literature suggests that primordial follicles do not grow, proliferate, or regenerate [3]. This population of primordial follicles present at birth is termed the primordial follicle pool. During a female's reproductive life, selected primordial follicles transition to primary follicles and develop until the pool is exhausted at menopause [4]. The developing primary follicle consists of a growing oocyte, a layer of quickly proliferating cuboidal epithelium called the granulosa, and newly sequestered mesenchymal cells called theca. After a follicle makes the primordial to primary follicle transition, it is destined either to ovulate or to degenerate through follicular atresia (i.e., apoptosis). Therefore, the rate of the primordial to primary follicle transition is one determinant of how long the primordial follicle pool will last. Once the primordial follicle pool is depleted, ovarian steroidogenesis ends and the series of changes called menopause begins [3]. In the event that the primordial to primary follicle transition is accelerated, a female would quickly lose her primordial follicle pool and enter a premature menopausal period. This is hypothesized to be the cause of the condition or conditions known as premature ovarian failure [5]. Elucidation of the coordinated events of the primordial to primary follicle transition may bring insight into these pathological conditions.

The coordination of the primordial to primary follicle transition is largely endocrine- and gonadotropin-independent. Ovaries cultured in serum-free medium contain primordial follicles that are fully competent to make the primordial to primary follicle transition [6–8]. Knockout mice who are null mutants for either FSH receptor or LH receptor are able to undergo the primordial to primary follicle transition [9, 10]. The primordial to primary follicle transition is coordinated primarily by locally produced regulatory factors [1]. The current study was designed to investigate one of these locally produced regulatory factors and the role of mesenchymal-epithelial cell interactions in this process.

A postnatal 4-day-old rat ovary culture system has been used to investigate local growth factors that promote the primordial to primary follicle transition. At this stage of development the ovary consists mostly of primordial follicles. Previously, kit ligand (KITL) [8] and leukemia inhibitory factor (LIF) [11] have been shown to be produced by the pregranulosa and to act on the oocyte to promote the primordial to primary follicle transition. Kit ligand also appears to function as a theca cell recruitment factor [12]. Kit ligand has been shown to interact with keratinocyte growth factor (KGF, also known as fibroblast growth factor-7 [FGF-7]) in the ovary to regulate ovarian surface epithelium [13] and large antral follicle growth [14]. Fibroblast growth factor-2 (FGF-2, also known as basic fibroblast growth factor [bFGF]) [15] has also been shown to be produced by the oocyte and acts on the pregranulosa to promote the primordial to primary follicle transition. Recently,

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bone morphogenic protein-4 (BMP4) has been found to be produced in the stroma of the ovary to act as a survival factor for the oocytes and primordial follicles [16]. Nerve growth factor also appears to influence primordial to primary follicle transition [17]. Interestingly, anti-Müllerian hormone (AMH) (i.e., Müllerian inhibiting substance) that is produced by granulosa cells of larger developing follicles can inhibit the primordial-to-primary follicle transition [18]. This allows developing follicles to regulate the onset of primordial follicle development.

An interesting question that arises in the study of primordial follicle development is, ''At what stage of folliculogenesis does the mesenchymal theca cell originate?'' In most species, morphologically distinct theca cells do not appear until an early preantral stage of folliculogenesis. In the rat, the theca become apparent during the transition to the primary follicle stage [4]. The assumption is that mesenchymal-epithelial interactions facilitate the primordial to primary follicle transition in all species, but the mesenchymal cells may not be morphologically distinct. The current study investigates a mesenchymal factor, KGF, for its ability to promote the primordial to primary follicle transition.

KGF, also termed FGF-7, is a prototypical mesenchymal factor [19]. KGF is a 28-kDa protein that is a member of the large fibroblast growth factor protein family [20, 21]. There are four receptors for the fibroblast growth factor family, with numerous splice variants. A splice variant of fibroblast growth factor receptor 2 is the unique receptor for KGF [22]. This splice variant is primarily expressed in epithelial cells. KGF has been shown to have a role in the developing antral ovarian follicle [23]. KGF is produced by the theca cells [23] and acts on the granulosa cells to promote KITL expression, which in turn acts back on the theca in a positive feedback loop [14]. This cell-cell interaction is coordinated by gonadotropins and steroids to facilitate the rapid growth of the antral follicle. The presence and action of KGF at earlier stages of folliculogenesis are unknown.

The hypothesis tested is that KGF from the stroma adjacent to the primordial follicle interacts with the pregranulosa cells. This sets up a mesenchymal-epithelial interaction that helps coordinate the primordial to primary follicle transition. Experiments in this study test whether KGF promotes the primordial to primary follicle transition, and whether KGF interacts with other paracrine growth factors to coordinate this developmental process.

MATERIALS AND METHODS

Histology and Organ Cultures

Sprague Dawley rats from the Washington State University breeder colony (breeder rats came from Charles River Laboratories, NC) were used in this study. All animal procedures were approved by the Washington State University Institutional Animal Care and Use Committee. Ovaries from 4-day-old rats were cultured for 14 days. Whole ovaries were cultured as previously described [12] on floating filters (0.4 μm Millicell-CM; Millipore, Bedford, MD) in 0.5 ml Dulbecco modified Eagle medium (DMEM)-Ham F-12 medium (1:1, vol/vol) containing 0.1% BSA (Sigma, St. Louis, MO), 0.1% albumax (Gibco BRL, Gaithersburg, MD), 2.75 μg/ml transferrin, and 0.05 mg/ml L-ascorbic acid (Sigma) in a 4-well culture plate (Nunc plate; Applied Scientific, South San Francisco, CA). Ovaries were cultured at 37°C in 5% CO_2 with medium and treatments replaced every 2 days. Ovaries may be cultured in this manner for 2 wk and maintain a healthy appearance. The total number of follicles present in the ovaries is unchanged over the culture period, suggesting that there is very little follicle atresia [8]. Approximately 45% of follicles in untreated (control) ovaries initiate primordial follicle transition in culture, compared to approximately 35% in age-matched in vivo ovaries [8]. Ovaries were randomly assigned to treatment groups, with 1–3 ovaries per floating filter. From 3 to 8 ovaries were examined per treatment group. Treatments during organ culture were: KGF (100 ng/ml; R&D Systems, Minneapolis, MN), KITL (100 ng/ml; R&D Systems), LIF (100 ng/ml; R&D Systems), FGF2/bFGF (100 ng/ml; R&D Systems), insulin 200 ng/ml, KGF neutralizing antibody (10 μg/ml; R&D Systems), and KIT (i.e., c-kit) neutralizing antibody (10 μg/ml; Gibco BRL) [8]. Medium was supplemented with penicillin, streptomycin, and gentamycin to prevent bacterial contamination. After culture, ovaries were fixed in Bouin fixative (Sigma) for 2 h. Ovaries were then embedded in paraffin, sectioned at 4μm, and stained with hematoxylin-eosin.

The number of follicles at each developmental stage was counted in two serial sections with the largest cross-section through the center of the ovary and averaged. Normally two ovaries were in each treatment group. Experiments were repeated three times, with n = 6 for each treatment group. Normally, 150–200 follicles were present in a cross-section. No change was found in total follicle number per section or ovary because of any treatment (data not shown). Ovaries cultured for 2 wk in this manner with or without treatment also show no loss in total follicle numbers [8]. Some variability exists between different experiments regarding the basal (i.e., endogenous) levels of primordial follicle development in the control. This accounts for the slight differences between the experiments in the degree of follicle development seen in controls and is why controls are run with each experiment.

Real-Time Polymerase Chain Reaction

Ovaries were isolated from 4-day-old rats and placed into culture as described above. Cultured ovaries were treated for 2 days with 50 ng/ml of either KGF or KITL, or were left untreated as controls. Three ovaries from the same culture well were pooled to make each RNA sample. RNA was extracted using the Trizol reagent (Sigma, St. Louis, MO). RNA samples were DNase treated using the TURBO-DNA-free kit (Ambion, Austin, TX). One to two micrograms of total RNA from each sample was reverse-transcribed to cDNA using a standard oligo-dT RT protocol in a reaction volume of 25μL. cDNA samples were diluted 1:10 and 2μL of diluted sample per well was used as template for real-time polymerase chain reaction (PCR) analysis. Each sample was run in triplicate. The Platinum SYBR Green qPCR Supermix kit (Invitrogen, Grand Island, NY) was used according to the manufacturer's instructions. The *Kitl* primers (NCBI: NM_021843) were r*Kitl*-720: 5′ATTTATGTTACCCCCTGTTG CAGCC3′ and r*Kitl*-859: 5′CAATTACAAGCGAAATGAGAGCCG3′. The *Kgf* primers (NCBI_022182) were r*Kgf*-1–670: 5′AGTGGGCCGTT TTTTGTTCTTT3′ and r*Kgf*-1–590: 5′GGGAAATGTTCGTGGCCTTA A3′. The *S2* housekeeping reference gene primers (NCBI: NM_031838) were r*S2*-F: 5′CTGCTCCTGTGCCCAAGAAG3′ and r*S2*-R: 5′AAGGT GG CCTTGGCAAAGTT3′. Real-time PCR was performed on an ABI-7000 real-time machine (Applied Biosystems, Inc., Foster City, CA). For *Kitl*, the protocol was 60°C for 2 min, 95° for 10 min, then 40 cycles of 95°C for 20 sec and 68°C for 90 sec. For *Kgf* the protocol was 40 cycles of 95°C for 20 sec, 64°C for 45 sec, and 72°C for 60 sec. Fluorescent detection data were analyzed and normalized for *Kitl* mRNA levels to *S2* mRNA levels, and then KGF-treated sample values were normalized to their own experiments' control *Kitl* mRNA levels.

Immunohistochemistry

Localization of KGF protein was determined by immunohistochemical analysis. Four-day-old rat ovaries were cultured for 2 wk and then fixed in Bouin solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid) for 1–2 h. Ovaries were paraffin-embedded and sectioned at 3–5 μm. Ovaries were deparaffinized in xylenes and hydrated through an ethanol series of 100%, 90%, and 70%. Antigens were exposed by boiling sections for 5 min in 0.01 M sodium citrate buffer, pH 6.0. A solution of 10% goat serum in phosphate-buffered saline (PBS) was used as a blocking agent before sections were incubated with primary antibody. Slides were incubated with polyclonal rabbit anti-human KGF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Secondary antibody (biotinylated goat anti-rabbit IgG, Vector, Burlingame, CA) was detected by using the Vectastsain kit (Vector).

Western blot analysis was preformed to evaluate the antibody used for immunohistochemical localization of KGF. Antibodies were tested against recombinant human KGF (R&D Systems) and rat ovary protein lysates. Ovary lysates were prepared by homogenizing adult rat ovaries in homogenization/lysis buffer (0.3M sucrose, 10 mM Tris pH 8.0, 400 mM NaCI2, 0.5% NP40) with complete Mini protease inhibitor cocktail tablets (Roche, Indianapolis, IN), centrifuging at 10 000 × *g* for 30 min at 4°C, and collecting supernatant. The protein concentration in the supernatants

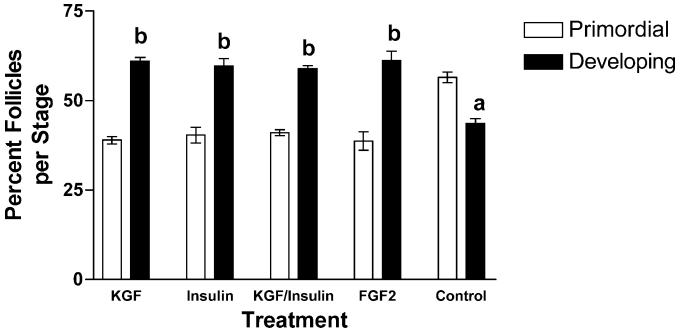

FIG. 1. Ovaries from 4-day-old rats were cultured for 2 wk in the absence or presence of KGF and/or insulin. FGF2/bFGF was used as a positive control. Data are displayed as the percentage of primordial or developing follicles per ovary in each treatment group. The developing follicle category contains predominantly primary follicles and some secondary follicles. Data from three different experiments with two ovaries per experiment were combined. The mean ± SEM is presented. Different letters represent significantly different treatment groups using a Dunnett test against a control ($P < 0.001$).

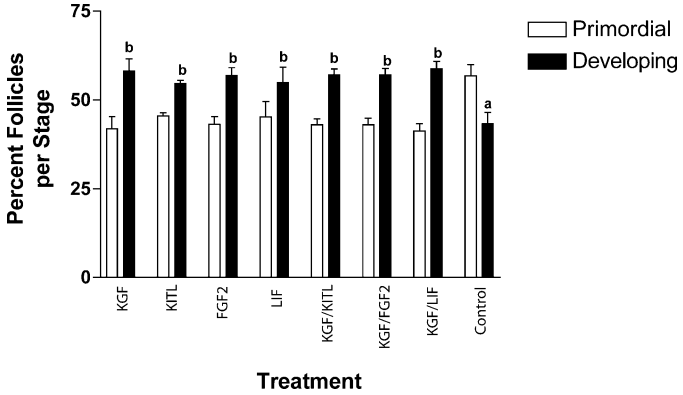

FIG. 2. Ovaries from 4-day-old rats cultured for 2 wk in the absence or presence of KGF, FGF2, LIF, KITL, and the combination of KGF and the above growth factors. Data are displayed as the percentage of primordial or developing follicles per ovary in each treatment group. The developing follicle category contains predominantly primary follicles and some secondary follicles. Data from three experiments with two ovaries per experiment were combined. The mean ± SEM is presented. Different letters represent significantly different treatment groups using a Fisher least significant difference test ($P < 0.001$).

was estimated using a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). Approximately 50μg protein lysates or 120 ng recombinant KGF in SDS sample loading buffer was boiled for 5 min and electrophoresed on a 10% SDS-polyacrilamide gel. The protein was subsequently transferred onto a PVDF membrane (Immobilon-P, Millipore) and probed with antibodies to KGF (Cat. Nos. sc-136 and sc-7882; Santa Cruz Biotechnology Inc.; and MAB251; R&D Systems). The specific antigen antibody complex was visualized using a chemiluminescent detection kit (Pierce, Rockford, IL).

Statistics

Treatment groups are compared using analysis of variance (ANOVA) followed by a Dunnett or Fisher LSD test where appropriate. In the case of real-time PCR experiments, the expression value for each treated group was normalized to its control, so all control values were equal to one. Therefore, the nonparametric Wilcoxon rank sums test was used to compare treatment groups. Groups were considered significantly different if $P \leq 0.05$. All statistics were calculated with the help of JMP v3.1 software (SAS Institute, Inc., Cary, NC).

RESULTS

The 4-day-old rat ovary is composed primarily of newly assembled primordial follicles. Therefore, the analysis of how many primordial follicles make the primordial to primary follicle transition is easily assessed. Briefly, ovaries were dissected from 4-day-old rat pups and placed on a membrane filter floating on serum-free medium. Ovaries were cultured for 2 wk with various treatments before morphological analysis. Previous time course studies have shown negligible induction of primordial follicle development after 2 days of culture, some development after 7 days of culture, and optimum development after 14 days of culture [8, 15]. The first organ culture experiment was to determine whether KGF promotes the primordial to primary follicle transition in vitro (Fig. 1). Ovaries were treated with 100 ng/ml KGF, 200 ng/ml insulin, and a combination of KGF and insulin. FGF2/bFGF was used as a positive control because it is known to stimulate primordial to primary follicle transition [15]. In the control untreated ovary cultures, approximately 45% of the follicles made the primordial to primary follicle transition. In the KGF treated ovaries, greater than 65% of the follicles made the primordial to primary follicle transition, in a manner similar to that of the positive FGF2 control (Fig. 1). KGF was able to significantly promote the primordial to primary follicle transition in vitro. Insulin was also able to promote the primordial to primary follicle transition as previously described [24]. The combined treatment demonstrated that the effect of KGF was not additive with that of insulin (Fig. 1). Similar results were observed with 25, 50, and 100 ng/ml KGF (data not shown). To further understand the mechanism of KGF action, the potential additive effects with other growth factors known to promote the primordial to primary follicle transition were investigated. Ovaries were cultured with KGF in combination with KITL, FGF2, or LIF (Fig. 2). None of these combinations produced an effect greater than that of KGF alone on the primordial to primary follicle transition. Previous studies have shown that hepatocyte growth factor (HGF), epidermal growth factor (EGF) [1, 24] and growth and differentiation factor-9 [25] do not promote primordial follicle transition in this organ culture system. Therefore, the stimulatory actions of KGF, FGF2 and KITL are specific when compared with other factors such as HGF and EGF.

The role of endogenous KGF was investigated with a neutralizing anti-KGF antibody used to treat 4-day-old ovaries in culture (Fig. 3A). KITL was used as a stimulator of follicle development because of its close relationship with KGF in other mesenchymal-epithelial systems, such as in the antral follicle [14]. Antral follicle theca cells produce KGF that can act on granulosa cells to increase KITL production to feedback on theca cells to increase KGF expression [14]. The KGF antibody was unable to attenuate spontaneous primordial to primary follicle transition in the ovary. Interestingly, the KGF antibody was able to attenuate the stimulatory action of KITL on primordial follicle transition (Fig. 3A). This observation suggests that KGF and KITL interact in a similar manner to that of the antral follicle mesenchymal-epithelial systems. The inverse of this experiment was performed to further investigate any interaction between KGF and KITL. Ovaries were cultured in the presence of KGF and a neutralizing KIT (i.e., c-kit) antibody that blocks actions of the KITL receptor (Fig. 3B). The KIT antibody used was found to block KITL actions, as previously described [8]. In contrast to the actions of the KGF antibody, the KIT antibody was unable to attenuate the stimulatory action of KGF on the primordial to primary follicle transition. Therefore, KGF antibody can block

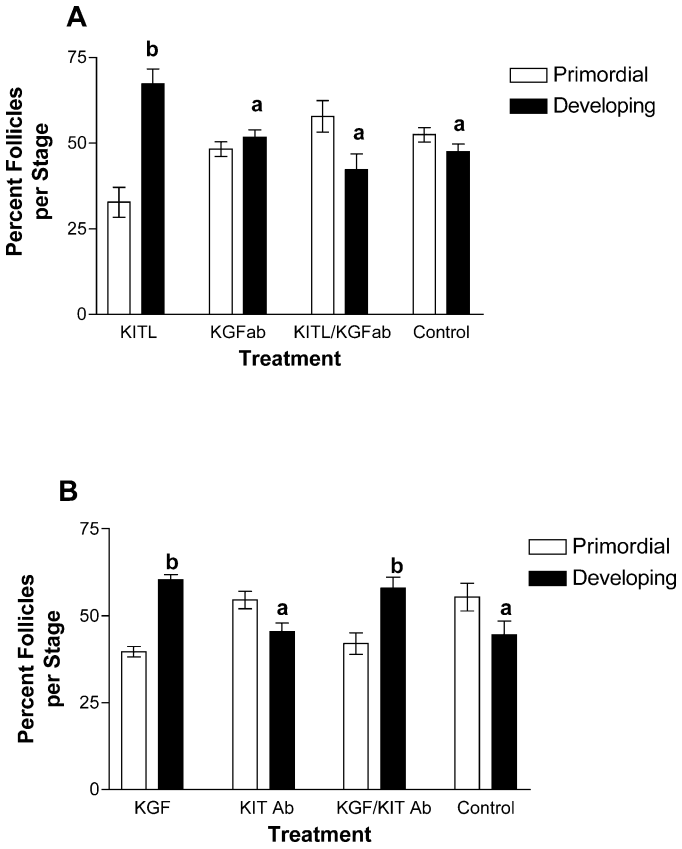

FIG. 3. **A**) Ovaries from 4-day-old rats cultured for 2 wk in the presence of KGF antibody (KGFab), KITL, and the combination of KGF antibody and KITL. **B**) Ovaries from 4-day-old rats cultured for 2 wk in the presence of KIT antibody (KIT Ab), KGF, and the combination of KIT antibody and KGF. Data are displayed as the percentage of primordial or developing follicles in each treatment group. The developing follicle category contains predominantly primary follicles and some secondary follicles. Data from three different experiments with three ovaries per experiment were combined. The mean ± SEM is presented. Different letters represent significantly different treatment groups using Dunnett's test against a control ($P < 0.001$).

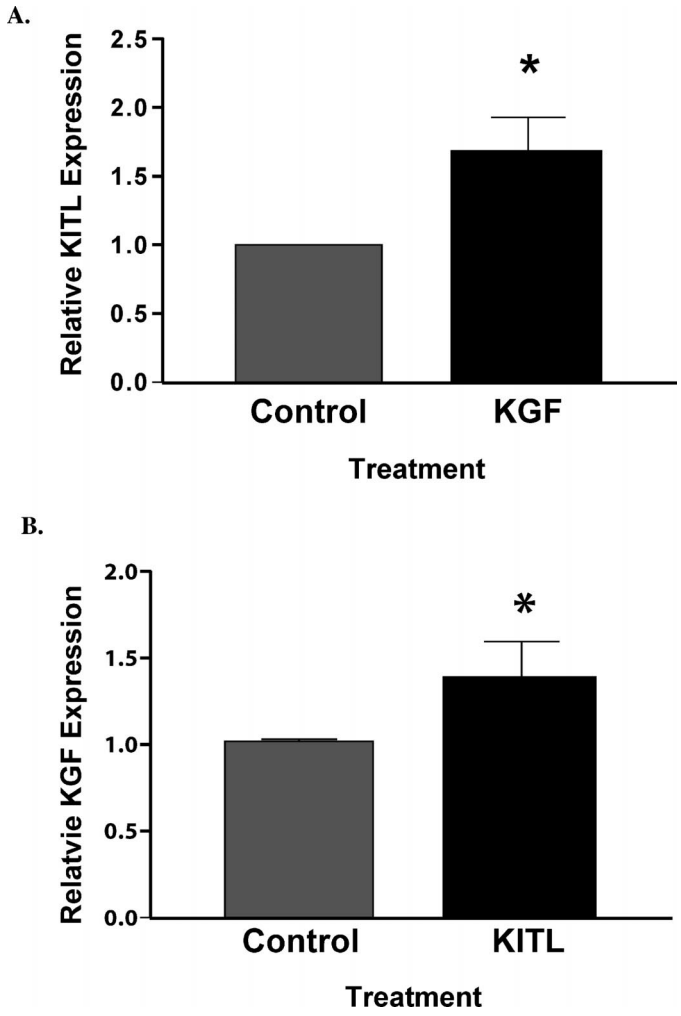

FIG. 4. Real-time PCR analysis of relative kit ligand (*Kitl*) (**A**) and *Kgf* (**B**) expression. Levels of mRNA in 4-day-old ovary cultures treated with 50 ng/ml of KGF or KITL for 2 days in vitro were determined. Data are expressed as the mean ± SEM of four pooled ovary samples per treatment group from two separate experiments (n = 4). The mRNA expression levels are normalized to the control with no treatment. *Significant difference from control ($P < 0.02$) as determined by a nonparametric Wilcoxon rank sums test.

KITL actions, but blocking KITL activity had no effect on KGF actions (Fig. 3).

A quantitative real-time PCR procedure was performed on RNA from whole postnatal 4-day-old ovaries to assess *Kitl* and *Kgf* expression in response to growth factors. As previously discussed, the neonatal ovary is primarily composed of early-stage primordial follicles. To investigate more direct actions of KGF, ovaries were incubated for 2 days in the absence or presence of KGF before RNA collection and analysis. KGF treatment was found to increase *Kitl* mRNA expression compared to controls ($P \leq 0.05$) (Fig. 4A). The inverse of the above experiment was also performed. Ovaries were incubated in the absence or presence of KITL and then *Kgf* mRNA expression was measured. KITL treatment was found to increase *Kgf* mRNA expression (Fig. 4B).

Immunohistochemistry was performed to localize KGF protein in freshly isolated 4-day-old neonatal rat ovaries. KGF staining was present in selected mesenchymal cells and focal stromal areas around developing primordial follicles (Fig. 5). Observations suggest KGF is produced by individual stromal cells adjacent to the granulosa layer of the developing primordial follicle. Western blot analysis was preformed to evaluate the specificity of KGF antibodies to recombinant KGF and rat ovary lysates. The primary antibody (Cat. No. sc-7882) showed the expected bands at 18 and 36 kDa against recombinant KGF, and the same bands were present in the ovary lysates (data not shown). Antibody sc-1365 showed no bands in the ovary lysates and antibody MAB251 did not detect the recombinant KGF (data not shown). These antibodies were deemed inappropriate for immunohistochemistry and were not subsequently used. Although immunohistochemical and/or neutralizing antibodies do not always work well on Western blot analysis, the antibody that showed specificity on the Western blot and worked in immunohistochemistry was selected. The negative control nonimmune IgG showed no staining (Fig. 5C). This was not a peptide antibody, so blocking peptide experiments could not be performed. The hematoxylin-eosin stain was found to mask the KGF immunostain, so a separate hematoxylin-eosin stained ovary section is shown in Figure 5D. The KGF receptor (i.e., FGFR2 IIIb splice variant) has been previously localized to the ovarian follicle [14, 26]. The KGF receptor immunohistochemistry and mRNA were localized to the developing granulosa

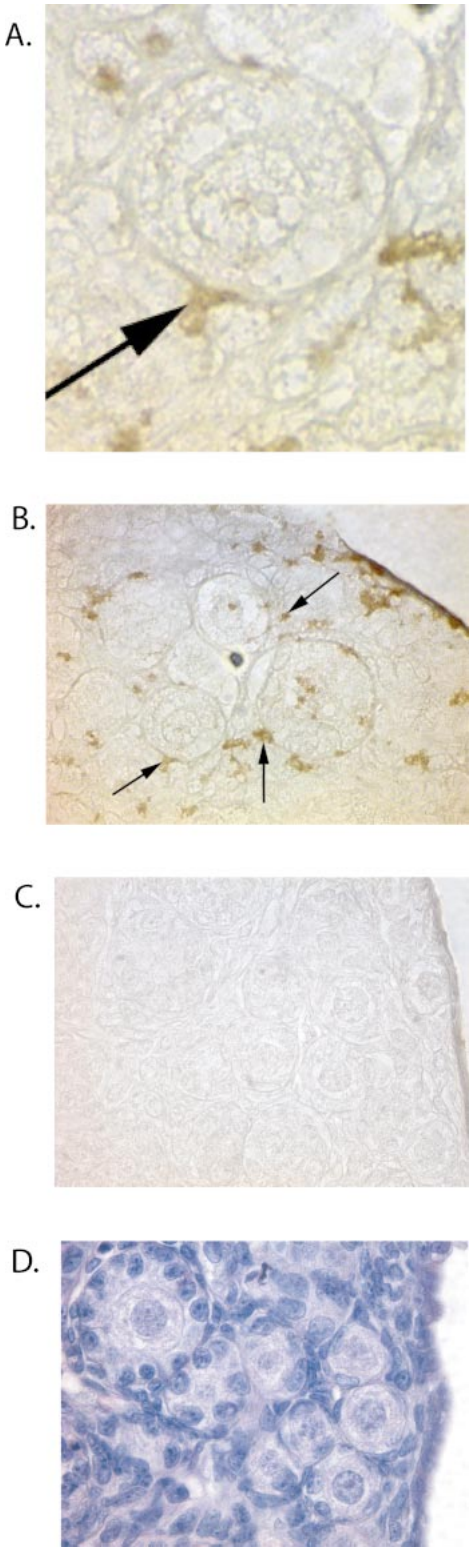

FIG. 5. Immunohistochemical localization of KGF was performed on freshly isolated 4-day-old neonatal rat ovaries using KGF antibody sc-7882. Brown staining indicates the presence of KGF. Staining localizes to stromal cells (arrows) around follicles. **A**) KGF immunostain. **B**) KGF immunostain. **C**) Nonimmune IgG immunostain control. **D**) Hematoxylin-eosin stain of ovary section. Data are representative of three different experiments. Original magnification **A** ×1000; **B**–**D** ×400.

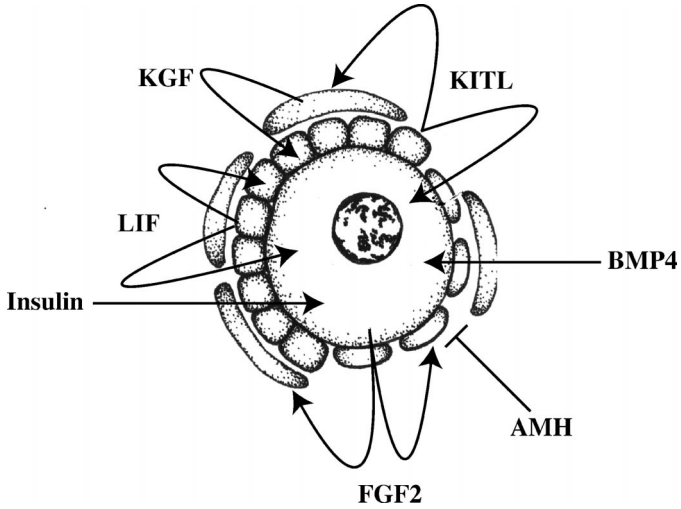

FIG. 6. Proposed model of cell-cell interactions that coordinate the primordial to primary follicle transition. Kit ligand (KITL) produced from the granulosa acts on the oocyte and theca. Leukemia inhibitory factor (LIF) from the granulosa acts on the oocyte and granulosa. Basic fibroblast growth factor-2 (FGF2/bFGF) from the oocyte acts on the granulosa and theca. Insulin acts as an endocrine agent on the oocyte. Bone morphogenic protein-4 (BMP4) from theca and surrounding stroma acts as a survival factor for the follicles. Anti-müllerian hormone (AMH), most likely from the larger follicles, represses the primordial to primary follicle transition. In response to KITL, the theca cells produce KGF to act on granulosa cells.

cells. Expression in granulosa cells increases as follicles develop into large antral follicles [14, 26].

DISCUSSION

The hypothesis tested was that KGF acts as a mesenchymal factor in a mesenchymal-epithelial interaction to promote the primordial to primary follicle transition. The observations presented suggest KGF is expressed by selected stromal (i.e., precursor theca) cells surrounding the primordial follicle and acts to promote the primordial to primary follicle transition. Although there are no morphologically distinct theca cells in the primordial follicle, these data suggest that there is communication between selected stromal cells bordering the primordial follicles and the granulosa of the primordial follicle. This communication involves an interaction between KITL and KGF in a manner that is similar to what occurs in other systems in the ovary such as the antral follicle [12, 14]. These stromal cells adjacent to the granulosa cells appear to represent a precursor theca population that is established surrounding the developing primordial follicle. Previous studies have shown KGF to be produced by the theca cells of larger antral follicles [14, 23, 26]. All developmental systems appear in part to involve coordinated interactions between mesenchymal cells and adjacent epithelial cells [27]. The developing primordial follicle is similar and appears to involve both precursor theca cells and granulosa cells. No other markers are currently known for this precursor theca cell population. Steroidogenic enzymes and hormone receptors are not expressed at this early stage of development. A previous study did identify BMP4 as being expressed in a similar cell population [16]. Further analysis of this putative precursor theca cell population will need to determine whether KGF and BMP4 colocalize and to identify additional markers of this cell population.

The organ culture experiments demonstrate that KGF

promotes the primordial to primary follicle transition in vitro. KGF did not have an additive effect with other growth factors known to promote the primordial to primary follicle transition (Fig. 6). The inability of KGF to have an additive effect with insulin suggests a lack of direct action on the oocyte. Other growth factors, such as KITL and LIF, have been shown to possess an additive effect with insulin [24] and these are factors that appear to act directly upon the oocyte (Figs. 2 and 6). Growth factors that do not act upon the oocyte, such as FGF2, do not display an additive effect with insulin. Observations suggest that KGF acts as a mesenchymal factor on the epithelial granulosa cells, which are the site of KITL expression (Fig. 6).

The neutralizing KGF antibody was unable to retard any endogenous KGF activity in the organ culture experiment. However, the KGF antibody was able to attenuate the stimulatory action that exogenous KITL had on the primordial to primary follicle transition. This suggests that the KITL from the pregranulosa promotes the production or action of KGF, presumably derived from the mesenchymal cells, to coordinate the primordial to primary follicle transition. This was confirmed with the results of real-time PCR analysis showing that KITL treatment of ovaries stimulates *Kgf* mRNA expression. Observations suggest that KGF is a mesenchymally-derived factor from a precursor theca cell that acts on the granulosa (Fig. 6). The inverse experiment was performed with KGF and neutralizing KIT antibody. The KIT antibody was unable to attenuate KGF promotion of the primordial to primary follicle transition. Although KGF was shown to stimulate *Kitl* mRNA expression, KGF must have a capacity to stimulate primordial to primary follicle transition that is independent of *Kitl* expression. KGF appears to have the ability to act directly on the primordial follicle through stimulating KITL expression, as well as having KITL-independent mechanisms. An alternative consideration is that the KIT antibody was not completely effective in blocking KITL actions. However, this KIT antibody was found to be active and to block KITL actions [8]. Further investigation is needed to clarify the mechanisms of KGF action.

Interestingly, both KITL and KGF were able to increase expression of each other, creating a positive feedback loop. This is similar to the interaction described for KITL and KGF in larger antral follicles [14]. The experiments to assess alterations in mRNA levels used ovary organ cultures. Therefore, changes in the populations of different follicle categories present could influence changes in mRNA. It is not possible to purify cell populations to assess direct effects on gene expression from these early stage follicles. To address this, only a 2-day culture period was used, in which no change in follicle development was detected (data not shown) [8]. This shorter incubation period for analysis of effects on mRNA levels corrected for the use of the organ cultures and supports a direct effect of these factors on gene expression.

The current model of the primordial to primary follicle transition involving KGF is presented in Figure 6. The granulosa produces LIF that acts upon the granulosa cells and the oocyte. The oocyte produces FGF2/bFGF that acts upon the granulosa and theca cells. Insulin acts as an endocrine agent on the oocyte. The theca and the surrounding stroma produce BMP4, which acts as a follicular survival factor. In this study, it is proposed that the granulosa cells produce KITL that acts upon the oocyte and also acts on the stroma to recruit theca cells that produce KGF, which then acts upon the granulosa to promote the primordial to primary follicle transition. This model of KITL and KGF action is consistent with a previous hypothesis of KITL acting as a theca cell recruiter and organizer [12]. The KITL acts to recruit theca cells, which, among other functions, begin to produce KGF that promotes the primordial to primary follicle transition (Fig. 6).

The regulation of the primordial to primary follicle transition is one of the critical factors in establishing the size of the primordial follicle pool and thus the length of a female's reproductive life. Elucidation of the factors that control the size of the primordial follicle pool provides insight into the pathological conditions that result in premature menopause or premature ovarian failure. The current study suggests communication occurs between the developing granulosa/oocyte unit and the immediately surrounding stroma to promote the primordial to primary follicle transition. Observations support a mesenchymal-epithelial interaction involving KGF and KITL in the initiation of primordial follicle development. Establishing this interaction provides valuable insight into the biology of the primordial follicle and may lead to a better understanding and design of therapeutics for the treatment of pathological conditions caused by primordial follicle dysfunction.

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