

Growth Differentiation Factor 9 Is Antiapoptotic during Follicular Development from Preantral to Early Antral Stage

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Growth Differentiation Factor 9 Is Antiapoptotic during Follicular Development from Preantral to Early Antral Stage

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Ovarian follicular atresia represents a selection process that ensures the release of only healthy and viable oocytes during ovulation. The transition from preantral to early antral stage is the penultimate stage of development in terms of gonadotropin dependence and follicle destiny (survival/growth vs. atresia). We have examined whether and how oocyte-derived growth differentiation factor 9 (GDF-9) and FSH regulate follicular development and atresia during the preantral to early antral transition, by a novel combination of *in vitro* gene manipulation (*i.e.* intraoocyte injection of GDF-9 antisense oligos) and preantral follicle culture. Injection of GDF-9 antisense suppressed basal and FSH-induced preantral follicle growth *in vitro*, whereas addition of GDF-9 enhanced basal and FSH-induced follicular development. GDF-9 antisense activated caspase-3 and induced apoptosis in cultured preantral follicles, a response attenuated by exogenous GDF-9. GDF-9 increased

phospho-Akt content in granulosa cells of early antral follicles. Although granulosa cell apoptosis induced by ceramide was attenuated by the presence of GDF-9, this protective effect of GDF-9 was prevented by the phosphatidylinositol 3-kinase inhibitor LY294002 and a dominant negative form of Akt. Injection of GDF-9 antisense decreased FSH receptor mRNA levels in cultured follicles, a response preventable by the presence of exogenous GDF-9. The data suggest that GDF-9 is antiapoptotic in preantral follicles and protects granulosa cells from undergoing apoptosis via activation of the phosphatidylinositol 3-kinase/Akt pathway. An adequate level of GDF-9 is required for follicular FSH receptor mRNA expression. GDF-9 promotes follicular survival and growth during the preantral to early antral transition by suppressing granulosa cell apoptosis and follicular atresia.

THE OVARIAN FOLLICLE, consisting of an oocyte surrounded by granulosa and theca cells, represents the basic functional unit of the ovary. Follicular growth can be classified into three phases according to their developmental stage and gonadotropin dependence (1): 1) follicular growth through primordial, primary, and secondary stages (gonadotropin-independent phase), 2) transition from preantral to early antral stage (gonadotropin-responsive phase). The

growth of these follicles is controlled primarily by intraovarian mechanisms and, although unaffected by the absence of gonadotropins (2, 3), is stimulated by the presence of FSH (1, 4, 5), and 3) continual growth beyond the early antral stage (gonadotropin-dependent phase), which includes follicle recruitment, selection, and ovulation (6). In mammals, a single or small number of germ cell(s) will ovulate during an ovarian cycle, whereas most follicles undergo atresia by follicle cell apoptosis (1, 7), a selection process that ensures the release of only the healthiest and most viable oocytes (8, 9).

Follicles selected for further development are thought to receive precise signals from gonadotropins and locally produced growth factors for survival, whereas follicular atresia is a consequence of inadequate growth support (10). Although apoptosis can occur at all stages of follicular development, the early antral follicles (diameter: 200–400 μm in rats; 2–5 mm in human) are most susceptible to atretogenic signals (1, 11). In contrast, minimal atresia or granulosa cell apoptosis is evident in preantral and the smallest an-

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Abbreviations: BMP-15, Bone morphogenetic protein-15; CTL MO, control MO; dnAkt, dominant negative Akt; eCG, equine chorionic gonadotropin; FSHR, FSH receptor; GDF-9, growth differentiation factor-9; HA, hemagglutinin; HRP, horseradish peroxidase; MO, morpholino antisense oligonucleotide; MOI, multiplicity of infection; p-Akt, phospho-Akt; PI3K, phosphatidylinositol 3-kinase; RT, room temperature; Smad, Sma- and Mad-related protein; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

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tral follicles (diameter: $<200 \mu\text{m}$ in rats; $<2 \text{ mm}$ in humans) (7, 12). Accordingly, the preantral to early antral transition is the penultimate stage of development in terms of gonadotropin dependence and follicle destiny (survival/growth vs. atresia). Although oocyte-somatic cell communication is believed to play a critical role in folliculogenesis, including activation of resting follicles, early growth, and terminal differentiation (13–17), whether oocytes play a role in the determining fate of the follicle, by regulating follicle cell apoptosis during the preantral to early antral transition, is not known. It is also not clear whether and by what means oocyte-derived factor(s) may influence the integrity of the granulosa layer via paracrine actions to promote follicular cell survival and growth during this stage of development.

Growth differentiation factor-9 (GDF-9) is an oocyte-derived factor and a member of the TGF- β superfamily, which includes TGF- β , activin, and bone morphogenetic proteins (BMPs) (18, 19). GDF-9 is expressed in the mammalian oocyte throughout follicular development (20–23). Deletion of the GDF-9 gene in mice blocked folliculogenesis at the primary stage, demonstrating the importance of this growth factor in early follicular development (21). Subsequent studies have shown that GDF-9 stimulates granulosa cell proliferation (24), preantral follicle growth (22), and cumulus cell expansion (25), whereas it suppresses FSH-induced cAMP production and steroidogenesis (24). Treatment with GDF-9 resulted in a higher proportion of viable human follicles in organ culture, suggesting a possible role of GDF-9 in follicular survival (26). Although GDF-9 promotes granulosa cell mitosis and preantral follicle growth, whether this latter response is mediated via suppressed apoptosis is not known.

In the present studies, we hypothesized that oocyte-derived GDF-9 is an antiapoptotic factor and promotes preantral follicular growth, in part, by suppressing granulosa cell apoptosis. We have examined whether and by what means GDF-9 and FSH regulate follicular development and atresia during the preantral to early antral transition, by a novel combination of *in vitro* gene manipulation and preantral follicle culture. We have demonstrated that GDF-9 is antiapoptotic in cultured preantral follicles and protects granulosa cells from undergoing apoptosis via activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. In addition, an adequate level of GDF-9 is required for FSH receptor (FSHR) mRNA expression in follicles during this early stage of the development.

RESULTS

GDF-9 and FSH Stimulate Preantral Follicle Growth *in Vitro*

To examine the effect of GDF-9 and FSH on preantral follicle growth, large preantral follicles ($150\text{--}180 \mu\text{m}$ in diameter) isolated from 14-d-old rats were cultured

with or without 100 ng/ml of GDF-9 and different concentrations of FSH (10 or 100 ng/ml) for 4 d. Preantral follicles cultured in the absence of GDF-9 and FSH exhibited minimal growth (follicular volume change at d 4: $30.7 \pm 2.0\%$; Fig. 1). Addition of GDF-9 to the culture media significantly increased the follicular growth and the increase at d 4 was $105.7 \pm 7.1\%$ ($P < 0.01$ vs. CTL; Fig. 1). FSH also stimulated preantral follicle growth, although it appeared more effective at 10 ng/ml ($115.7 \pm 6.9\%$) than 100 ng/ml ($89.1 \pm 4.6\%$) ($P = 0.09$). Moreover, GDF-9 significantly enhanced follicular growth induced by 10 ng/ml ($164.6 \pm 15.7\%$; $P < 0.05$) but not 100 ng/ml ($101.7 \pm 11.2\%$; $P > 0.05$) of the gonadotropin (Fig. 1).

Effect of GDF-9 and FSH on Follicle Cell Apoptosis in Cultured Follicles

Preantral follicles cultured in the absence of GDF-9 and FSH did not reach $200 \mu\text{m}$ in diameter, remained at the preantral stage on d 4 (Fig. 2A), and exhibited minimal apoptosis as determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) (Fig. 2B). Significant apoptosis was noted in follicles that grew to more than $200 \mu\text{m}$ in diameter (corresponding to early antral stage) in the presence of 10 ng/ml of FSH (Fig. 2, C and D). Apoptosis was absent in follicles treated with 100 ng/ml FSH (Fig. 2, E and F) or GDF-9 (Fig. 2, G and H) even in early antral stage. The granulosa cells of 100 ng/ml FSH-treated follicles appeared to be detached from each other when observed under bright-field imaging

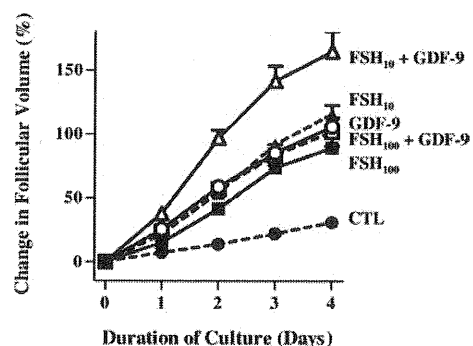


Fig. 1. Effects of GDF-9 and FSH on Preantral Follicle Growth *in Vitro*

Rat preantral follicles ($150\text{--}180 \mu\text{m}$ in diameter) were cultured for 4 d with or without GDF-9 (100 ng/ml) and different concentrations of FSH [10 ng/ml (FSH₁₀) or 100 ng/ml (FSH₁₀₀)]. CTL, Preantral follicles cultured in the absence of GDF-9 and FSH. Follicular diameter was measured daily, and results were expressed as change in follicular volume. The percentage change of follicular volume on d “n” of culture is defined as the volume difference between d “n” and d 0 (the day of isolation) expressed as a percentage of the volume at d 0. Results are represented as means \pm SEM of a total of 40 follicles from eight to 10 independent experiments. Note that GDF-9 stimulated basal and FSH (10 ng/ml)-induced follicular growth.

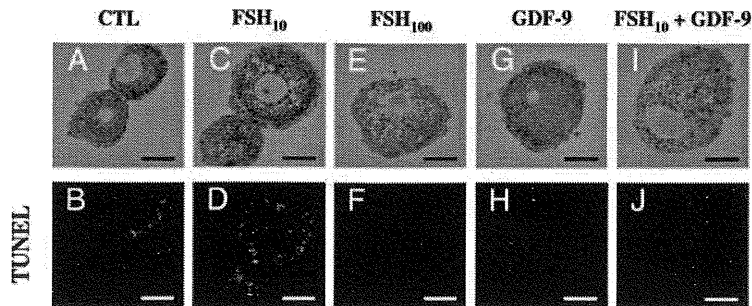


Fig. 2. Effects of GDF-9 and FSH on Follicle Cell Apoptosis *in Vitro*

Preantral follicles (150–180 μm in diameter) were cultured in the absence (A and B) and presence (G and H) of GDF-9 (100 ng/ml) and different concentrations of FSH [10 ng/ml (FSH₁₀; panels C and D) or 100 ng/ml (FSH₁₀₀; panels E and F)]. CTL, Preantral follicles cultured in the absence of GDF-9 and FSH. Cell death in the follicles on d 4 of culture was assessed by TUNEL. An image from 10 representative follicles is shown for each treatment group. Note that, although follicle cell apoptosis was noted in follicles which grew to more than 200 μm in diameter in the presence of 10 ng/ml of FSH (D), this apoptotic response was markedly decreased with GDF-9 cotreatment (J). Scale bar, 100 μm .

(Fig. 2E). Although apoptosis was noted in follicles cultured in the presence of 10 ng/ml of FSH, this response was markedly decreased with GDF-9 cotreatment (Fig. 2, I and J).

Intraocyte Injection of GDF-9 Morpholino Induces Follicle Cell Apoptosis and Caspase-3 Activation in Cultured Follicles

To assess the role of GDF-9 on follicular development and atresia during the preantral to early antral transition, GDF-9 Morpholino antisense oligos (GDF-9 MO, 10 μM) or its control Morpholino (CTL MO, 10 μM) was injected into the oocyte of cultured preantral follicles. Figure 3, panels Aa and Ab, show GDF-9 positive staining and negative control (preincubated with blocking peptide), respectively. Injection of GDF-9 MO, but not CTL MO, markedly decreased oocyte GDF-9 content (Fig. 3, panels Ba, Bc, and C; $P < 0.01$) and induced follicle cell apoptosis at d 4 (Fig. 3, panel B, a–d) and at d 6 (Fig. 3, panel B, e–h) *in vitro*.

To further examine the apoptotic response by GDF-9 down-regulation, caspase-3 activity in CTL MO- and the GDF-9 MO-injected follicles was assayed, using a specific bioluminescent substrate of caspase-3. The GDF-9 MO-injected follicles exhibited significant decreased bioluminescence on d 4 ($78.0 \pm 4.8\%$; $P < 0.05$), when compared with the CTL MO-injected follicles, indicating that GDF-9 down-regulation in cultured follicles increased caspase-3 activity (Fig. 3D). Although this increased caspase-3 activity appears small, this is significant considering that granulosa cell apoptosis is heterogeneous in the follicle and a continuous process, and represents an early stage of follicular atresia.

Intraocyte Injection of GDF-9 Morpholino Suppresses Preantral Follicle Growth *in Vitro*

Intraocyte injection of GDF-9 MO suppressed basal and FSH-induced preantral follicle growth during a 4-d culture period (Fig. 4A). Whereas basal follicular vol-

ume in the CTL MO group was significantly increased by d 4 ($36.8 \pm 3.4\%$), a decrease was noted in the GDF-9 MO group ($-19.5 \pm 1.2\%$), resulting in a significant difference between the two experimental groups ($P < 0.01$; Fig. 4, A and B). In the presence of FSH (10 ng/ml), GDF-9 down-regulation also suppressed follicular growth [$77.1 \pm 8.8\%$ (CTL MO + FSH₁₀) vs. $16.6 \pm 4.7\%$ (GDF-9 MO + FSH₁₀), $P < 0.01$; Fig. 4A]. The effect of GDF-9 MO appeared to be specific to GDF-9, because the addition of GDF-9 to the culture media prevented the follicular growth arrest caused by GDF-9 MO injection. No significant difference in the change in follicular volume was observed between the CTL MO ($36.8 \pm 3.4\%$) and the GDF-9 MO + GDF-9 ($28.7 \pm 10.1\%$) group ($P > 0.05$; Fig. 4B). No significant difference in the follicular volume change was observed between the CTL MO + FSH₁₀ ($77.1 \pm 8.8\%$) and the GDF-9 MO + GDF-9 + FSH₁₀ ($72.7 \pm 11.5\%$) group ($P > 0.05$).

To assess the role of GDF-9 on oocyte growth during the preantral to early antral transition, oocyte diameter was also measured daily. No significant difference was observed in the increase in oocyte volume between the CTL MO- and the GDF-9 MO-injected follicles [at d 4: $27.6 \pm 5.0\%$ (CTL MO) vs. $32.7 \pm 3.5\%$ (GDF-9 MO); $P > 0.05$]. Addition of FSH (10 ng/ml) to the culture medium failed to alter oocyte growth in the CTL MO ($30.8 \pm 4.7\%$; $P > 0.05$) or GDF-9 MO ($36.1 \pm 6.6\%$; $P > 0.05$) groups.

GDF-9 Protects Granulosa Cells from Ceramide-Induced Apoptosis

To determine whether GDF-9 is antiapoptotic on granulosa cells during early antral development, granulosa cells from follicles primarily at the early antral [24 h post-equine choriogonadotropin (eCG)] and large antral/preovulatory (48 h post-eCG) stages of development were cultured with different concentrations of GDF-9 (0, 10, and 100 ng/ml) and ceramide [an inducer of cell cycle arrest and apoptosis (27); 30 μM ,

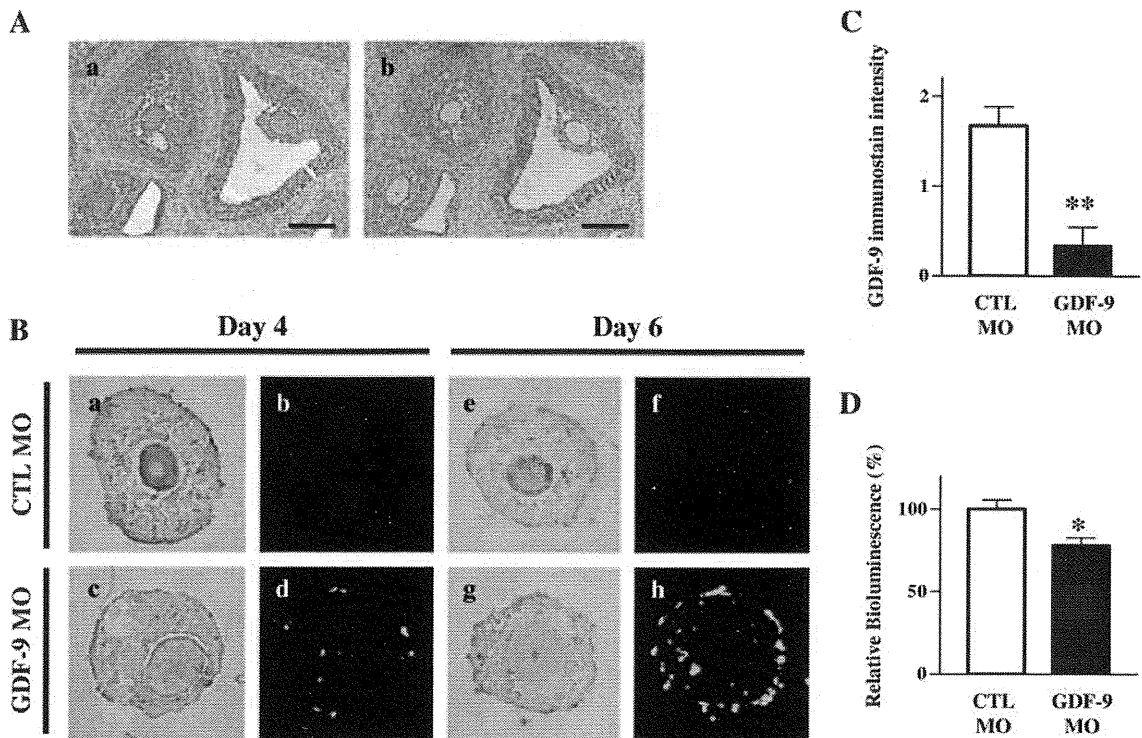


Fig. 3. Effect of Intraoocyte Injection of CTL/GDF-9 MO on Oocyte GDF-9 Expression, Follicular Caspase-3 Activity, and Apoptosis *in Vitro*

GDF-9 MO (10 μM) or CTL MO (10 μM) was injected into the oocyte of cultured preantral follicles (150–180 μm in diameter) at d 0. Successful injection was confirmed by visualization of fluorescence in the oocyte. A, GDF-9-positive staining (panel a) and negative control (panel b) in rat ovary. Scale bar, 100 μm . B, GDF-9 expression (immunohistochemistry) and apoptosis (TUNEL) were monitored on d 4 (panels a–d) and d 6 (panels e–h). An image from 10 representative follicles is shown for each treatment group. Note that injection of GDF-9 MO, but not CTL MO, decreased oocyte GDF-9 content and induced follicle cell apoptosis. C, Quantitative result of GDF-9 immunostaining intensity in cultured follicular oocytes ($n = 6$ in each group) injected with CTL MO or GDF-9 MO from three independent experiments. Note that oocytes injected with GDF-9 MO exhibited significantly lower GDF-9 immunostain intensity compared with those with CTL MO (**, $P < 0.01$). D, Caspase-3 activity in CTL MO- and GDF-9 MO-injected follicles on d 4 was assayed. Three follicles from the same treatment group were pooled and served as one sample. Results are represented as means \pm SEM of five independent experiments. Note that GDF-9 MO-injected follicles exhibited decreased bioluminescence, indicating that GDF-9 MO down-regulation in cultured follicles increased caspase-3 activity. *, $P < 0.05$ vs. CTL MO-injected follicles.

added 4 h after GDF-9] and the effect of GDF-9 on ceramide-induced apoptosis was determined by nuclear morphology (Hoechst staining). Treatment with ceramide significantly increased apoptosis in granulosa cells ($P < 0.01$) from both early antral (Fig. 5A) and large antral/preovulatory follicles (Fig. 5B). The ceramide-induced apoptosis was significantly attenuated by the presence of 100 ng/ml of GDF-9 in cells from early ($P < 0.05$; Fig. 5A) but not late ($P > 0.05$; Fig. 5B) stage of follicular development, suggesting that GDF-9 is an antiapoptotic factor for granulosa cells and that its action is dependent on follicular stage.

Activation of PI3K/Akt Pathway Is Involved in the Antiapoptotic Action of GDF-9

It is well established that activation of the PI3K/Akt pathway protects granulosa cells from proapoptotic signals (28). To determine whether GDF-9 activates PI3K pathways, granulosa cells from early antral folli-

cles were incubated with different concentrations of GDF-9 (0, 10, and 100 ng/ml) for various durations (0.25, 1, and 4 h), and phospho-Akt (p-Akt) and total Akt content were examined by Western blotting. A very low level of p-Akt was detected in the absence of GDF-9 (Fig. 6A). Preliminary data indicated that FSH (100 ng/ml) significantly increased p-Akt content within 15 min in our culture system (data not shown). GDF-9 (100 ng/ml) significantly increased p-Akt content in granulosa cells after 4 h treatment ($P < 0.01$; Fig. 6B).

To determine whether the antiapoptotic effect of GDF-9 is mediated via activation of the PI3K/Akt pathway, granulosa cells were pretreated with the PI3K inhibitor LY294002 before the addition of GDF-9 (0 or 100 ng/ml) and ceramide (30 μM), and apoptosis was assessed. Pretreatment with LY294002 blocked the protective effect of GDF-9 against ceramide-induced apoptosis ($P < 0.05$; Fig. 7A). In addition, the above studies were extended to examine whether down-regulation of Akt function by dominant negative Akt

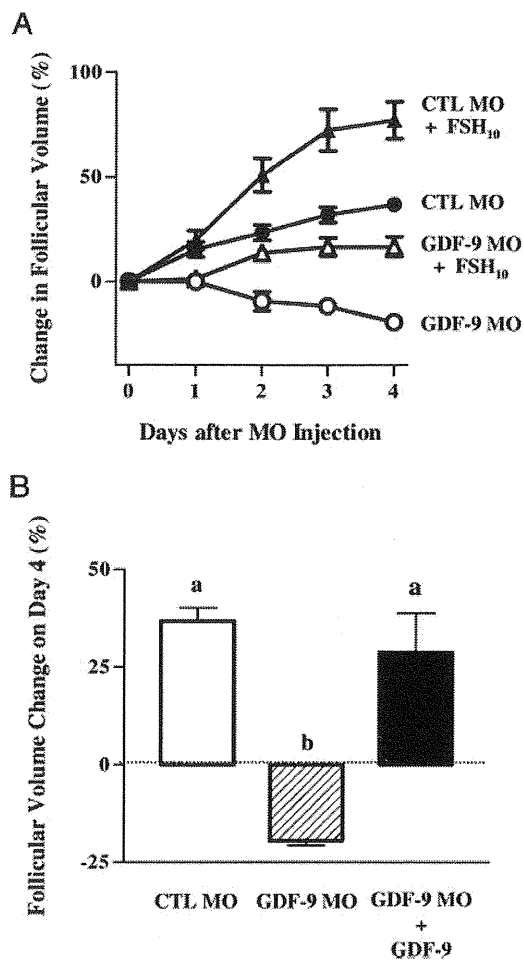


Fig. 4. Intraoocyte Injection of CTL/GDF-9 MO Suppresses Basal and FSH-Induced Preantral Follicle Growth *in Vitro*. GDF-9 MO (10 μ M) or CTL MO (10 μ M) was injected into the oocyte of isolated preantral follicles (150–180 μ m in diameter) at d 0. At d 1, the follicles were treated with or without FSH (10 ng/ml; panel A) and GDF-9 (100 ng/ml; panel B) and cultured for another 3 d. Follicular diameter was measured daily, and results were expressed as change in follicular volume. The percentage change of follicular volume on d “n” of culture is defined as the volume difference between d “n” and d 0 (the day of injection) expressed as a percentage of the volume at d 0. Results represent the means \pm SEM of a total of 16 follicles from four or five independent experiments. A, GDF-9 MO injection suppressed basal and FSH-induced follicle growth during a 4-d culture period. B, Addition of GDF-9 to the culture media prevented the growth arrest on d 4 caused by GDF-9 antisense injection. Bars with different superscripts are significantly different at $P < 0.01$.

(dnAkt) expression would attenuate the antiapoptotic action of GDF-9. Granulosa cells from early antral follicles were infected with adenoviral hemagglutinin (HA)-tagged dnAkt or LacZ [as control; multiplicity of infection (MO) = 40; 12 h]. HA content, as determined by Western blotting, was elevated in cells infected with dnAkt, but not with LacZ (Fig. 7C), confirming the infection was successful. Ceramide induced apoptosis in

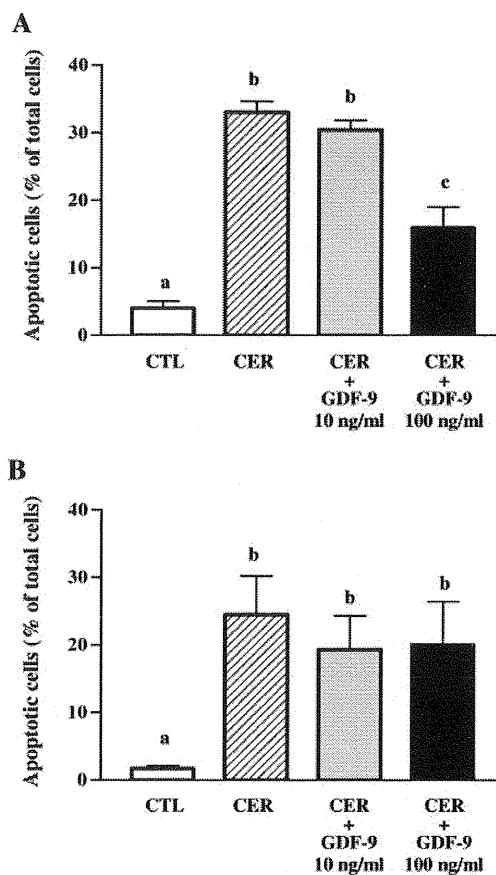


Fig. 5. GDF-9 Attenuates Ceramide-Induced Granulosa Cell Apoptosis

Granulosa cells from follicles mainly at the early antral (24 h post-eCG; panel A) and large antral/preovulatory (48 h post-eCG; panel B) stages of development were cultured with different concentrations of GDF-9 (0, 10, and 100 ng/ml) and ceramide (CER: 30 μ M; added 4 h after GDF-9). CTL, Granulosa cells cultured in the absence of ceramide and GDF-9. The effect of GDF-9 on ceramide-induced apoptosis at 48 h was determined by nuclear morphology. Results represent means \pm SEM of three or four independent experiments. Note that ceramide-induced apoptosis was attenuated by the presence of GDF-9 (100 ng/ml) in cells from early (A) but not late (B) stage of follicular development. Within each panel, bars with different superscripts are significantly different at $P < 0.05$.

granulosa cells infected with LacZ ($P < 0.01$), and this response was attenuated by the presence of GDF-9 ($P < 0.01$; Fig. 7B). In granulosa cells infected with dnAkt, the protective effect of GDF-9 against ceramide-induced apoptosis was blocked (Fig. 7B). Taken together, these results indicate that activation of PI3K/Akt pathway is involved in the antiapoptotic action of GDF-9 in granulosa cells of the early antral follicles.

GDF-9 Is Required for the Maintenance of Follicular FSHR mRNA Expression

To determine whether and what means GDF-9 regulates FSH action during the preantral to early antral

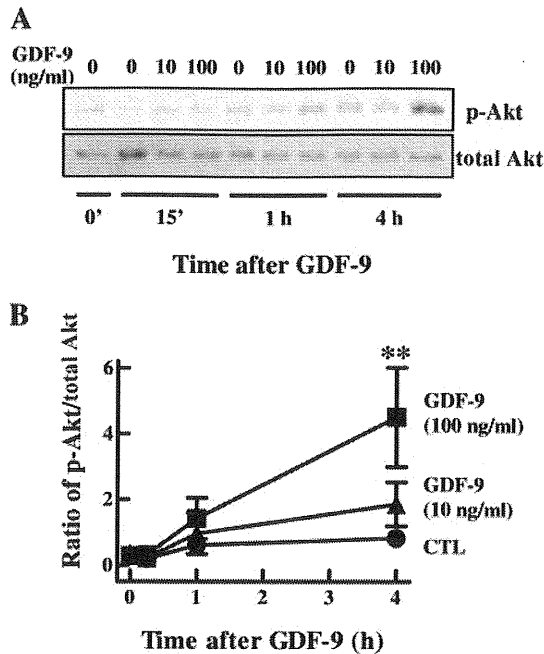


Fig. 6. GDF-9 Increases p-Akt Content in Granulosa Cells
Granulosa cells from early antral follicles were incubated with different concentrations of GDF-9 (0, 10, and 100 ng/ml) for various durations (0.25, 1, and 4 h). CTL, Granulosa cells cultured in the absence of GDF-9. p-Akt and total Akt content were examined by Western blotting. Representative images (A) and densitometric data of p-Akt contents (B), expressed as ratio of p-Akt to total Akt, are shown. Results are represented as means \pm SEM of three independent experiments. Note that GDF-9 (100 ng/ml) increased p-Akt content in granulosa cells after 4 h treatment. **, $P < 0.01$ vs. control (no GDF-9).

transition, real-time quantitative PCR analysis of FSHR was performed on total RNAs from the MO-injected follicles. Whereas treatment with GDF-9 did not alter FSHR mRNA levels in the follicles injected with CTL-MO, GDF-9 MO markedly decreased this response (85%, $P < 0.01$; Fig. 8A). The down-regulation of FSHR transcript by GDF-9 MO was prevented by exogenous GDF-9 ($P < 0.01$; GDF-9 MO + GDF-9 vs. GDF-9 MO + CTL). FSHR mRNA abundance in GDF-9 MO + GDF-9 was not different from those in CTL MO + CTL or CTL MO + GDF-9 ($P > 0.05$; Fig. 8B). FSH (10 ng/ml) decreased FSHR mRNA levels in the CTL MO-injected follicles after 4 d culture irrespective of the presence of GDF-9, but had no effect on FSHR mRNA abundance after its down-regulation by GDF-9 MO ($P < 0.05$; Fig. 8A).

DISCUSSION

In the present study, we have demonstrated that the oocyte-derived factor GDF-9 controls the destiny of the ovarian follicle (growth vs. atresia) during follicular

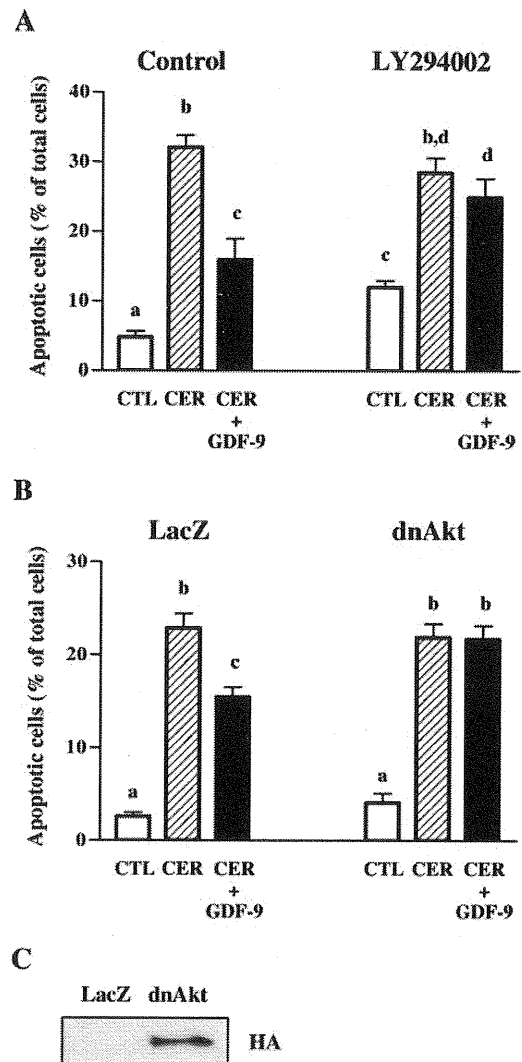


Fig. 7. Effects of the PI3K Inhibitors LY294002 and dnAkt Adenoviral Expression on GDF-9-Mediated Protection from Ceramide-Induced Apoptosis in Granulosa Cells

A, Granulosa cells from early antral follicles were pre-treated with the PI3K inhibitor LY294002 ($t = -0.5$ h) before the addition of GDF-9 (0 or 100 ng/ml, $t = 0$ h) and ceramide (CER: $30 \mu\text{M}$, $t = 4$ h). B, Granulosa cells were infected with adenoviral HA-tagged dnAkt or LacZ (as control; MOI = 40; 12 h), and then treated with GDF-9 (0 or 100 ng/ml; $t = 0$ h) and ceramide ($30 \mu\text{M}$; $t = 4$ h). C, To assess infection efficiency, HA in adenovirus-infected cells was assayed at 24 h by Western blotting, using an anti-HA antibody. CTL, Granulosa cells cultured in the absence of ceramide and GDF-9. Apoptosis was assessed by nuclear morphology at 48 h. Results are represented as means \pm SEM of three independent experiments. Note that the PI3K inhibitor (A) and dnAkt (B) prevented the protective effect of GDF-9 against ceramide-induced apoptosis. Within each panel, bars with different superscripts are significantly different at $P < 0.05$.

development from the preantral to early antral stage by regulating granulosa cell apoptosis. We have shown that intraoocyte injection of GDF-9 MO antisense sup-

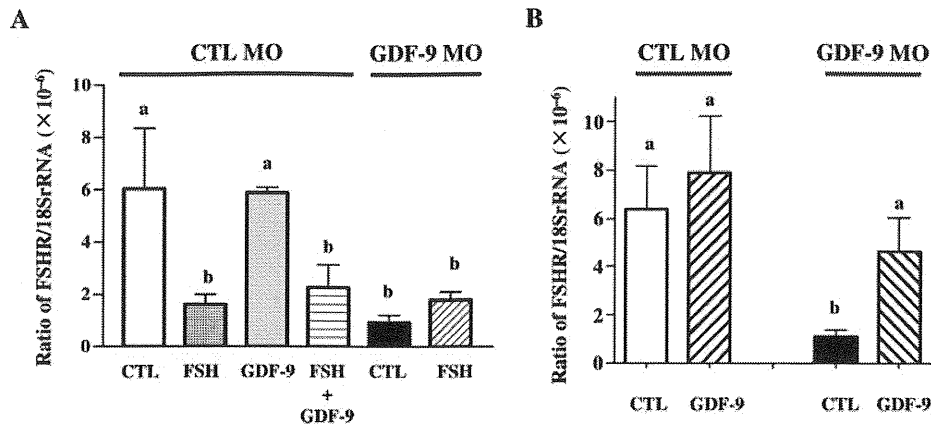


Fig. 8. Effect of Intraoocyte Injection of CTL/GDF-9 MO on FSHR Expression *in Vitro*

GDF-9 MO (10 μ M) or CTL MO (10 μ M) was injected into the oocyte of cultured preantral follicles (150–180 μ m in diameter). The follicles were cultured for 4 d with or without FSH (10 ng/ml) and GDF-9 (100 ng/ml), and real-time quantitative PCR analysis of FSHR was performed. CTL, Preantral follicles cultured in the absence of GDF-9 and FSH. Total RNAs from three follicles (pooled from the same treatment group) were extracted, and the cDNAs were amplified as one sample. Results are represented as means \pm SEM of three or four (A) or four to seven (B) independent experiments. Note that follicular FSHR mRNA abundance in GDF-9 MO was markedly lower than those in CTL MO and GDF-9 MO + GDF-9. Bars with different superscripts are significantly different at $P < 0.05$.

pressed basal and FSH-induced preantral follicle growth *in vitro*, whereas GDF-9 enhanced basal and FSH (10 ng/ml)-induced follicular development. GDF-9 MO activated caspase-3 and induced apoptosis in cultured preantral follicles, a response attenuated by exogenous GDF-9. GDF-9 protected granulosa cells of early antral follicles from ceramide-induced apoptosis via activation of PI3K/Akt signaling pathway. These results suggest that GDF-9 promotes follicular survival and growth during the preantral to early antral transition by suppressing granulosa cell apoptosis and follicular atresia.

The process of follicular atresia represents a selection system that ensures the release of only healthy and viable oocytes during ovulation (8, 9). Although intraovarian regulation, together with the action of FSH, is important for follicular selection, the ovarian factor(s) responsible and precisely when in the developmental process is follicle destiny (growth/ovulation or atresia) determined are not clear. The destiny of the follicle is dependent on a delicate balance in the expression and actions of factors promoting follicle cell proliferation, differentiation, and apoptosis (8, 29). The transition of the developing follicle from the preantral to early antral stage is primarily controlled by intraovarian regulators (e.g. GDF-9), but is also responsive to FSH (1). However, continual growth beyond the early antral stage is gonadotropin dependent. The latter contention is supported by the observation that antral follicles cultured in the absence of FSH exhibited abundant apoptosis and follicular growth arrest, a response that could be attenuated by FSH supplementation (30). In addition, as shown in the present study, preantral follicles continued to grow and exhibited minimal apoptosis in the absence of FSH but not of GDF-9 (GDF-9 MO groups), suggesting that this

oocyte-derived factor plays a more significant role in promoting cell survival and follicular growth during this early stage of development. In addition, GDF-9 protected granulosa cells from preantral, but not antral, follicles against ceramide-induced apoptosis, suggesting that GDF-9 is necessary for granulosa cell survival during the preantral to early antral transition but is insufficient for antral follicle development, which is dependent on FSH support. Although BMP-15 (GDF-9B), another oocyte-specific member of the TGF- β superfamily, is also an important regulator of ovarian function (19, 31, 32), whether its action in granulosa cells is antiapoptotic and important in protecting the preantral follicles from undergoing atresia remains unknown. Our observation that exogenous GDF-9 is capable of protecting the follicles from GDF-9 MO-induced growth arrest suggests that the antisense was specific to GDF-9.

GDF-9 signals through a complex of type I (activin-like receptor kinase-5) and type II (BMP receptor type II) membrane serine/threonine kinase receptors (33, 34), resulting in the phosphorylation and activation of Sma- and Mad-related protein (Smad)2 and Smad3 in granulosa cells (33, 35, 36). Smad3 knockout mice exhibited increased atretic follicles and impaired follicle growth (37). Our present finding that intraoocyte injection of GDF-9 MO induced follicle cell apoptosis and suppressed follicular growth is consistent with the above findings but is in contrast with the report that GDF-9-deficient ovaries did not show increased apoptosis (38). The reason for the latter response is unclear although this may be a consequence of growth arrest at the primary stage, which prevented further development to the early antral stage, when apoptosis is most common.

Gonadotropins and a number of growth factors are known to activate the PI3K/Akt pathway and prevent apoptosis in granulosa cells and cultured follicles (28, 30, 39). PI3K converts phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-triphosphate, leading to activation of downstream kinases including Akt, which in turn phosphorylates Bad, forkhead in rhabdomyosarcoma, Fas-associated death domain-like IL-1 β -converting enzyme-like inhibitory protein, and X-linked inhibitor of apoptosis protein (40). Activation of the PI3K/Akt pathway promotes cell survival and proliferation (10, 40–42). In the ovary, gonadotropins increase granulosa cell p-Akt and X-linked inhibitor of apoptosis protein content and suppress apoptosis *in vivo* and *in vitro*, whereas withdrawal of gonadotropin support decreases Akt phosphorylation and induces cell apoptosis (30, 39). In the present study, GDF-9 activated the PI3K/Akt pathway in granulosa cells of early antral follicles, as evidenced by increased p-Akt content. In addition, the PI3K inhibitor LY294002 and a dominant negative form of Akt prevented the protective effect of GDF-9 against apoptosis induced by ceramide, an intracellular intermediate that directs cell cycle arrest and apoptosis in granulosa cells and ovarian follicles *in vitro* (28, 43, 44) and directly antagonizes the PI3K/Akt cell survival pathway (27, 45, 46). These findings suggest that activation of Akt is necessary for maintenance of granulosa cell survival by GDF-9 and are consistent with the report that Akt overexpression can block ceramide generation and protect cells from ceramide-induced apoptosis in neuron cells (47). The effects of GDF-9 on the PI3K/Akt pathway may be indirect, because p-Akt content was increased only after 4 h of incubation, whereas FSH can significantly increase p-Akt content within 15 min in our culture system. A similar phenomenon has been reported by Eppig and colleagues (48), whereby GDF-9 activates MAPK in mouse cumulus cells after 4 h incubation. Although the precise mechanism for the activation of this pathway by GDF-9 is not known, it is possible that the growth factor-induced p-Akt up-regulation may involve Smad3-mediated down-regulation of phosphatase and tensin homolog (a tumor suppressor that negatively regulates phosphoinositide phosphorylation). In this context, it has been shown that Smad3 interacts with phosphatase and tensin homolog and that TGF- β -mediated cellular motility and invasion are up-regulated by the loss of this phosphatase (49). Whether this indeed is the case in the cumulus/granulosa cells awaits further investigation.

It is worth noting that although the cell survival action of GDF-9 is at variance with the reported proapoptotic effects of Smad2/3 activation induced by other members of TGF- β family in hepatocytes and trophoblast cells (50, 51), the possibility exists that, once activated, Akt may bind and sequester Smad3 in the cytoplasm and thus render it incapable of gene activation (52). Thus, the responsiveness of a particular

cell type to pro- or antiapoptotic signal is dependent on the ligand as well as the pathway involved.

Although transgenic mouse models have proven to be valuable in assessing the role of genes of interest in mammalian oogenesis and folliculogenesis (53), examination of the specific functions of these genes at late stages of development can sometime be problematic. In this context, we hereby describe the novel establishment of a follicle culture system that is coupled to an *in vitro* gene manipulation procedure for the assessment of oocyte-granulosa interaction in the growing follicle. This model would provide a useful approach in assessing the role of specific genes in follicular development and atresia in a tissue-specific and time (stage)-controlled manner.

Previous studies have shown that GDF-9-knockout mice exhibited increased oocyte growth relative to controls (21). Although intraoocyte injection of GDF-9 MO suppressed basal and FSH-induced preantral follicle growth in the present study, it had no effect on oocyte size compared with control, irrespective of the presence of FSH. Although the reason for this apparent difference is not clear, it is of interest to note that the influence of GDF-9 on oocyte growth appeared to be dependent on follicular stage [being more significant in smaller follicles than in larger ones (54)]. It is possible that the lack of effect of GDF-9 MO on oocyte growth could be due to the more advanced stage of development at which our studies were conducted. Nonetheless, our results suggest that GDF-9 is involved in the regulation of follicular development from the preantral to early antral stage, but not in that of oocyte growth. Whether GDF-9 MO injection affects oocyte maturation remains to be elucidated.

Although the transition of preantral to early antral follicle can be stimulated by FSH (1, 5), our knowledge on the precise role of FSH at this period remains incomplete. It has been suggested that gonadotropins are not cell survival factors at early stages of folliculogenesis, because preantral follicles in serum-free cultures undergo apoptosis despite exposure to FSH (55). In the present study, whereas preantral follicles (<200 μ m in diameter) exhibited only minimal apoptosis, significant apoptotic cell death was noted in follicles that grew to the early antral stage (>200 μ m in diameter) in the presence of FSH (10 ng/ml). This result is consistent with the fact that the vast preponderance of atretic follicles *in vivo* is present at this stage of development and that atretic preantral follicles are rarely observed (1, 12). Although 10 ng/ml of FSH was insufficient to suppress apoptosis in cultured follicles at the early antral stage, a higher concentration (100 ng/ml) was antiapoptotic. In contrast, the lower concentration of FSH appeared more effective in promoting preantral follicle development than the higher concentration, and follicles cultured in the presence of high concentrations of the gonadotropin exhibited features of cumulus expansion. These results are consistent with the reports that a high FSH concentration promoted *in vitro* premature granulosa cell differenti-

ation in oocyte-granulosa cell complexes from mouse preantral follicles (56) and that a low FSH concentration significantly improved oocyte fertilization rate and blastocyst development (57). Low, but not high, FSH concentrations promoted mouse oocyte growth *in vitro* (58).

Although BMP-15 is known to inhibit FSH activities by suppressing FSHR mRNA expression in granulosa cells irrespective of the presence of FSH (59), precisely how GDF-9 interacts with FSH remains unclear. It has been demonstrated that GDF-9 inhibits FSH-dependent LH receptor expression, cAMP production, and estradiol and progesterone synthesis (24) but markedly enhanced FSH-induced preantral follicle growth *in vitro* (22) and granulosa cell inhibin A and B production (35). To determine whether the enhancement of FSH-induced follicular growth by GDF-9 observed by Hayashi *et al.* (22) and in the present study was mediated via increased FSHR expression, we determined FSHR mRNA abundance in preantral follicles after GDF-9 down-regulation. Injection of GDF-9 MO decreased FSHR mRNA levels in the cultured follicles, suggesting that GDF-9 is required for FSHR mRNA expression in preantral follicles. Although exogenous GDF-9 failed to alter FSHR mRNA levels in CTL MO-injected follicles, this might be due to the presence of sufficient endogenous GDF-9 to maintain maximal FSHR mRNA expression during the period. This notion is consistent with the significant increase in FSHR mRNA levels in GDF-9 MO-injected follicles treated with GDF-9 (Fig. 8B). Moreover, GDF-9 enhanced FSH-induced preantral follicle growth and cotreatment with GDF-9 suppressed the apoptosis in follicles cultured in the presence of FSH. This result suggests that GDF-9 is involved in the regulation of FSHR mRNA expression, although the detailed mechanism is not clear. However, the fact that exogenous GDF-9 did not affect the down-regulation of FSHR mRNA by FSH suggests that the observed effect of the growth factor on preantral follicle growth and atresia may also involve an interaction downstream of the receptor.

It is well established that FSHR mRNA expression is up-regulated by FSH (60). However, in the present study, addition of FSH (10 ng/ml) decreased FSHR mRNA levels in the CTL MO-injected/cultured follicles. Although the reason(s) for this difference remains to be determined, gonadotropin receptors are known to be desensitized or down-regulated after prolonged stimulation (61) or after challenge with a single high dose of FSH (62). Luteinization is also associated with decreased expression of FSHR mRNA (63–65). Our finding that 10 ng/ml of FSH was insufficient to prevent apoptosis in cultured follicles could be due to down-regulation of FSHR mRNA and decreased gonadotropin sensitivity.

In summary, we have established a novel combination of *in vitro* gene manipulation in the oocyte and preantral follicle culture. Using this *in vitro* model in combination with primary granulosa cell cultures, we have shown that GDF-9 plays an important role in

promoting preantral follicular growth by suppressing atresia. GDF-9 is antiapoptotic and suppresses granulosa cell apoptosis through activation of the PI3K/Akt pathway. GDF-9 is required for FSHR expression during follicular development from preantral to early antral stage.

MATERIALS AND METHODS

Materials

All culture media and supplements were purchased from Life Technologies Inc. (Burlington, Ontario, Canada). Bovine insulin, human transferrin, ascorbic acid, sodium selenite anhydrous, L-glutamine, agarose (low gelling temperature), eCG, trypsin, trypsin inhibitor, PI3K inhibitor (LY294002), and Hoechst 33258 compound were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human FSH was obtained from the National Hormone & Peptide Program, Harbor-UCLA Medical Center (Torrance, CA). MOs for CTL and GDF-9 were purchased from Gene-Tools, LLC (Philomath, Oregon). ICSI (Intracytoplasmic Sperm Injection) micropipets (no. MIC-35–30) were from Humagen (Charlottesville, VA). Goat antihuman GDF-9 antibody (C-18) and its blocking peptide, as well as goat ImmunoCruz staining system were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TUNEL enzyme and TUNEL label mix were from Roche Diagnostics (Indianapolis, IN). CleavaLite caspase-3 activity assay kit was from Chemicon International, Inc. (Temecula, CA). C8-ceramide (*N*-octanoylsphingosine, D-erythro) was from Biomol International LP (Plymouth Meeting, PA). Deoxyribonuclease I and RevertAid Enzyme (H Minus M-MuLV RT) were from Fermentas International, Inc. (Burlington, Ontario, Canada). Rabbit antimouse p-Akt (Ser473) and total Akt antibodies were from Cell Signaling Technology (Oakville, Ontario, Canada). Horseradish peroxidase (HRP)-conjugated antirabbit antibodies were from Bio-Rad Laboratories, Inc. (Mississauga, Ontario, Canada). Adenoviral construct with LacZ cDNA was prepared by the Adenovirus Core Facility, University of Ottawa Neuroscience Research Institute (Ottawa, Ontario, Canada). Enhanced chemiluminescence Western blotting detection reagents were from Amersham Biosciences (Baie d'Urfe, Quebec, Canada). RNeasy Micro kit and QuantiTect SYBR Green PCR kit were purchased from QIAGEN, Inc. (Mississauga, Ontario, Canada). PCR primers for FSHR and 18S rRNA were from Invitrogen Canada, Inc. (Burlington, Ontario, Canada). Random Decamer Primers were from Ambion, Inc. (Austin, TX). Recombinant rat GDF-9, adenoviral dnAkt construct containing HA-tagged, triple-A mutation (K179A, T308A, and S473A; kinase dead) were generously provided by Dr. Aaron J. W. Hsueh (Stanford University School of Medicine, Stanford, CA) and Dr. Kenneth Walsh (Cardiovascular Research, St. Elizabeth's Medical Centre, Boston, MA), respectively.

Preantral Follicle Isolation and Culture

All the animal work was carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Ottawa Health Research Institute Animal Care Committee. Female Sprague Dawley rats were obtained from Charles River Canada (Montreal, Quebec, Canada) and maintained under standard conditions. Large preantral follicles (diameter, 150–180 μ m) from 14-d-old rats known to exhibit negligible apoptosis (55) were isolated in Leibowitz L-15 medium with BSA (0.1%, wt/vol) at d 0, using 28.5-gauge needles (Becton Dickinson and Co., Franklin Lakes, NJ). Only round follicles with intact basement membrane and theca

layer were selected for the present studies because follicles lacking basement membrane or theca layer were broken or ruptured at a high rate, after the treatment with FSH/GDF-9 or microinjection. Follicles were cultured individually for 4 d in a 96-well plate (Sarstedt, Inc., Newton, NC; no. 83.1837.50) in 100 μ l of α -MEM supplemented with HEPES (10 mM), BSA (0.1%, wt/vol), bovine insulin (5 μ g/ml), transferrin (2 μ g/ml), ascorbic acid (25 μ g/ml), sodium selenite anhydrous (2 ng/ml), L-glutamine (3 mM), sodium pyruvate (100 μ g/ml), streptomycin (100 μ g/ml), and penicillin (100 U/ml) with or without 100 ng/ml of GDF-9 and different concentrations of FSH (10 or 100 ng/ml). Preliminary data indicated that 100 ng/ml of GDF-9 or 5 ng/ml of FSH are the minimal effective concentrations for inducing a significant increase in preantral follicle growth in our culture system. Follicular diameter was measured daily as the average distance between the outer edges of the basement membrane in two perpendicular planes, and results were expressed as change in follicular volume. The percentage change of follicular volume on d "n" of culture is defined as the volume difference between d "n" and d 0 (the day of isolation) expressed as a percentage of the volume at d 0. The culture medium was changed every other day, and the spent media were kept at -20 C for further analysis. At the end of the 4-d culture period, follicles were fixed in buffered formalin phosphate solution [10%, wt/vol; 24 h, room temperature (RT)], embedded in agarose (2%, wt/vol), stained with Nuclear Fast Red solution (4 h, RT; to facilitate visualization of follicles during sectioning; Sigma-Aldrich, St. Louis, MO), processed, and embedded in paraffin.

Intraoocyte Injection of Morpholinos in Cultured Preantral Follicles

Expression of MOs is effective in suppressing translation of target genes in zebrafish (66) and *Xenopus* (67) embryos. To assess the role of GDF-9 on follicular development and atresia during the preantral to early antral transition, GDF-9 content in the cultured follicles was manipulated by intraoocyte microinjection of GDF-9 MO. The GDF-9 MO sequence was designed based on its rat cDNA sequence (5'-ACAG-GAATCTGCTGGGAAATGCCAT-3'; not homologous with BMP-15/GDF-9B cDNA), whereas the control MO (CTL MO) sequence (5'-CCTCTTACCTCAGTTACAATTTATA-3') was designed by Gene-Tools. Large preantral follicles were isolated from 14-d-old rats and cultured individually in a 96-well plate without GDF-9 or FSH. After 16–24 h, only the follicles (diameter, 150–180 μ m) with intact basement membrane and thecal layer were selected for the microinjection. CTL MO or GDF-9 MO (10 μ M) was injected into the oocyte of the preantral follicles at d 0. The volume of MO injected (3 μ l) was less than 5% (vol/vol) of the oocyte volume. Successful injection was confirmed by visualization of fluorescence (Lisamine tag). At d 1 (i.e. 24 h after intraoocyte injection), the oocyte morphology was evaluated microscopically, and only follicles with morphologically intact oocytes were cultured with or without GDF-9 (100 ng/ml) and FSH (10 ng/ml) for another 3 d. Follicular diameter was measured daily, and the culture medium was changed every other day. The percentage change of follicular volume on d "n" of culture is defined as the volume difference between d "n" and d 0 (the day of microinjection) expressed as a percentage of the volume at d 0. At the end of the culture period, follicles were collected for further analyses.

TUNEL and GDF-9 Immunohistochemistry

To detect cell death in follicles on d 4 and 6 of culture, TUNEL was performed as described previously (30). Briefly, paraffin-embedded follicle sections (thickness, 5 μ m) were mounted on positively charged slides (VWR International Ltd., Mississauga, Ontario, Canada; no. 48311-703), deparaffinized, hydrated, and immersed in PBS with 3% (vol/vol) H₂O₂ (10 min,

RT; to inhibit endogenous peroxidase activity). The sections were then incubated in 50 μ l of the TUNEL mixture (47.5 μ l of TUNEL label containing fluorescein isothiocyanate-conjugated dUTP and 2.5 μ l of TUNEL enzyme) in a humidified chamber (60 min, 37 C). The sections were photographed on a Leica microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with epifluorescent optics. As a negative control, sections were incubated with 50 μ l of TUNEL-label solution containing no TUNEL enzyme.

After TUNEL observation, the same slides were immediately used for immunolocalization of GDF-9 in the cultured follicles. Antigen retrieval by microwave treatment was performed in 10 mM sodium citrate buffer (pH 6.0; 10 min). Sections were sequentially incubated in H₂O₂ (3%; 10 min, RT), blocking serum (30 min, RT), goat antihuman GDF-9 antibody (1:200 of dilution; overnight, 4 C), biotinylated secondary antibody (30 min, RT), and HRP-streptavidin (30 min, RT) in a humidified chamber. Final visualization of antigen was achieved with diaminobenzidine/H₂O₂ solution for 8 min. The sections were then mounted for photography. Primary antibody preabsorbed with its blocking peptide (10 \times ; overnight, 4 C) was used for negative control. The intensity of GDF-9 immunostain in six oocytes for each group at d 4 of culture was semiquantified using a relative scale: 0, 1, and 2 for no (Fig. 3B, panel g), weak (Fig. 3B, panel e), and strong (Fig. 3B, panel a) staining, respectively.

Activated Caspase-3 Assay for Cultured Follicles

To provide a quantitative assessment of apoptosis in MO-injected cultured follicles, caspase-3 activity was examined by the CleavaLite caspase-3 activity assay kit. Three follicles on d 4 from the same treatment group were pooled for analysis. In accordance with the manufacturer's instructions, caspase-3 activity in follicular lysates was determined by measuring the amount of specific bioluminescent substrate, which upon cleavage by caspase-3, exhibits decreased bioluminescence. Fold-increase in caspase-3 activity was calculated as relative bioluminescence by comparing the results from the GDF-9 MO-injected follicles with the level of the CTL MO-injected follicles.

Granulosa Cell Isolation and Culture

Immature female Sprague Dawley rats (21–22 d old) were injected sc with eCG (10 IU). Ovaries were collected in Leibowitz L-15 medium with BSA (0.1%, wt/vol) 24 h thereafter, and granulosa cells from mainly early antral follicles were harvested by follicle puncture. Oocytes were removed from the cell preparations by filtering the cell suspensions through a nylon cell strainer (40 μ m; Becton Dickinson and Co., no. 352340). At d 0, 5 \times 10⁵ viable granulosa cells (determined by Trypan blue dye-exclusion test), were cultured for 24 h in a 12-well plate with 1 ml of RPMI 1640 medium [supplemented with HEPES (10 mM), streptomycin (100 μ g/ml), penicillin (100 U/ml), and fungizone (0.625 μ g/ml)] containing fetal bovine serum (10%, wt/vol). The media were then replaced with serum-free RPMI 1640 supplemented as above, and cells were treated with different concentrations of GDF-9 (0, 10, or 100 ng/ml). Four hours thereafter, C8-ceramide (30 μ M; a cell-permeable, short-chain ceramide analog) or dimethylsulfoxide (vehicle control), were added. In some experiments, cells were pretreated with the PI3K inhibitor LY294002 (10 μ M; 0.5 h before GDF-9). Floating cells and cells attached to the growth surface (collected by trypsin treatment) were pooled 2 d thereafter and assessed for apoptosis.

To obtain a granulosa cell preparation with minimal non-viable cells for Western blot analysis, granulosa cells were then treated sequentially in serum-free medium containing trypsin (20 μ g/ml; 1 min), trypsin inhibitor (300 μ g/ml; 6 min), and deoxyribonuclease I (100 μ g/ml; 6 min) at 37 C, as previously described (68). After two rinses, 4–5 \times 10⁵ viable

cells were incubated in 1 ml of serum-free RPMI 1640 medium (supplemented as described above) with different concentrations of GDF-9 (0–100 ng/ml) for various durations (0.25, 1, and 4 h).

Adenoviral Infection of Granulosa Cells

Viable granulosa cells (5×10^5) were cultured overnight in a 12-well plate with 1 ml of RPMI 1640 medium (supplemented as above) containing fetal bovine serum (10%). They were then infected (MOI = 40) with adenoviral dnAkt or LacZ (as control) in RPMI 1640 containing 2% fetal bovine serum. Media were replaced 12 h thereafter with serum-free RPMI (supplemented as above) with or without ceramide and/or GDF-9. Cells were collected 48 h later for subsequent analyses. At MOI = 10, the adenoviral LacZ infection efficiency over 48 h (as determined by X-gal assay) was more than 90%.

Analysis of Granulosa Cell Apoptosis

At the end of the culture period, floating cells and cells attached to the growth surface were pooled, as described earlier, and stained with Hoechst staining buffer (62.5 μ g/ml, in 10% formalin; overnight, 4 C). Apoptotic cells were identified based on their typical apoptotic nuclear morphology (e.g. nuclear fragmentation, presence of apoptotic bodies) under fluorescent microscope, as previously reported (30, 69). At least 200 cells in a randomly selected area in each treatment group were assessed. To avoid experimental bias, the "counter" was not aware of the treatment.

Western Blot Analysis for Phospho-Akt

Combined floating and attached cells collected at the end of the culture period were centrifuged (10 min, $250 \times g$), and the resulting pellets were suspended in an ice-cold lysis buffer [HEPES (50 mM), NaCl (150 mM), EGTA (1 mM), sodium pyrophosphate (10 mM), $MgCl_2$ (1.5 mM), NaF (100 mM), glycerol (10%), and Triton X-100 (1%) in PBS] containing a protease inhibitor cocktail [PMSF (1 mM), aprotinin (10 μ g/ml), and sodium orthovanadate (1 mM)]. Cells were sonicated, and lysates were frozen until further analysis. Protein content of cell lysates was determined with the Bio-Rad DC Protein Assay Reagent. Protein lysates (50 μ g) were separated by 12% SDS-PAGE and electrotransferred to nitrocellulose membranes. Nonspecific binding to the membranes was blocked with dehydrated nonfat milk (5%, 1 h, RT). Blots were incubated with anti-p-Akt antibody (dilution 1:1000; overnight, 4 C) and then with HRP-conjugated secondary antibody (1 h, RT). Peroxidase activity was visualized with the enhanced chemiluminescence kit, and membranes were exposed to x-ray film (Kodak Canada, Inc., Toronto, Ontario, Canada). After p-Akt detection, membranes were stripped and then reprobated with anti-Akt antibody (dilution 1:1000) for total Akt. Signals were quantified densitometrically using Scion Image Software (Scion Corp., Frederick, MD), and ratio of p-Akt to total Akt was calculated.

Real-Time PCR Analysis of FSHR Expression

Total RNAs from three MO-injected follicles (pooled from the same experiment group) were extracted, using RNeasy Micro kit according to manufacturer's instructions. RNAs were reverse transcribed in a final volume of 20 μ l solution containing 1 \times First-Strand Buffer (3 mM $MgCl_2$; 75 mM KCl; 50 mM Tris-HCl, pH 8.3), 500 μ M each deoxynucleotide triphosphate, 10 mM dithiothreitol, 100 U of RevertAid Enzyme, 100 ng Random Decamer Primers, and total RNA. Real-time quantitative PCR analysis for FSHR was performed on the follicular cDNAs, using a LightCycler 2.0 System (Roche Di-

agnostic Corp.). The FSHR primers used for amplification were a 5'-forward primer (5'-CATCACTGTGTCCAAGGCCA-3') and a 3'-reverse primer (5'-TGCGGAAGTCTTGGT-GAAAA-3'), as previously described by Romero *et al.* (70). FSHR transcript level was normalized on the basis of the level of transcripts for 18S rRNA (5'-forward primer; 5'-CGCGGT-TCTATTTTGTGGT-3', 3'-reverse primer; 5'-AGTCG-GCATCGTTTATGGTC-3'). Amplification reaction was then performed using the QuantiTect SYBR Green PCR kit. The thermal cycling conditions comprised an initial denaturation step at 95 C for 15 min and 40 cycles at 95 C for 15 sec, 58 C (FSHR) or 56 C (18S rRNA) for 20 sec, and 72 C for 30 sec. The level of FSHR mRNA was expressed as a ratio to 18S rRNA values.

Statistical Analysis

Results are presented as means \pm SEM of at least three independent experiments, as detailed in the figure legends. All data were subjected to one- or two-way (repeated-measure) ANOVA, except unpaired *t* test for caspase-3 activity assay and immunostaining intensity of GDF-9 in oocytes (Prism 3.0 statistical software; GraphPad Software, Inc., San Diego, CA). Differences between experimental groups were determined by the Tukey or Bonferroni post test. Statistical significance was inferred at $P < 0.05$.

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