Effects of growth differentiation factor 9 on cell cycle regulators and ERK42/44 in human granulosa cell proliferation

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GDF-9 is a member of the transforming growth factor-β (TGFβ) superfamily that includes activins and bone morphogenetic proteins (BMPs), is expressed mainly in growing oocytes from the primary follicle stage to just after ovulation and plays key roles during folliculogenesis as in ovine, bovine, and mouse. GDF-9 stimulates granulosa cell proliferation and plays important roles during folliculogenesis. However, its molecular mechanisms are still far from clear, particularly its roles in human granulosa cells around the periovulatory stage. Therefore, we investigated the effects of GDF-9 on cell cycle distribution, regulatory molecules, and signaling pathways involved in human luteinized granulosa (hLG) cells in vitro. Primary cultures of hLG cells obtained from women undergoing IVF and treated with and without recombinant GDF-9 were evaluated with and without a specific inhibitor to activin receptor-like kinase 5 (ALK5; SB-431542), ERK42/44 (PD-098059), or Smad3 (SIS3). Cell proliferation, cell cycle distribution, mRNA expression, and protein expression of relevant cell cycle molecules were determined by [3H]thymidine incorporation, flow cytometry, quantitative PCR, and immunoblotting, respectively. GDF-9 stimulated [3H]thymidine incorporation, enhanced cell transition from G0/G1 to S and G2/M phases (whereas both SB-431542 and PD-098059 attenuated these changes), increased mRNA and protein expression of cyclin D1 and E, and decreased those of the cyclin-dependent kinase (CDK) inhibitors p15INK4B and p16INK4A. GDF-9 also activated Rb protein (a critical G1 to S-phase regulator), ERK42/44, and Smad3. PD-098059 blocked Rb protein phosphorylation and the increase in cyclin D1 and E but not the decrease in p15INK4B and p16INK4A induced by GDF-9. In contrast, SIS3 reversed the decrease in p15INK4B and p16INK4A but not the increase in cyclin D1 and E induced by GDF-9. GDF-9 stimulates hLG cell proliferation by stimulating cyclin D1 and E and suppressing p15INK4B and p16INK4A via both Smad-dependent and Smad-independent pathways.

GDF-9 are infertile because folliculogenesis is arrested at the primary follicle stage (6). In mutant mice lacking connexin-43, impaired granulosa cell proliferation can be restored with recombinant GDF-9 (11). However, the molecular basis of GDF-9 as a paracrine factor modulating granulosa cell functions is unknown. Furthermore, the roles of GDF-9 in regulating human ovarian physiology around the periovulatory transition are unclear.

Intracellular signaling by TGFβ superfamily is generally divided into two main groups: the TGFβ/activin and BMP signaling pathways that activate Smad2/3 and Smad1/5/8, respectively, GDF-9 lacks the cysteine residue present in most TGFβ superfamily ligands. In rodents and humans, GDF-9 utilizes a hybrid of these two signaling pathways as it first binds to the BMP-activated type II receptor, but downstream, it involves the TGFβ type I receptor (ALK-5) to activate Smad2/3 (14, 17, 30). Although GDF-9 is an important signaling molecule, recent studies also implicate that maturation of the mouse oocyte-cumulus cell complex induced by GDF-9 requires activation of mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinases (ERK42/44) (27). To further characterize the effects of GDF-9 on granulosa cell cycle progression, we undertook the following study in primary human granulosa cell cultures.

We studied the effect of GDF-9, with and without a specific inhibitor to ALK5 (SB-431542), ERK42/44 (PD-098059), or Smad3 (SIS3), on granulosa cell proliferation, cell cycle progression, and distribution using [3H]thymidine incorporation and flow cytometry and mRNA and protein expression of cell cycle regulatory protein such as cyclin D1 and E and cyclin-dependent kinase (CDK) inhibitors p15INK4B and p16INK4A of the INK family and p21Cip1 and p27Kip1 of the Cip/Kip family using quantitative PCR, Western blot analysis, and immunofluorescence staining. We also evaluated the effects of GDF-9 on phosphorylation of Rb protein (a critical G1 to S-phase regulator), ERK42/44, and Smad3 in relation to some of these cell cycle regulators. Our study reveals, for the first time, the presence of GDF-9-responsive molecules in the cell cycle machinery of human granulosa cells and provides insights into the mechanisms by which GDF-9 regulates human granulosa cell proliferation.

MATERIALS AND METHODS

Materials. DMEM-Ham’s F10 was obtained from Hyclone Laboratories (Logan, UT). Recombinant human GDF-9 was from PeproTech (Rock Hill, NJ). ERK42/44-synthetic inhibitor PD-098059 and Smad3 inhibitor SIS3 were purchased from Calbio-
chem (San Diego, CA). ALK4/5/7 inhibitor SB-431542 and all other chemicals, unless otherwise stated, were from Sigma Chemical (St. Louis, MO).

**Tissue collection and cell culture.** Human luteinized granulosa (hLG) cells were obtained from women undergoing in vitro fertilization (IVF) treatment in the IVF Program of the University of British Columbia. The study was approved by the university and hospital ethics review committees. Freshly isolated granulosa cells were prepared and cultured as described previously (15). Briefly, after ovum retrieval, the aspirated follicular fluid was centrifuged at 300 g for 10 min, and the pellet was resuspended by PBS. Percoll was used to separate the granulosa cells from the red blood cells. The isolated hLG cells were washed with DMEM-Ham’s F-10 (1:1) to remove the remaining red blood cells and cultured in the same medium containing 10% FBS and 0.12 mM gentamycin for 48 h before the various experiments related to the effects of GDF-9 were conducted.

**[3H]thymidine incorporation.** As described previously (21), cells were seeded in 24-well plates and cultured in DMEM-Ham’s F-10 (1:1) with 10% FBS for 24 h. Cells were washed with PBS, starved in serum-free medium for 4 h, and incubated with GDF-9 at concentrations from 0 to 400 ng/ml for another 30 h in DMEM-Ham’s F-10 (1:1) containing 1% FBS. One microcurie of [3H]thymidine (American (St. Louis, MO). Standard (San Diego, CA). ALK4/5/7 inhibitor SB-431542 and all other chemicals, unless otherwise stated, were from Sigma Chemical (St. Louis, MO).

**Flow cytometry.** Cells were synchronized by starvation and treated with different concentrations of GDF-9, as described above. Cells were harvested and washed with PBS twice, then fixed by dropwise addition of 1 ml of ice-cold 70% ethanol, and stored at −20°C before being stained. For fluorescence-activated cell sorting (FACS), cells were centrifuged and washed with PBS, the supernatant was discarded, and the pellets were resuspended and stained with propidium iodide (PI) staining solution (0.1 mg of PI and 0.5 mg/ml RNase A in PBS) at room temperature for 30 min. Cells were passed through a Falcon 70-μm cell strainer cap to remove aggregated cells immediately before FACS. Using the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), measurements of forward scatter, side scatter, and PI fluorescence were obtained for ≥10,000 individual events per reaction. Data acquisition and analysis were performed using CellQuest (version 3.3) software and ModFit LT software (Version 2.0), respectively, for Macintosh (Verity Software House, Topsham, ME).

**RNA isolation and reverse transcription.** Total RNA was isolated using Mini Plus RNeasy Kit (Qiagen, Mississauga, ON, Canada) and reverse transcribed using First Strand cDNA Synthesis Kit (Amer sham Pharmacia Biotech). Briefly, 1 μg cDNA was placed in 24 μl of reaction mixture containing 1 U Taq polymerase (Invitrogen, Burlington, ON, Canada). The efficiency of cDNA synthesis from each sample was estimated by a GAPDH amplimer. All PCR primers span the introns to detect specific mRNA sequences.

**Quantitative (real-time) PCR.** Primers (see Table 1) were added to 25 μl of total reaction volume containing SYBR Green PCR reagents (Applied Biosystems, Foster City, CA). Each reaction was run in triplicate under amplification conditions on the ABI 7700 thermocycler (Applied Biosystems), including 15-min denaturing at 95°C followed by 15-s denaturing at 95°C, 30-s annealing at 60°C, and 30-s extension at 72°C for 40 cycles. The threshold cycle value was determined for each reaction using Sequence Detection Software (Applied Biosystems), whereas quantification was accomplished using the 2−ΔΔCT method, which assumed 100% efficient target and standard gene PCR reactions and results conforming to criteria as described previously (16).

**Immunofluorescence staining.** hLG cells were cultured in 12-well plates with coverslips. After 200 ng/ml GDF-9 treatment for 12 h, the cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. The coverslips were blocked with Dako Protein Block solution (Dako, Mississauga, ON, Canada) for 30 min and incubated overnight at 4°C with 1:500 diluted anti-p15INK4B or p16INK4A primary antibodies in protein block solution. Texas Red-labeled goat anti-rabbit antibody diluted 1:800 (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody. Cell nuclei were stained with 0.5 μg/ml Hoechst 33258, rinsed with PBS, and mounted with Gelvatol. Fluorescence signals were examined using a Zeiss Axio phot microscope equipped with a digital camera (Q Imaging, Burnaby, BC, Canada).

**Western blot analysis.** After treatment with GDF-9, cells were washed with ice-cold PBS and lysed with ice-cold lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in PBS, pH 7.4) with freshly added 1% protease inhibitor cocktails. The extracts were placed on ice for 30 min, harvested, and centrifuged for 15 min at 4°C to remove cell debris. Protein concentration was determined using the Bradford assay. The samples were boiled for 30 min before SDS-PAGE was run with 10–12% separating gels. Proteins were then electrotransferred to a PVDF membrane. The membrane was blocked with 5% skim milk or BSA in Tris-buffered saline (TBS) and incubated with primary antibodies diluted in 5% skim milk or BSA. The antibodies used were anti-phospho-p44/p42 MAPK (1:3,000 dilution), anti-p44/p42 MAPK (1:3,000 dilution), anti-phospho-Rb (1:1,000 dilution), anti-Smad3 (1:1,000 dilution), and anti-p15INK4B (1:1,000 dilution) from Cell Signaling Technology (Danvers, MA) and anti-p16INK4A (1:1,000 dilution), anti-p16INK4A (1:1,000 dilution), anti-cyclin D1 (1:2,000 dilution), and anti-cyclin E (1:1,000 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA). After extensive washing with TBS containing 0.1% Tween-20, immunoreactive bands were visualized after being probed with peroxidase-conjugated secondary antibodies (1:3,000 diluted anti-rabbit antibody, 1:2,000 diluted anti-mouse antibody, or 1:1,000 goat antibody) and signal development with an enhanced chemiluminescence kit (Amer sham Pharmacia Biotech).

**Statistical analysis.** Data were means ± SE from at least three sets of experiments (each from a separate patient), and in each set, cells

Table 1. Real-time PCR primer sequences

<table>
<thead>
<tr>
<th>Genes</th>
<th>Reverse Primer</th>
<th>GenBank No.</th>
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<tbody>
<tr>
<td>p15INK4B</td>
<td>CCGCATGCGCTGCGT</td>
<td>NM_078478.2</td>
</tr>
<tr>
<td>p16INK4A</td>
<td>AGTGCAGGCTCCCGA</td>
<td>NM_000077.3</td>
</tr>
<tr>
<td>p21Cip1</td>
<td>GAGGAGCCAGCTAGA</td>
<td>NM_0000007.9</td>
</tr>
<tr>
<td>p27Kip1</td>
<td>TCCATGGTGGTGGTG</td>
<td>NM_000406.2</td>
</tr>
<tr>
<td>CDK4</td>
<td>AGACTGGGGAGCGCGAC</td>
<td>NM_000057.2</td>
</tr>
<tr>
<td>CDK6</td>
<td>GTCATGGGCTGCAAA</td>
<td>NM_001259.5</td>
</tr>
<tr>
<td>cyclin A</td>
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<td>NM_003914.2</td>
</tr>
<tr>
<td>cyclin E</td>
<td>ATCGCGCAACGACGAC</td>
<td>NM_078478.2</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>ATCGCGCAACGACGAC</td>
<td>NM_001759.3</td>
</tr>
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CDK, cyclin-dependent kinase.
were cultured in triplicate at each dose or treatment combination. Data were analyzed by one-way factorial ANOVA, and corresponding significant results were further assessed by Tukey’s multiple comparison tests. A P value <0.05 was considered statistically significant.

RESULTS

Granulosa cell proliferation and cell cycle progression. Recombinant GDF-9 promoted granulosa cell proliferation in a dose-dependent manner, with significant increases at concentrations of 100–400 ng/ml (Fig. 1A), peaking at 200 ng/ml. These stimulatory effects of GDF-9 (200 ng/ml) were attenuated in cells pretreated with SB-431542, an ALK4/5/7 specific inhibitor that blocked ALK5. This was not due to a nonspecific effect of SB-431542 since there were no significant changes on cell proliferation at the chosen dose of 0.5 μM (Fig. 1B), which was derived from a separate dose-response experiment on the effects of SB-431542 on [3H]thymidine incorporation (Fig. 1B, inset). Furthermore, SB-431542 only partially blocked [3H]thymidine incorporation induced by GDF-9, although cell proliferation was significantly greater than that observed in untreated cells or cells treated with SB-431542 alone, suggesting that GDF-9 might act through other signaling pathways.

After treatment with 100 ng/ml GDF-9, FACS analysis showed a significant decrease, a significant increase, and an insignificant rise in the percentages of cells in G1/G0 phase (70.1 ± 4.0%, P < 0.05), S phase (8.6 ± 1.2%, P < 0.05), and G2/M phase (19.5 ± 2.3%), respectively, relative to control values (Fig. 2A). Treatment with 200 ng/ml GDF-9 further enhanced these changes and significantly increased the percentage of cells in G2/M phase (P < 0.001; Fig. 2A) as well. As for [3H]thymidine incorporation, SB-431542 inhibited these GDF-9 effects on cell cycle distribution (Fig. 2B) and significantly dampened the transition of cells from G1/G0 phase to S and G2/M phases. These results indicate that GDF-9 indeed promotes cell cycle progression.

Expression of cyclins and CDKs. Expression of selective positive cell cycle regulators cyclin A, cyclin D1, and cyclin E and CDK4 and -6 after GDF-9 (200 ng/ml) treatment was assessed by real-time PCR and Western blot analysis. No significant changes of cyclin A mRNA and protein expression were observed between treated and untreated cells (data not shown). After GDF-9 treatment, cyclin D1 mRNA levels increased from baseline at 2 h and significantly so to peak at 4 h before falling toward baseline level by 12 h (Fig. 3A). A similar, but insignificant, change was observed in untreated cells; hence, the level was significantly greater in treated cells at 4 h. Cyclin E mRNA expression increased significantly from 2 to 8 h in both GDF-9-treated and untreated cells before leveling off at 12 h (Fig. 3A). GDF-9 increased cyclin E mRNA expression, which was significantly greater than that of untreated cells at 12 h. In contrast, GDF-9 did not change CDK4 and CDK6 mRNA expression. However, intrinsic changes related to cell cycle dynamics were observed with levels falling from 2 h to a trough at 4–8 h for CDK4 and at 4 h for CDK6 (Fig. 3A) before rising above baseline values in both treated and untreated cells. Following GDF-9 treatment (200 ng/ml), cyclin D1 protein levels increased at 8 h, peaked at 12 h, and then declined but were still significantly above baseline at 24 h. Cyclin E protein expression was also significantly increased by GDF-9 but, corresponding to its expression later than cyclin D1, from 2 to 8 h in both GDF-9-treated and untreated cells before falling toward baseline level by 12 h (Fig. 3A). A similar, but insignificant, change was observed in untreated cells; hence, the level was significantly greater in treated cells at 4 h. Cyclin E mRNA expression increased significantly from 2 to 8 h in both GDF-9-treated and untreated cells before leveling off at 12 h (Fig. 3A). GDF-9 increased cyclin E mRNA expression, which was significantly greater than that of untreated cells at 12 h. In contrast, GDF-9 did not change CDK4 and CDK6 mRNA expression. However, intrinsic changes related to cell cycle dynamics were observed with levels falling from 2 h to a trough at 4–8 h for CDK4 and at 4 h for CDK6 (Fig. 3A) before rising above baseline values in both treated and untreated cells. Following GDF-9 treatment (200 ng/ml), cyclin D1 protein levels increased at 8 h, peaked at 12 h, and then declined but were still significantly above baseline at 24 h. Cyclin E protein expression was also significantly increased by GDF-9 but, corresponding to its expression earlier than cyclin D1, in the cell cycle. peaked at 18 h instead (Fig. 3B). There was a dose-dependent increase in both cyclin D1 and E protein expression (Fig. 3C). Similarly, GDF-9 significantly enhanced Rb protein phosphorylation at both 100 and 200 ng/ml (Fig. 3C). These results indicate that GDF-9 upregulates cyclin D1 and E, which leads to Rb protein phosphorylation and, consequently, granulosa cell cycle progression and cell proliferation.

Expression of cell cycle inhibitors. In untreated cells, p15INK4B mRNA expression decreased significantly at 4 h and then increased from 8 to 12 h. GDF-9 (200 ng/ml) further decreased p15INK4B mRNA expression at 4 h and attenuated its rise from 8 to 12 h. Thus, at 4 and 8 h, p15INK4B mRNA expression levels were only 0.48- and 0.71-fold, respectively,
of baseline level and were significantly lower than corresponding values in untreated cells (Fig. 4A). Whereas the expression levels of p16INK4A mRNA without GDF-9 treatment continued to rise from 4 to 12 h, GDF-9 significantly blunted these rises at 8 and 12 h to 0.67- and 1.66-fold, respectively, of baseline value, accentuating the differences between GDF-9-treated and untreated cells (Fig. 4A). Interestingly, both p21Cip1 and p27Kip1 mRNA expression levels with and without GDF-9 treatment were not significantly different, and both showed no significant time-dependent changes. Following immunofluorescence staining, both p15INK4B and p16INK4A showed strong fluorescent density (red color) in untreated cells, which was markedly attenuated in cells treated with 200 ng/ml GDF-9 (Fig. 4B). These suppressive effects of GDF-9 were significantly attenuated when cells were pretreated with SB-431542 to block ALK5 activation (Fig. 4C). Thus, GDF-9 restrains mRNA and protein expression of p15INK4B and p16INK4A in the INK family of CDK inhibitors but has no effects on those of p21Cip1 and p27Kip1 in the Cip/Kip family of CDK inhibitors.

Effects of ERK42/44 on GDF-9-modulated cell cycle regulators. PD-098059, a specific inhibitor for ERK42/44 of the MAPK pathway, partially attenuated GDF-9-stimulated thymi-

Fig. 2. Effects of GDF-9 on cell cycle progression and distribution as measured by flow cytometry. A: cell cycle distribution after hLG cells were cultured with and without GDF-9 at 100 and 200 ng/ml and harvested at 30 h for analysis. B: changes in cell cycle distribution with and without preincubating hLG cells with 0.5 μM of SB-431542 for 1 h before GDF-9 treatment, as in Fig. 1. Flow cytometry analysis was performed in 5 separate experiments (from 5 different patients). Data are means ± SE of the 5 experiments. The carrier, DMSO, at a vol/vol dose equivalent to 0.5 μM SB-431542, did not affect granulosa cell proliferation induced by GDF-9. FL2-A, fluorescence 2-area.
Fig. 3. Expression of cell cycle positive regulators. A: hLG cells were treated with and without 200 ng/ml GDF-9, and cells were extracted for mRNA analysis at the different time points shown. Cyclin D1, cyclin E, cyclin-dependent kinase (CDK)4, and CDK6 mRNA levels were measured by real-time PCR relative to the internal standard, GAPDH. Significant differences relative to 0, 1, 2, 4, and 8 h are indicated by a, b, c, d, and e, respectively. The P value above a specific time point represents significant between-treatment differences in mRNA expression. B: cyclin D1 and cyclin E protein expression at different time points were measured by Western blot analysis from protein extracted at 0, 8, 12, 18, and 24 h after treatment with 200 ng/ml GDF-9. Significant differences are indicated by a, b, and c relative to 0, 8, and 12 h, respectively. C: cyclin D1, cyclin E, and phospho-Rb protein expression after GDF-9 treatment at 0, 100, and 200 ng/ml. Protein was extracted for analysis at 12 h for cyclin D1, 18 h for cyclin E, and 16 h for phospho-Rb. †Significant dose-related differences relative to 0 ng/ml; ‡significant dose-related differences relative to 100 ng/ml. Data are means ± SE from 3 sets of experiments with cells cultured in triplicate at each dose or treatment combination.
Fig. 4. Expression of cell cycle negative regulators. A: hLGs were treated with and without 200 ng/ml GDF-9, and cells were extracted for mRNA analysis at the different time points shown. p15INK4B, p16INK4A, p21Cip1, and p27Kip1 mRNA expression was measured by real-time PCR relative to the internal standard, GAPDH. Significant differences relative to 0, 1, 2, 4, and 8 h are indicated by a, b, c, d, and e, respectively. The P value above a specific time point represents significant between-treatment differences in mRNA expression.

B: p15INK4B and p16INK4A protein expression on immunofluorescence staining with anti-p15INK4B and anti-p16INK4A antibodies. hLG cells were cultured using coverslips with and without 200 ng/ml GDF-9 for 16 h, fixed, and stained as described in MATERIALS AND METHODS. Cells were double-stained with antibodies for p15INK4B or p16INK4A (shown in red) and Hoechst 33258 (1:2,000) for nuclei (shown in blue).

C: GDF-9-induced changes of p15INK4B and p16INK4A protein expression with and without 0.5 μM SB-431542 (an ALK4/5/7-specific inhibitor) were measured by Western blot analysis. †Significant differences relative to untreated cells; ‡significant differences relative to cells treated with 200 ng/ml GDF-9 alone. Data are means ± SE from 3 separate experiments.
dine incorporation (Fig. 5A). At the dose of 10 μM, PD-098059 alone had no significant effects on cell cycle distribution but attenuated the effects of GDF-9 (200 ng/ml) on cell cycle progression. Compared with GDF-9 (200 ng/ml) alone, PD-098059 (10 μM) attenuated the percentage decrease of cells in the G0/G1 phase (from 64.2 ± 2.2 to 53.2 ± 3.6%, P < 0.05), the percentage increase of cells in S phase (from 12.0 ± 0.8 to 18.1 ± 1.3%, P < 0.05), and the percentage increase of...
cells in G2/M phase (from 19.4 ± 2.1 to 24.2 ± 1.0%, P > 0.05). However, these results showed that PD-098059 could only partially block the effects induced by GDF-9. This was not surprising, given that we demonstrated that GDF-9 could also act through the ALK pathway.

GDF-9 markedly stimulated ERK42/44 phosphorylation (Fig. 5C) in parallel with increased protein expression of cyclin D1 and E and decreased protein expression of p15Ink4B and p16Ink4A. In comparison, total ERK42/44 showed no changes under similar conditions. PD-098059 significantly blocked phosphorylation of ERK42/44 and stimulation of cyclin D1 and E protein expression but, interestingly, not suppression of p15Ink4B and p16Ink4A protein expression induced by GDF-9 (Fig. 5C). GDF-9 induced Smad3 phosphorylation (Fig. 5D), but SIS3, a Smad3 inhibitor, significantly suppressed these changes (Fig. 5D). Furthermore, SIS3 partially reversed the decrease in p15Ink4B and p16Ink4A expression (Fig. 5D) but not the increase in cyclin D1 and E expression induced by GDF-9. Thus, GDF-9 regulates cyclin D1 and E expression via ERK42/44 activation but regulates p15Ink4B and p16Ink4A expression via Smad3 activation.

**DISCUSSION**

GDF-9 is required to sustain the physiological growth and differentiation of the follicle after the primary one-layer stage (7). Our data show that GDF-9 can stimulate [3H]thymidine incorporation in primary cultures of human luteinized granulosa cells in the periovulatory stage at doses of 100–400 ng/ml. Although statistically significant, these increases were relatively small, reflecting the differentiated status of these cells. This study also validates previous observations by our group and others that GDF-9 can increase [3H]thymidine incorporation in immortalized human granulosa cells (21) and promote human ovarian follicle growth and development in organ cultures (12). These data are also consistent with those observed in other species (e.g., rodents and cattle), although the effective GDF-9 doses vary, e.g., 150–600 ng/ml in bovine follicles (26), 30–150 ng/ml in hamster follicles (28), and, in our study, 100–400 ng/ml with maximal responses generally at 200 ng/ml. Variations in the effective GDF-9 doses among these studies can be due to differences in cell developmental stage, species biology, culture conditions, and/or the bioactivity of the various recombinant GDF-9 preparations obtained from different sources. However, the physiologic GDF-9 levels for human oocyte-granulosa cell interactions remain unknown.

To our knowledge, the current study represents the first to evaluate GDF-9 regulation of human granulosa cell cycle progression and distribution and related cell molecules and cell-signaling pathways. Progression from one phase of the cell cycle to the next is controlled by an orderly activation of a CDK in complex with its corresponding cyclin and is regulated by growth factors, nutrients, and other cell-cell and cell-matrix interactions. The most important mammalian cyclin-CDK complexes known to date are cyclin D and CDK4/6 in mid-to late G1 phase, cyclin E and CDK2 in late G1 and early S phase, cyclin A and CDK2 in G2 phase, and cyclin B and CDK1 in M phase (5, 9). In the present study, we confirmed that GDF-9 could promote human luteinized granulosa cell transition from G0/G1 phase to S and G2/M phases (Fig. 2). Indeed, quantitative PCR and Western blot analysis showed that GDF-9 up-regulated both cyclin D1 and E mRNA and protein expression (Fig. 3) but not that of cyclin A. These observed differences likely reflect the cell-cycle sequence of activation of cyclin D-CDK4/6, then cyclin E-CDK2, and so on, relative to the duration of hLG cells in culture conditions and the differentiated stage of luteinized granulosa cells.

Although CDK4/6 expression was not found to be higher in treated cells, GDF-9 significantly increased cyclin D1, Rb protein phosphorylation, and cyclin E expression. Cyclin D-CDK4/6 complex formation is required to phosphorylate Rb protein, which in turn allows E2F to stimulate transcription of the genes encoding cyclin E, CDK2, and E2F itself (18) and facilitates cell progression from G1 to S phase. However, whether GDF-9 directly stimulates cyclin E expression and Rb protein phosphorylation or acts through increased cyclin D1 expression and complex formation with CDK4/6 is unknown. Further studies will be needed to investigate the effects of GDF-9 on these interactions among cyclin D1, cyclin E, Rb protein, and other related factors.

Interestingly, our study found an intrinsic pattern of basal mRNA expression of cyclin D1, cyclin E, CDK4, and CDK6 in our primary human luteinized granulosa cell cultures independent of any GDF-9 treatment. Both cyclin D1 and cyclin E showed a slight and transient increase from 2 to 12 h, which might contribute to the proliferative capacity of human luteinized granulosa cells. However, both CDK4 and CDK6 mRNA expression decreased from 2 h to a trough at 4–8 h and at 4 h, respectively, before rising above baseline values in both treated and untreated cells (Fig. 3). mRNAs are constantly being degraded in the cytoplasm, and their degradation and regulation are major mechanisms that help govern cellular mRNA levels. The half-lives of mRNAs vary with different genes, species, and microenvironments (22). The mRNA half-lives of the cell cycle regulators and factors that influence them in granulosa cells are unknown. It is tempting to speculate that shorter mRNA half-lives of CDK4/6 mRNA in periovulatory granulosa cells might be one reason why CDK4/6 mRNA expression did not rise in parallel with that of cyclin D1 and E following GDF-9 treatment and for the slow rate of cell proliferation in these more differentiated granulosa cells. Further studies are of interest to understand the intrinsic expression patterns of granulosa cell cycle regulators and the regulatory machinery involved.

Cell cycle regulation is a complex event and involves not only positive regulators such as cyclins and CDKs but also negative regulators such as CDK inhibitors. The effects of p15Ink4B and p16Ink4A of the INK family and p21Cip1 and p27Kip1 of the Cip/Kip family (24, 25) on rodent folliculogenesis have been well studied (2). TGFβ1 supported p27Kip1 degradation, thus removing a block to DNA synthesis in granulosa cells from hamster preantral follicles (31). In GDF-9-deficient mouse ovaries, p21Cip1 and p27Kip1 failed to block folliculogenesis at the primary follicle stage (10). Data from our study also showed no difference in the expression of p21Cip1 and p27Kip1 following GDF-9 treatment and no difference from baseline in both treated and untreated human granulosa cells in the periovulatory period. In contrast, mRNA and protein expression of p15Ink4B and p16Ink4A were attenuated by GDF-9. In mice, expression of p15Ink4B has been associated with luteinization and withdrawal of granulosa cells from the

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cell cycle (4), whereas decreased p16INK4A expression as demonstrated by immunohistochemistry has been shown to promote follicle growth (19). Hence, the attenuated effects of GDF-9 on p15INK4B and p16INK4A expression compared with untreated cells in our study would contribute to granulosa cell progression and proliferation as observed by [3H]thymidine incorporation and FACS (Fig. 1 and 2). It is also tempting to speculate that, with release of the oocyte following ovulation, withdrawal of GDF-9 secreted by the oocyte may contribute to luteinization of granulosa cells together with factors that are known to influence granulosa cell luteinization, such as LH and vascular endothelial growth factor (1).

As demonstrated earlier, the effect of GDF-9 was only partially blocked by the specific ALK inhibitor SB-431543, which has been shown to have no effects on ERK, p38, or JNK of the MAPK pathway (13). To obtain additional insights into the signaling pathways involved in GDF-9-regulated granulosa cell cycle progression, ERK42/44 and Smad3 were evaluated in our study. Indeed, ERK42/44 was activated by GDF-9 in this study, consistent with similar observations in cumulus cells from mice (27). Our data showed that the ERK42/44 inhibitor PD-098059 significantly attenuated GDF-9-stimulated thymidine incorporation and cell cycle transition from G0/G1 phase to S and G2/M phases (Fig. 5, A and B). This was further demonstrated by increased phosphorylated ERK42/44 (Fig. 5C), concomitantly with increased protein expression of cyclin D1 and E, and decreased protein expression of p15INK4B and p16INK4A. However, PD-098059 significantly blocked stimulation of cyclin D1 and E protein expression induced by GDF-9 but not suppression of p15INK4B, and p16INK4A protein expression induced by GDF-9 would suggest the involvement of different signaling pathways. Indeed, the effects of the Smad3 inhibitor SIS3 on these GDF-induced changes provide additional evidence in this regard. In our study, GDF-9 induced Smad3 phosphorylation (Fig. 5D) as well as cyclin D1 and E expression but suppressed p15INK4B and p16INK4A expression. SIS3, although it significantly reversed the changes induced by GDF-9 on Smad3 phosphorylation and p15INK4B and p16INK4A expression (Fig. 5D), had no effects on cyclin D1 and E expression. Interactions between Smad3, Myc-Max heterodimers, and p15INK4B promoter in the TGFβ-signaling pathway have also been implicated in the tight control of p15INK4B activation (23). Taken together, our findings suggest that GDF-9 regulates cyclin D1 and E expression via ERK42/44 activation but regulates p15INK4B and p16INK4A expression via Smad3 activation. Further studies using siRNA and immunoprecipitation assays will be of interest to further characterize the effects of GDF-9 on these signaling pathways.

There are limitations to extrapolating our results derived from luteinized granulosa cells obtained from women undergoing IVF treatment to normal ovarian physiology. These cells have been exposed to pharmacological doses of exogenous gonadotropins and, depending on the ovarian stimulation protocol used, to GnRH agonist or GnRH antagonist treatment as well. Furthermore, these cells are in the process of undergoing differentiation to luteal cells and have relatively limited capacity for cell proliferation. Nevertheless, in the absence of granulosa cells from the unstimulated, normal ovaries that are easily accessible for research, findings from our cell culture model do provide interesting hypotheses for further evaluation of cell cycle regulators and related mechanisms involved in human granulosa cells.

With these caveats, our data indicate that GDF-9 regulates granulosa cell proliferation through a combination of Smad-dependent and Smad-independent signaling pathways. Interestingly, GDF-9 protects granulosa cells against apoptosis via activation of the phosphatidylinositol 3-kinase/Akt pathway (20). Confirmation of our findings in unstimulated human granulosa cells and further identification of other Smad-independent GDF-9 signals involved in human granulosa cell cycle regulation will be of interest and will provide further insight into ovarian follicle development that is still not clearly understood in humans.

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