Growth and Differentiation Factor-9 Stimulates Progression of Early Primary but Not Primordial Rat Ovarian Follicle Development[1]

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ABSTRACT

The ovary contains a pool of primordial follicles containing oocytes arrested in meiosis that are the source of developing follicles for the female. Growth and differentiation factor-9 (GDF-9) is a member of the transforming growth factor beta superfamily of growth factors, and follicles of GDF-9 knockout mice arrest in the primary stage of development. The effect of GDF-9 treatment on the primordial to primary follicle transition and on subsequent follicle progression was examined using a rat ovary organ culture system. Ovaries from 4-day-old rats were cultured under serum-free conditions in the absence or presence of growth factors. GDF-9 treatment caused a decrease in the proportion of stage 1 early primary follicles and a concomitant increase in the proportion of stage 2 mature primary follicles. GDF-9 did not effect primordial follicles or stage 0 to stage 1 follicle transition. GDF-9 also did not influence stage 3 or 4 secondary follicle numbers. Isolated antral follicle granulosa and theca cell cultures were used to analyze the actions of GDF-9. GDF-9 treatment did not directly influence either granulosa or theca cell proliferation. The ability of GDF-9 to influence the expression of another growth factor was examined. GDF-9 treatment increased kit ligand (KL) mRNA expression in bovine granulosa cells after 2 days of culture. Ovaries from 4-day-old rats were also cultured with or without GDF-9 treatment, and total ovary expression of KL mRNA was increased by GDF-9. In summary, GDF-9 was found to promote the progression of early primary follicle development but did not influence primordial follicle development. The actions of GDF-9 on specific stages of follicle development may in part be mediated through altering the expression of KL.

developmental biology, follicle, granulosa cells, growth factors, ovary

INTRODUCTION

Female mammals have a pool of oocytes arrested in primordial follicles that are the sole source for ovulated eggs over their reproductive life. A primordial follicle is composed of an oocyte arrested in the diplotene stage of prophase one of meiosis and is surrounded by a single layer of squamous pregranulosa cells. When follicles leave the resting pool, they undergo a primordial to primary follicle transition and the surrounding squamous pregranulosa cells become cuboidal granulosa and begin to proliferate [1–4]. As follicles continue to develop through the primary, secondary, and preantral stages, they gain successive layers of granulosa cells, the oocyte increases in size, and theca cells surround the follicle. The orderly progression of oocytes and their follicles from this arrested pool to finally become ovulatory follicles is essential for reproduction. When the available pool of primordial follicles is depleted, reproductive function ceases and humans enter menopause [5–8]. An understanding of the role of growth factors in controlling this orderly follicular progression may lead to improved therapies for infertility and control of the menopausal transition.

Several growth factors have been implicated as acting locally within the ovary to promote the primordial to primary follicle transition. Kit ligand (KL) promotes the primordial follicle transition and also can directly stimulate theca cell proliferation [9]. KL can also promote recruitment of theca cells from the stroma surrounding the primordial follicle [10]. Basic fibroblast growth factor (bFGF) also promotes the primordial to primary follicle transition [11]. Actions of bFGF that may contribute to follicular transition and development include the capacity to increase proliferation of granulosa, theca, and ovarian stromal cells [11–14]. Additionally, bone morphogenetic protein-7 (BMP-7) has been shown to promote the primordial to primary follicle transition and to increase granulosa cell proliferation [15]. Bone morphogenic protein-15 (BMP-15) is a growth factor expressed in the oocytes of developing follicles that plays a role in early follicle progression [16, 17]. BMP-15 stimulates proliferation of preantral granulosa cells and inhibits FSH-stimulated progesterone production in later stage granulosa [18]. Leukemia inhibitory factor (LIF) has also recently been shown to promote the primordial to primary follicle transition and to up-regulate granulosa cell expression of KL [19].

Growth and differentiation factor-9 (GDF-9) has been proposed as another candidate to promote the primordial to primary follicle transition [20, 21]. GDF-9 is a member of the large transforming growth factor beta (TGFβ) superfamily of growth factors and is most homologous with BMP-15 [16, 17]. Data from several studies suggest that GDF-9 is an important regulator of early follicle development. The GDF-9 mRNA transcript and protein are expressed in the oocytes of primary, but not primordial, follicles in mice [22, 23], rats [24], and humans [25]. The presence of GDF-9 in primary and later stage follicles suggests that GDF-9-mediated signaling events may be important in the primary and later stages of follicle development. In contrast, GDF-9 mRNA is expressed in the primordial and subsequent stages of developing follicles in cattle and sheep [26]. The potential role of GDF-9 in the primordial to primary follicle transition in the cow and sheep will need to be elucidated. Ovarian development has been examined in mice with the GDF-9 gene knocked out. Primordial follicles undergo primordial to primary follicle transition in GDF-9 knockout mice, but follicle development is then arrested at the primary stage [27]. The oocytes in these arrested follicles continue to grow and develop according to

the morphologic determinants of the germinal vesicle chromatin pattern, but eventually oocyte viability is lost [28]. No recognizable theca cell development occurs around these arrested follicles [29]. These gene knockout experiments indicate that GDF-9 is not essential for the primordial to primary follicle transition. However, considering the potential compensatory role of other growth factors, it is possible that GDF-9 is able to indirectly promote and facilitate the primordial to primary follicle transition. A similar phenomenon exists for bFGF. Basic FGF promotes the primordial to primary follicle transition [11], but bFGF knockout mice still are able to undergo some primordial follicle transition because they are fertile [30]. One question examined in the present study is whether GDF-9 has specific effects on the primordial follicle transition. These effects may be separate and distinct from those on later stage follicle progression, where the knockout mouse studies indicate there is a required role for GDF-9.

In the current study, the ability of GDF-9 to promote the progression of each of the preantral stages of follicular development, including the primordial to primary follicle transition, is examined in an in vitro ovary culture system. Potential mechanisms of action by which GDF-9 may exert its effects are investigated by determining the ability of GDF-9 to influence theca and granulosa cell proliferation and the ability of GDF-9 to induce the expression of KL.

MATERIALS AND METHODS

Histology and Organ Cultures

All animals were used in accordance with the requirements of the Washington State University Institutional Animal Care and Use Committee and NIH guidelines for the care and use of laboratory animals. Ovaries were removed from postnatal Day 4 rat pups and dissected free of the ovarian bursa. Whole ovaries were cultured for 14 days as previously described [31] 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, v/v) containing 0.1% bovine serum albumin (Sigma, St. Louis, MO), 0.1% albumax (Gibco BRL, Gaithersburg, MD), 27.5 μg/ml transferrin, 0.05 mg/ml L-ascorbic acid (Sigma) and 1 μg/ml insulin (human recombinant, Sigma) in a four-well culture plate (Nunc plate; Applied Scientific, South San Francisco, CA). Ovaries were randomly assigned to treatment groups with approximately three ovaries per floating filter. Seven ovaries were examined per treatment group in each of three experiments. During organ culture, ovaries were treated with 50 ng/ml GDF-9 (Dr. Aaron Hseuh, Stanford, CA) or were left untreated. Culture medium was replaced and ovaries were treated every 2 days. Medium was supplemented with penicillin, streptomycin, and gentamycin to prevent bacterial contamination. Ovaries cultured in this way have been shown to have no change in the total number of oocytes compared with uncultured controls [31]. The cultured ovaries are viable and cells have a healthy appearance as previously reported [11, 31]. After culture, ovaries were fixed in Bouin solution for 1–2 h, embedded in paraffin, sectioned (3–5 μM), and stained with hematoxylin and eosin.

The number of follicles at each developmental stage was counted in each of two serial sections from the largest cross-section through the center of the ovary and averaged in each category. In two serial sections, many of the same follicles and oocytes are present in each section. This provides an opportunity to get a representative count of the same follicles from two perspective sections. Normally, 150–250 follicles (i.e., containing complete transected nucleus) are present in a cross-section. In order to be counted, a follicle section must transect the nucleus of the oocyte. The person counting and categorizing follicles was blinded about the treatment group of the ovaries. Follicles were classified as either primordial (stage 0) or as one of the developing preantral stages (stages 1–4) as described previously [1, 31]. Briefly, stage 0 (primordial) follicles consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Stage 1 (early primary) follicles have some cuboidal granulosa cells and some squamous pregranulosa cells in a single layer around the oocyte. Stage 2 (primary) follicles have a complete single layer of cuboidal granulosa cells around an enlarging oocyte. Stage 3 follicles are characterized by the presence of a partial second layer of granulosa cells around

a further enlarged oocyte. Stage 4 (secondary) follicles have at least two complete granulosa cell layers. All these developmental stages are prior to the formation of a fluid-filled antrum in the follicle. Follicles were classified primarily on the basis of granulosa cell shape and number and secondarily on oocyte size.

Cell Culture

Theca cells and granulosa cells were isolated from freshly obtained bovine ovaries as previously described [32]. Cells were plated and cultured either at subconfluent densities for use in growth assays (in DMEM plus 0.1% bovine serum albumin plus 0.1% calf serum) or at confluent densities for use in assays of mRNA expression (in Ham F-12 plus 0.1% bovine serum albumin).

Cell Growth Assays

Cells were cultured at subconfluent densities (growth permissive) in DMEM plus 0.1% bovine serum albumin plus 0.1% calf serum for 24 h; then, cells were treated for 20 h with GDF-9 (50 ng/ml) or 10% calf serum. This was followed by a 6-h incubation with [3H]thymidine (10 μCi/ml). Counts per minute (CPM) of [3H]thymidine incorporated into DNA were determined by scintillation assay as described previously [11] and normalized to the total DNA per well. DNA levels were assayed by incorporation of SYBR-green dye (Molecular Probes Inc., Eugene, OR) at 1: 5250 dilution. Samples were excited at 300 nm and fluorescence measured at 520 nm. Fluorescence was compared with that of a standard curve of known calf thymus DNA.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

To assay for KL mRNA expression, granulosa cells were plated at confluent density in Ham F-12 plus 0.1% bovine serum albumin for 24 h; then cells were treated for 2 days with GDF-9 (50 ng/ml) or 10% calf serum. At the end of treatment, total RNA was prepared from cultured cells using Trizol reagent (Gibco BRL, Rockville, MD). RNA was stored at $-70°$C until use. As described previously [33], the RNA samples were reverse transcribed to cDNA by adding 1 μg RNA to 1 μl each of 1 μM 3' KL and cyclophilin primers. Primer sequences are detailed below. Before reverse transcription, tubes containing total RNA and 3'-primers were heated to 65°C for 10 min and cooled to room temperature. Total RNA was reverse transcribed for 1 h at 42°C using the following conditions: 1 μg total RNA, 1 μM specific 3'-primers, 0.1 mM dNTP, 10 mM DTT, 40 units RNAse inhibitor (Promega, Madison, WI), and 200 units M-MLV reverse transcriptase (Gibco BRL) in 20 μl RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KC1, 3 mM MgCl$_2$). After 1 h, samples were heated to 95°C for 5 min to inactivate the reverse transcriptase enzyme. Steady-state levels of KL and cyclophilin (i.e., 1B15) mRNAs were analyzed using a specific quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay for each gene [33]. These quantitative RT-PCR assays have previously been described in detail [9]. The primers used were KL, 5'-GGACAAGTTTTCGAATATTTCTGAAGGCTTGAGTAATTATTG-3' (5'-primer; 42-mer), and 5'-AGGCCCCAAAAGCAAACCCGATCA-CAAGAG-3' (3'-primer; 30-mer), which generated a specific 452-base pair (bp) L PCR product, and 1B15, 5'-ACACGCCATAATGGCACT-GGTGGCAAGTCCATC-3' (5' primer, 33-mer), and 5'-ATTTGCCAT-GGACAAGATGCCAGGACCTGTATG-3' (3' primer, 33-mer), which generated a specific 105-bp product. Amplification was performed using the following conditions: 1.5 mM MgCl$_2$, 0.4 μM each primer, 25 μM dNTP, and 0.5 units Taq polymerase in 25 μl Fermante PCR buffer. Each PCR amplification consisted of an initial denaturing reaction (5 min, 95°C), 26–30 cycles of denaturing (1 min, 95°C), annealing (1 min, 60°C for 1B15 and 2 min, 55°C for KL), and elongation (2 min for 1B15 and 3 min KL, 72°C) reactions and a final elongation reaction (10 min, 72°C). At least 1.0 μCi of 32P-labeled dCTP (Redivue, Amersham Life Sciences, Arlington Heights, IL) was included in each sample during amplification for detection purposes. Specific PCR products were quantified by electrophoresis of all samples on 6% polyacrylamide gels, exposing the gels to a phosphor screen for 8–24 h, followed by quantifying the specific bands on a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Each gene was assayed in separate PCR reactions from the same RT. All KL data were normalized for 1B15. The optimal number of cycles for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity (i.e., logarithmic phase of PCR reactions). KL PCR products were amplified for 30 cycles, and 1B15 PCR products were amplified for 26 cycles. The sensitivity of each quantitative PCR assay
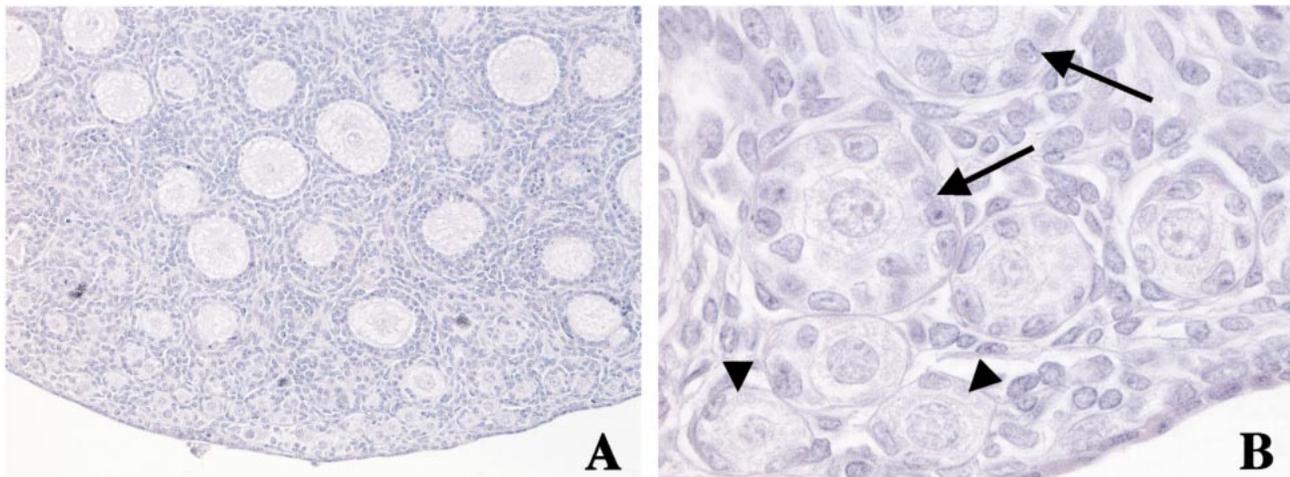
FIG. 1.   Morphology of 4-day-old rat ovaries cultured for 14 days. Sections with (**A**) ×100 magnification and (**B**) ×400 magnification are shown. The primordial follicles are indicated with an arrow head and primary follicles with an arrow.

was below 1 fg, which corresponds to less than 125 fg target mRNA/µg total RNA.

Statistics

Pair comparisons were performed using a Student t-test. Groups were considered significantly different for a two-tailed P-value if $P \leq 0.05$. For the ovary culture experiments, data were subjected to a two-way ANOVA with follicle stage and GDF-9 treatment being the independent variables. Bonferroni post hoc tests were also performed to more rigorously test for GDF-9 treatment effects at each stage of follicle development while controlling for type 2 error. All statistics were calculated with the help of either JMP v3.1 (SAS Institute, Inc., Cary, NC) or Graphpad Prism v3.0a software (Graphpad Software, Inc., San Diego, CA).
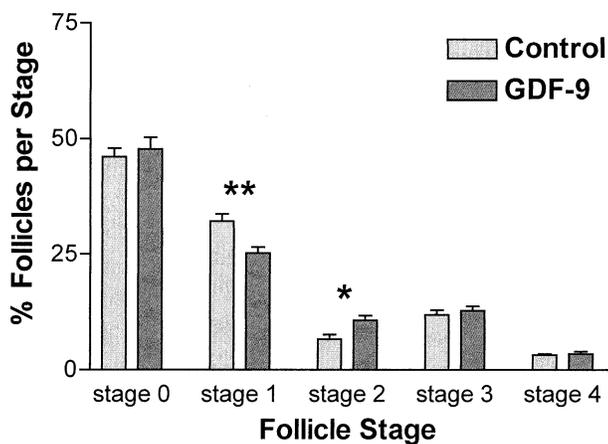

FIG. 2.   GDF-9 promotes the progression of early primary but not primordial follicles in cultured rat ovaries. Ovaries from 4-day-old rats were placed into culture for 14 days. Cultured ovaries were treated with no factors (control) or 50 ng/ml GDF-9. After culture, all ovaries were fixed, stained, and subjected to morphologic analysis. The follicles per ovary cross-section were counted and placed into stage 0 (primordial), stage 1 (early primary), stage 2 (primary), stage 3, or stage 4 (secondary) categories. Bar height indicates the proportion of follicles in each stage for each treatment group. Data are the mean ± SEM from three separate experiments with two or three replicates per experiment. **Control significantly different from GDF-9 treated at $P < 0.01$ by Student t-test and $P < 0.05$ by Bonferroni post hoc test; *$P < 0.05$ by Student t-test and $P < 0.1$ by Bonferroni post hoc test.

RESULTS

Ovaries from neonatal rats were cultured in the presence or absence of 50 ng/ml GDF-9 for 2 wk. The effect of GDF-9 on the primordial to primary follicle transition and on subsequent preantral follicle development was examined. After culture, the ovaries were fixed, sectioned, and stained with hematoxylin and eosin for morphologic analysis (Fig. 1). The follicles in each ovary were categorized as stage 0 (primordial), stage 1 (early primary), stage 2 (primary), stage 3, or stage 4 (secondary). The morphology of primordial and primary follicles is shown in Figure 1 and demonstrates the viability of the organ after 14 days of culture. As previously documented [9, 31], no change in total follicle number was observed with any of the treatments utilized, only a change in the follicle composition and stages (data not shown).

The results of GDF-9 treatment are presented in Figure 2. There was no difference in the proportion of primordial (stage 0) follicles between GDF-9-treated and control, untreated ovaries. This indicates that GDF-9 treatment has no effect on the primordial to primary follicle transition. GDF-9 treatment resulted in a statistically significant decrease in the proportion of stage 1 early primary follicles (25% stage 1) compared with controls (32% stage 1) with $P \leq 0.006$ by the Student t-test and $P < 0.05$ by the Bonferroni post hoc test. Conversely, GDF-9 treatment resulted in a significant increase in stage 2 primary follicles (10.5% vs. 6.5% for controls) with $P \leq 0.02$ by the Student t-test and $P < 0.1$ by the Bonferroni post hoc test. These data suggest that a larger proportion of stage 1 follicles is stimulated to progress to later developmental stages when treated with GDF-9 (Fig. 2).

One mechanism by which GDF-9 might affect follicle growth would be if GDF-9 directly stimulated proliferation of granulosa or theca cells. To test for this possibility, granulosa and theca cells from bovine ovaries were cultured in vitro in the presence or absence of 50 ng/ml GDF-9. [^{3}H]thymidine incorporation was measured as an indicator of granulosa and theca cell proliferation. Cultured cells were treated with 10% calf serum as a positive control. No significant difference was seen in the proliferation of either granulosa or theca cells when they were treated with GDF-9 for 20 h compared with untreated controls (Fig. 3). Treat-
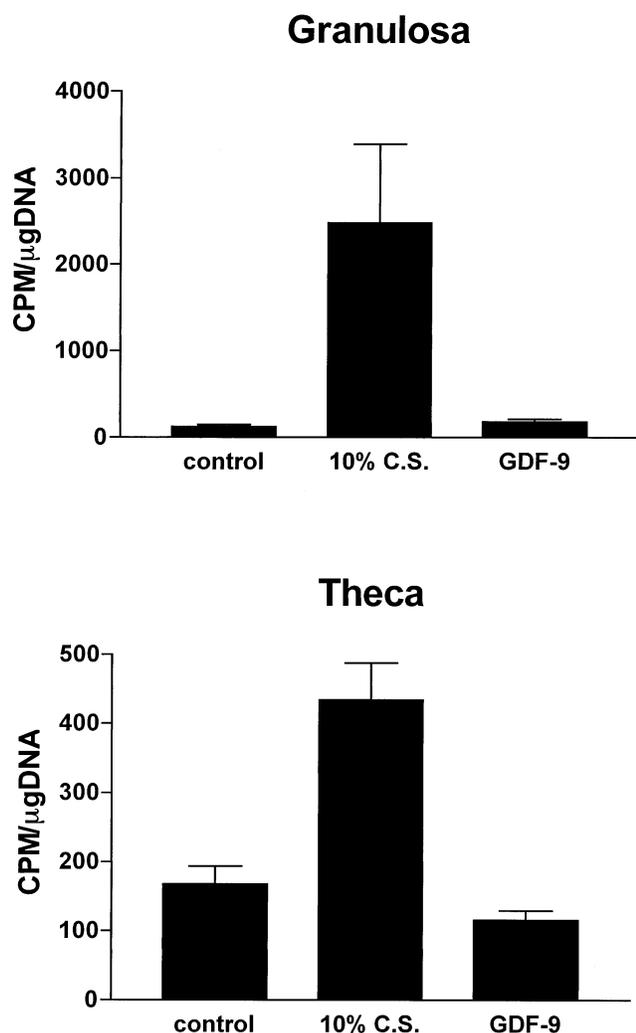
Granulosa

Theca

FIG. 3. GDF-9 does not promote proliferation of either theca or granulosa cells in culture. Granulosa (top panel) and theca cells (bottom panel) were isolated from antral bovine follicles and placed into culture. Cells were treated with 50 ng/ml GDF-9 or 10% calf serum as a positive control or were untreated. Cell proliferation was measured by [³H]thymidine incorporation. Data are mean ± SEM from three separate experiments with four replicates per experiment.

ment with 10% calf serum resulted in increased [³H]thymidine incorporation.

Because GDF-9 does not directly stimulate granulosa or theca cell proliferation, it is possible that GDF-9 signaling may induce production of other growth factors that in turn promote the events that make up follicle growth and development. KL is a growth factor known to promote growth of granulosa and theca cells as well as to stimulate recruitment of theca from the stroma surrounding follicles [9, 10]. To test whether GDF-9 induces KL expression, bovine granulosa cells were treated in vitro with 50 ng/ml GDF-9 for 2 days. A 48-h culture duration was used to allow sufficient time for transcription to be increased and mRNA levels to accumulate. The level of KL mRNA in these cells was then measured using a quantitative RT-PCR assay. KL mRNA levels were normalized to the levels of the constitutively expressed gene cyclophillin (1B15). Results are presented in Figure 4. GDF-9 treatment induces a significant ($P \leq 0.009$) increase in KL mRNA expression.

Further experiments were performed to test if GDF-9 can induce KL expression in neonatal rat ovaries. Four-day-old
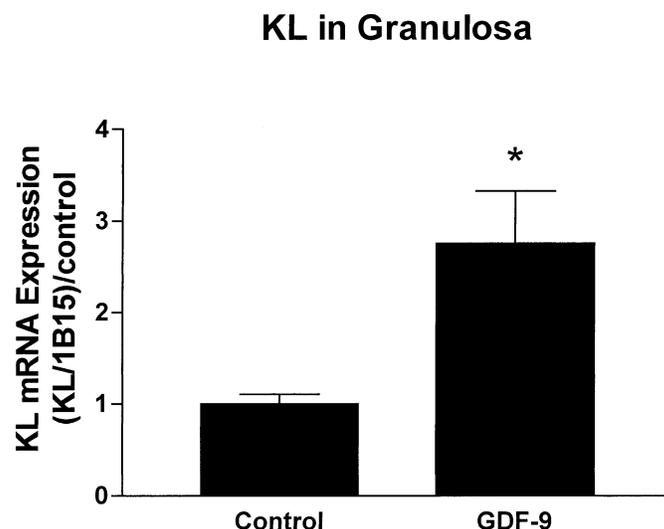
KL in Granulosa

FIG. 4. GDF-9 induces KL mRNA expression in cultured granulosa cells. Granulosa cells were isolated from antral bovine follicles and placed into culture. Cells were treated with 50 ng/ml GDF-9 or were untreated for 3 days. RT-PCR was performed to determine levels of KL mRNA expression. Data are expressed as KL mRNA/1B15 mRNA (pg/well) normalized to untreated control values. Data are mean ± SEM from three separate experiments with two replicates per experiment. Asterisk indicates a significant difference ($P < 0.05$) between treatments by Student t-test.

rat ovaries were cultured in vitro for 3 days in the presence or absence of 50 ng/ml GDF-9. After culture, mRNA was isolated from each ovary and levels of KL mRNA production were measured using a quantitative RT-PCR assay. KL mRNA levels were normalized to the levels of cyclophillin (1B15). Results are presented in Figure 5. GDF-9 treatment induces a significant ($P \leq 0.03$) increase in KL mRNA expression in the cells of neonatal rat ovaries. Therefore, GDF-9 can in part mediate its effects indirectly through a local network of growth factors such as KL (Fig. 6).
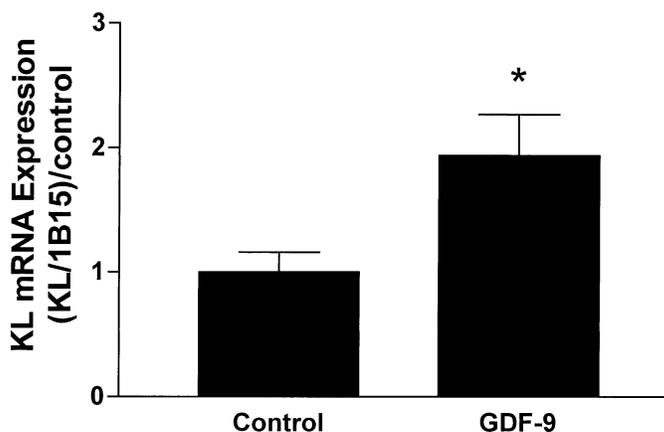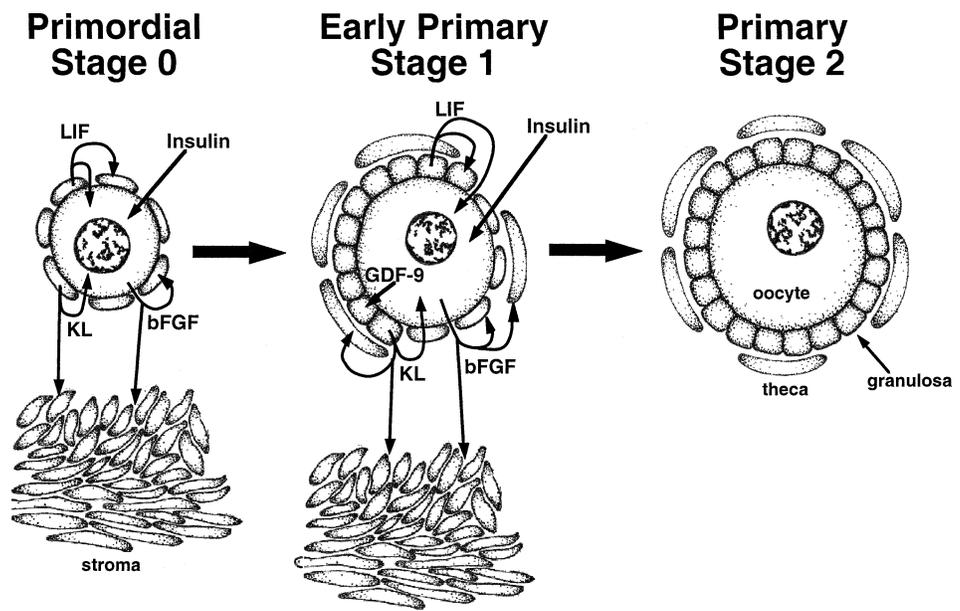
KL in Cultured Ovaries

FIG. 5. GDF-9 induces KL mRNA expression in cultured neonatal rat ovaries. Whole ovaries were treated in vitro with 50 ng/ml GDF-9 or were untreated for 3 days. RT-PCR was performed to determine levels of KL mRNA expression. Data are expressed as KL mRNA/1B15 mRNA (pg/well) normalized to untreated control values. Data are mean ± SEM from three separate experiments with two replicates per experiment. Asterisk indicates a significant difference ($P < 0.05$) between treatments by Student t-test.

FIG. 6. Model of potential paracrine signaling interactions in primordial, early primary, and primary follicles. Theca and granulosa cells and the oocyte are labeled on the primary follicle. The entire follicle is surrounded by stromal/interstitial cells with which paracrine interactions can occur. Arrows indicate proposed signaling interactions with growth and differentiation factor-9 (GDF-9), kit ligand (KL), basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), and insulin. Signaling interactions are not diagrammed on the primary follicle.

Primordial Stage 0 — LIF, Insulin, KL, bFGF, stroma

Early Primary Stage 1 — LIF, Insulin, GDF-9, KL, bFGF

Primary Stage 2 — oocyte, theca, granulosa

DISCUSSION

In these studies, it was demonstrated that GDF-9 did not promote the primordial to primary follicle transition in rat ovaries in vitro. However, GDF-9 treatment did lead to a decrease in the proportion of stage 1 early primary follicles and a concomitant increase in the proportion of stage 2 primary follicles. This suggests that GDF-9 promotes the progression of stage 1 follicles to develop into stage 2 follicles. No statistically significant change was seen in the number of stage 3 or 4 follicles with treatment. Recent in vivo studies [34] in the rat also found that GDF-9 treatment stimulates an increase in the number of primary follicles. In contrast with the current study, Vitt et al. [34] finds that GDF-9 treatment in vivo results in a decrease in primordial follicle number compared with controls. This would suggest that GDF-9 promotes primordial follicle transition in that in vivo model system. A concern raised is that there is no detectable GDF-9 expression in the primordial follicle oocytes of rats, mice, or humans [23–26, 35]. One possibility is that GDF-9 expression levels are too low to detect but are still adequate to promote primordial follicle transition in an untreated animal. In cattle and sheep, primordial follicles do express low but detectable levels of GDF-9 [26]. However, if GDF-9 could act on primordial follicles to promote transition to primary follicles, then one would expect the ovaries of the current experiment to show a decrease in their proportion of primordial follicles when cultured and treated with GDF-9 in vitro. This was not found to be the case. GDF-9 did not promote the primordial to primary follicle transition in rat ovaries in vitro.

Another possibility to explain the effect of GDF-9 treatment on rat primordial follicles in vivo when GDF-9 is not normally expressed in these follicles was proposed by Vitt et al. [34]. It was proposed that the principal action of GDF-9 was to promote progression of primary follicles to later preantral stages. This increased progression of primary follicles would indirectly stimulate the primordial follicle pool to increase primordial to primary follicle transition. This proposed mechanism is consistent with the result of the current study, in which it was found that GDF-9 treatment stimulated early primary (stage 1) follicles to progress to primary (stage 2) follicles. No significant change in stage

3 or 4 follicles was found with GDF-9 treatment. Because there was a slight trend toward increased stage 3 and 4 follicles, perhaps a longer in vitro treatment with GDF-9 or examination of a larger sample population might show significant follicle progression of these later stages. The proposed indirect stimulation of primordial follicles is further supported by the fact that, in GDF-9 knock-out mice, the primordial follicle transition occurs but the follicles fail to progress past the primary follicle stage [27]. Interestingly, if GDF-9 treatment acts indirectly in vivo to promote primordial follicle transition and because the GDF-9 treatment effect on primordial transition is not seen in the current in vitro study, this suggests that a cofactor possibly extrinsic to the ovary may be required for successful signaling between the developing primary follicles and the primordial follicle pool. Such extrinsic cofactors might be of neural or hormonal origin. In the in vitro ovary culture system of the current experiment, no extrinsic signaling cofactors are present and so no signaling would be possible from the primary follicles back to the primordial pool. Therefore, there would be no increase in primordial follicle transition, which is consistent with the current results. Alternatively, a cofactor produced in the ovary may be required for signaling from the primary to the primordial follicles. Under conditions of culture, this cofactor may be washed out or its expression altered, causing no increase in primordial follicle transition. An example of a growth factor that has been shown to interact with GDF-9 is BMP-15 [36]. This oocyte-derived growth factor is not required for fertility, but mice homozygous for the mutant knock-out allele (BMP-15$^{-/-}$) are subfertile and have minor ovarian histopathologic defects. Mice heterozygous for the GDF-9 knock-out allele (GDF-9$^{+/-}$) are apparently normal. However, if female mice are both BMP-15$^{-/-}$ and GDF-9$^{+/-}$, they have markedly reduced fertility and morphologically obvious defects in folliculogenesis and cumulus oopherus physiology. BMP-15 may thus be thought of as a cofactor to GDF-9 and their interaction as being necessary for normal folliculogenesis. Whether BMP-15 or any other signaling factors are needed for GDF-9 to affect primordial follicle transition will require further investigation.

Another possible mechanism by which GDF-9 may act

to promote primordial follicle transition rests on the idea that GDF-9 receptors may be present in the cells surrounding the oocyte of primordial follicles before that oocyte begins to express GDF-9. Experimental treatment with GDF-9 could then prematurely induce the primordial to primary follicle transition. However, there was no GDF-9 treatment effect on primordial follicles detected in the current in vitro experiment. So this preexisting receptor mechanism also would require the presence of a necessary extrinsic cofactor to mediate signaling to primordial follicles. Further investigation will be necessary to determine what exactly the mechanisms are by which GDF-9 affects follicle progression. The preponderance of data indicates that GDF-9 does not affect the primordial follicles and does stimulate the progression of early primary and primary follicles. One caveat to this theory arises from the idea that some early primary (stage 1) follicles with a mixture of flattened and cuboidal granulosa around the oocyte do not promptly progress to later stages and so should actually be classified as part of the arrested primordial pool [37]. If this proves to be the case, then GDF-9 may be promoting primordial to primary follicle transition in that portion of the arrested pool characterized as stage 1. The previous studies using neonatal rat ovary cultures and the timing of follicle development do not suggest a subpopulation of follicles but rather a progression of primordial to primary follicles [9, 11].

One mode of action by which oocyte-derived GDF-9 may stimulate primary follicle progression would be if GDF-9 could directly stimulate the proliferation of the granulosa and theca cells of the follicle. To test this hypothesis, bovine granulosa and theca cells were cultured with and without GDF-9 treatment and [^{3}H]thymidine incorporation was measured. GDF-9 treatment was found to have no effect on the proliferation of either granulosa or theca cells compared with controls. In an earlier study, GDF-9 was found to stimulate [^{3}H]thymidine incorporation in rat granulosa cells [38]. It is possible that rat granulosa cells proliferate in response to GDF-9 while bovine granulosa cells do not. However, it is shown that the GDF-9 used for treatment is bioactive in the bovine system because GDF-9 treatment induced an increase in KL mRNA expression. Procedural differences in the [^{3}H]thymidine incorporation assay could explain the differences seen between the rat and bovine models. In the work of Vitt et al., granulosa cells were cultured in the presence of GDF-9 and [^{3}H]thymidine for 24 h prior to the scintillation assay [38]. In 24 h, [^{3}H]thymidine uptake can be affected by thymidine transport or metabolism distinct from S-phase DNA synthesis. The genes regulating any of these things may be affected by GDF-9 treatment. In the current study, [^{3}H]thymidine was only present for 6 h of incubation before assay. These results may be more reflective of S-phase DNA synthesis.

GDF-9 may also act indirectly to stimulate primary follicle progression by inducing the expression of other growth factors. These indirect factors could in turn act on the cells of the follicle to promote the critical proliferation and differentiation events of follicle progression. One such candidate growth factor is KL. KL is produced by granulosa cells and acts on the oocyte and can also directly stimulate theca cell proliferation [9]. In addition, KL promotes recruitment of theca cells from the stroma surrounding the primordial follicle [10]. Experiments were performed to examine whether GDF-9 could induce KL expression. In one set of experiments, bovine granulosa cells in vitro were treated with GDF-9 for 2 days and KL mRNA expression measured. GDF-9 treatment caused an increase in granulosa cell expression of KL mRNA compared with controls. Because GDF-9 treatment did not directly increase granulosa cell proliferation, GDF-9 could indirectly influence proliferation through an increase in KL expression. It is likely that the 20 h of GDF-9 treatment performed in the growth assays is insufficient for enough KL transcription and translation to occur to affect cell proliferation. In addition, KL does not influence granulosa cell proliferation but acts on the oocyte and theca cells.

In another set of experiments, whole ovaries from 4-day-old rats were cultured in the presence or absence of GDF-9 for 3 days. These ovaries contain mostly primordial and primary follicles. GDF-9 treatment increased the KL mRNA content in these cultured ovaries. These observations support the hypothesis that one of the ways in which GDF-9 produced by the oocyte promotes follicle progression is to stimulate granulosa cells to express KL. KL may in turn promote theca cell recruitment from the surrounding stroma, increase theca cell proliferation, and directly act on the oocyte [39–42]. This hypothesis is also supported by a previous study in mice in which granulosa cells isolated from preantral follicles were cocultured with or without oocytes isolated from preantral follicles [43]. The presence of an oocyte in these cocultures increased KL expression in these granulosa cells [43]. GDF-9 is one of the growth factors expressed in preantral oocytes. Other actions of GDF-9 that promote follicle progression are currently under investigation.

An increasing list of paracrine growth factors has been implicated in regulating the primordial to primary follicle transition and the progression of early preantral follicle development. KL, bFGF, BMP-7, LIF, GDF-9, and the hormone insulin have all been demonstrated to be involved in the orderly progression of follicles through the early stages of preantral development [9–15, 19, 24, 27]. Figure 6 presents the current hypothesis of primordial and primary follicle development and the proposed actions of this network of growth factors. In summary, GDF-9 was found to promote the progression of early primary but not primordial follicles. One mechanism by which this may be accomplished is to induce the expression of KL from surrounding granulosa cells. An improved understanding of the factors controlling the orderly progression of follicle development could lead to improved therapies for some types of infertility and for control of the menopausal transition.

ACKNOWLEDGMENTS

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