Growth Differentiation Factor 9 Promotes Rat Preantral Follicle Growth by Up-Regulating Follicular Androgen Biosynthesis

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The transition from preantral to early antral stage is the penultimate stage of ovarian follicular development in terms of gonadotropin dependence and follicle destiny. Although oocyte-somatic cell communication is important in early follicular development, our knowledge of the precise role of the oocyte-derived growth differentiation factor (GDF)-9 during preantral follicle growth is incomplete. We examined whether and by what means oocyte-derived GDF-9 controls follicular development and steroidogenesis during the preantral to early antral transition, by a combination of in vitro gene manipulation (i.e. intraoocyte injection of GDF-9 antisense oligos) and preantral follicle culture. Intraoocyte injection of GDF-9 antisense suppressed rat preantral follicle growth in vitro, whereas GDF-9 enhanced follicular development. GDF-9 augmented testosterone production in preantral follicles. GDF-9 antisense suppressed androgen production and CYP17A1 mRNA expression in cultured follicles, a response attenuated by exogenous GDF-9. The nonaromatizable androgen 5α-dihydrotestosterone rescued the follicular growth arrest caused by GDF-9 down-regulation. The specific androgen receptor antagonist flutamide suppressed GDF-9-induced preantral follicle growth in vitro. The data suggest that GDF-9 plays an important role in promoting preantral follicle growth by up-regulating follicular androgen biosynthesis. GDF-9 is essential for CYP17A1 expression during follicular development from the preantral to the early antral stage. (Endocrinology 150: 2740–2748, 2009)
enhance rat preantral follicle growth (13), although its precise mechanism(s) is obscure.

Ovarian steroids, which include progesterone, androgen, and estrogen, act via specific nuclear receptors and are essential for normal folliculogenesis and ovulation (14). Progesterone receptor (15) or estrogen receptor (16) null mice are infertile, and androgen receptor (AR) null mice culminate in reduced fertility and premature ovarian failure (17), indicating that these steroids are essential for reproductive function and fertility. Although the exact role of GDF-9 on follicular cell differentiation during the transition of the follicle from preantral to early antral stage is not clear, GDF-9 is known to stimulate basal estradiol synthesis and suppress FSH-induced progesterone and estradiol production in undifferentiated rat granulosa cells (12). The role of GDF-9 on thecal cell androgen production is less clear. Whereas Solovyeva et al. (18) showed that GDF-9 stimulates androstenedione production in rat theca-interstitial cell, Spicer et al. (19) reported that GDF-9 inhibits androstenedione production by bovine thecal cells from small antral follicles. Although GDF-9 promotes granulosa cell mitosis and preantral follicle growth, whether the latter response is mediated via follicular steroidogenesis is not known.

In the present studies, we hypothesized that oocyte-derived GDF-9 stimulates preantral follicle growth in part by up-regulating follicular steroidogenesis. We examined whether and by what means GDF-9 and FSH regulate follicular development and steroid production during preantral-early antral transition by a combination of in vitro gene manipulation and preantral follicle culture. We have demonstrated that GDF-9 promotes preantral follicle growth by stimulating follicular androgen biosynthesis.

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, and agarose (low gelling temperature) were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human FSH was obtained from the National Hormone and Peptide Program, Harbor-UCLA Medical Center (Torrance, CA). Morpholino antisense oligos (MOs) for control (CTL) and GDF-9 were purchased from Gene-Tools, LLC (Philomath, OR). Intracytoplasmic sperm injection micropipettes (no. MIC-35-30) were from Gene-Tools, LLC (Philomath, OR). Intracytoplasmic sperm injection micropipettes (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). Progesterone and testosterone enzyme immunoassay (EIA) kit were from R&D Systems, Inc. (Minneapolis, MN), whereas the estradiol EIA kit was from Calbiotech, Inc. (Spring Valley, CA). 5α-Dihydropotestosterone (DHT), estradiol, and flutamide, a specific AR antagonist, were from Sigma-Aldrich Co. (St. Louis, MO). RNAspecific micro and QuantiTect SYBR Green PCR kit were purchased from QIAGEN, Inc. (Mississauga, Ontario, Canada). Random degeneramer primers were from Ambion, Inc. (Austin, TX). PCR primers for CYP17A1, AR, and 18S rDNA were from Invitrogen Canada, Inc. (Burlington, Ontario, Canada). Recombinant rat GDF-9 was generously provided by Dr. Aaron J. W. Hsu (Stanford University School of Medicine, Stanford, CA).

Preantral follicle isolation and culture

All animal procedures were 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, 150–170 μm) were isolated from 14-d-old rats in Leibowitz L-15 medium with BSA (0.1%, wt/vol) at 0, using 28.5-gauge needles (Becton Dickinson and Co., Franklin Lakes, NJ). Only round follicles with intact basement membrane and thecal layer were selected for the present studies. Follicles were cultured individually in a 96-well plate (Sarstedt, Inc., Newton, NC; no. 83.1837.50) in 100 μl of α-MEM supplemented with HEPES (10 mM), BSA (0.1%, wt/vol), bovine insulin (5 μg/ml), transferrin (2 μg/ml), ascorbic acid (25 μg/ml), sodium selenite anhydrous (2 ng/ml), L-glutamine (3 mM), sodium pyruvate (100 μg/ml), streptomycin (100 μg/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 10 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 of 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 day n of culture is defined as the volume difference between day 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 steroid assays.

Intraoocyte injection of MO oligonucleotides in preantral follicles

Expression of MOs is effective in suppressing translation of target genes in zebrafish (20) and Xenopus (21) embryos. As described previously (4), to assess the role of GDF-9 on follicular development and steroidogenesis during preantral-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′-ACAGGAATCTGGCGGGGAATGTCCAT-3′, not homologous with BMP-15/GDF-9B cDNA). The standard CTL MO sequence (5′-CCTCTACCTCAGTTACAACTTATA-3′) designed by Gene-Tools was not expressed in the follicles and caused no phenotype on follicular growth and steroidogenesis in the injected/cultured preantral follicles. The MOs were fluorescent tagged with Lissamine, which was visible by fluorescence microscopy. Large preantral follicles isolated from 14-d-old rats were cultured individually in a 96-well plate without GDF-9 or FSH. After 16–24 h, only the follicles (diameter 150–170 μm) with intact basement membrane and thecal layer were selected for the microinjection. CTL MO or GDF-9 MO (10 μM) was injected into the oocyte of the preantral follicles at d 0. The volume of MO injected (3 pl) was less than 5% (vol/vol) of the oocyte volume. Successful injection was confirmed by visualization of fluorescence (Lissamine 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 (d 1 to d 4). Follicular diameter was measured daily and the culture medium was changed every other day. The percentage change of follicular volume on day n of culture is defined as the volume difference between day 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 fixed and embedded, as described previously (7), for GDF-9 immunohistochemistry. The spent media were kept at −20°C for steroid assays. The follicle were also pooled and kept at −80°C for real-time PCR analyses.

To assess the role of androgen and estrogen on GDF-9-induced preantral follicle growth, the CTL MO- and GDF-9 MO-injected (at d 0) follicles were cultured with different concentrations of DHT (0 to 10 μM) or estradiol (0 to 10 μM) for 3 d (d 1 to d 4). Moreover, to ascertain the
direct effect of androgens, the CTL MO- and GDF-9 MO-injected follicles were preincubated for 1 h (at d 1) with different concentration of the AR antagonist flutamide (0–10 μM) before the addition of GDF-9 (100 ng/ml) and cultured for another 3 d (d 1 to d 4). Follicular diameter was measured daily, and results were expressed as change in follicular volume.

GDF-9 immunohistochemistry

The GDF-9 content in the injected/cultured follicles was examined by immunohistochemistry, according to the previous protocol (7). The intensity of GDF-9 immunostain in 10 oocytes for each group at d 4 of culture was semiquantified using a relative scale: 0, 1, and 2 for no (see Fig. 2Ad), weak, and strong (see Fig. 2Ac) staining, respectively.

Steroid assays

The levels of progesterone, testosterone, and estradiol in the spent media were measured using the respective EIA kits (as described in Materials and Methods) according to the manufacturer’s instructions. The intraassay coefficient of variation for progesterone, testosterone, and estradiol was 6.0, 9.5, and 10.9%, respectively, whereas the interassay coefficient of variation for progesterone, testosterone, and estradiol was 5.9, 11.7, and 12.5%, respectively. The sensitivity of the progesterone, testosterone, and estradiol assays was 9, 6, and 10 pg/ml, respectively.

Real-time PCR analysis of CYP17A1 and AR expression

Total RNAs from three MO-injected follicles (pooled from the same experiment group) were extracted, using RNasey microkit according to manufacturer’s instructions. Real-time quantitative PCR analysis for CYP17A1 and AR was performed on the follicular cDNAs, using a LightCycler 2.0 system (Roche Diagnostic Canada, Laval, Quebec, Canada). The primers of CYP17A1 used for amplification were a 5’-forward primer (5’-ACTGAGGTATCGGTATGC-3’) and a 3’-reverse primer (5’-TC GAACCTTCCTCGACATT-3’), whereas those of AR were a 5’-forward primer (5’-GGGTGACTTCTCCTGCGT-3’) and a 3’-reverse primer (5’-AACGTGTTCTCGTATCG-3’). The transcript levels of CYP17A1 and AR were normalized against those of 18S rRNA (5’-forward primer; 5’-CGGCGTCTTATTTTTGTTGT-3’, 5’-reverse primer; 5’-AGTCCGACCTGTTATGCT-3’). Amplification reaction was then performed using the QuantiTect SYBR Green PCR kit (QIAGEN). The thermal cycling conditions comprised an initial denaturation step at 95°C for 15 min and 50 cycles (CYP17A1) or 40 cycles (AR and 18S rRNA) at 95°C for 15 sec, 56°C for 20 sec, and 72°C for 30 sec. The levels of CYP17A1 and AR mRNA were expressed as a ratio to 18S rRNA values.

Statistical analysis

Results are presented as means ± SEM of at least three independent experiments. All data were subjected to one- or two-way (repeated measure) ANOVA, except unpaired t test for immunostaining intensity of GDF-9 in oocytes (Pirson 4.0 and InStat 3.0 statistical software; GraphPad Software, Inc., San Diego, CA). Differences between experimental groups were determined by the Tukey or Bonferroni posttest. Statistical significance was inferred at P < 0.05.

Results

Effects of GDF-9 and FSH on preantral follicular growth and steroidogenesis in vitro

To examine the effect of GDF-9 and FSH on preantral follicular growth, rat large preantral follicles were cultured with or without 100 ng/ml of GDF-9 and different concentrations of FSH (10 ng/ml [FSH10] or 100 ng/ml [FSH100]). CTL Preantral follicles were 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 day n of culture is defined as the volume of follicles on day n minus the volume at d 0 (the day of isolation) expressed as a percentage of the volume at d 0. Results are presented as means ± SEM of a total of 40 follicles from six to eight independent experiments. ND, Not detected. Bars with different superscripts are significantly different at P < 0.05. Note that GDF-9 and FSH stimulated preantral follicular growth (A). B–D, Concentrations of progesterone (B), testosterone (C), and estradiol (D) in the spent media on d 2 and d 4 were measured. Note that GDF-9 augmented testosterone and estradiol production in preantral follicles, whereas FSH stimulated progesterone and estradiol production in the follicles.

An increase in follicular volume was seen as early as d 2 after treatment with either GDF-9 or FSH (P < 0.05 vs. CTL). Addition of GDF-9 to the culture media significantly increased follicular growth at d 4 (P < 0.01 vs. CTL; Fig. 1A). Because 10 ng/ml of FSH appeared more effective on preantral follicle growth than 100 ng/ml of FSH at d 4 (P < 0.01), 10 ng/ml of FSH was used for the later microinjection studies.

To determine the influence of GDF-9 and FSH on follicular steroidogenesis during preantral follicle growth, the concentrations of progesterone, testosterone, and estradiol in the spent media were determined. Although 100 ng/ml of FSH augmented progesterone production in preantral follicles (P < 0.05 vs. CTL), neither 10 ng/ml of FSH nor GDF-9 significantly influences progesterone biosynthesis (Fig. 1B). Treatment with GDF-9 stimulated testosterone production in the preantral follicles (P < 0.01 vs. CTL), whereas FSH did not significantly affect testosterone level in the follicles (Fig. 1C). GDF-9 and FSH augmented estrogen production in the preantral follicles. We found
100 ng/ml of FSH to be more effective in stimulating estradiol production than GDF-9 in the follicles at d 4 (P < 0.01), although no significant difference was observed between 10 ng/ml of FSH and GDF-9 treatment group (Fig. 1D). It appeared that GDF-9 augmented testosterone and estradiol production in preantral follicles, whereas FSH stimulated progesterone and estradiol production in the follicles.

Effect of intraoocyte injection of GDF-9 MO on preantral follicle growth in vitro

To assess the role of GDF-9 on follicular development and steroidogenesis during the preantral to early antral transition, GDF-9 MO or its control MO (CTL MO) was injected into the oocyte of cultured preantral follicles (Fig. 2Aa). Successful injection was confirmed by visualization of fluorescence (Fig. 2Ab). The relative intensity of GDF-9 immunostain in GDF-9 MO group (0.33 ± 0.21; Fig. 2Ad) was significantly lower than those of CTL MO group (1.67 ± 0.21; Fig. 2Ac), indicating that GDF-9 MO markedly decreased oocyte GDF-9 content in vitro (P < 0.01).

In the absence of GDF-9 and FSH, the CTL MO-injected preantral follicles exhibited minimal growth [follicular volume change at d 4: 60.4 ± 7.8%; (CTL MO + CTL); Fig. 2Ba, C, and D]. Addition of GDF-9 (100 ng/ml) to the culture media significantly increased the follicular growth (CTL MO + GDF-9; Fig. 2Bb), and the increase at d 4 was 129.2 ± 13.0% (P < 0.01 vs. CTL MO + CTL; Fig. 2, C and D). Intraoocyte injection of GDF-9 MO suppressed basal preantral follicle growth during 4-d culture period (Fig. 2Bc). Whereas basal follicular volume in the CTL MO + CTL group was significantly increased by d 4 (P < 0.05), a decrease was noted in the GDF-9 MO + CTL group (−19.8 ± 5.1%), resulting in a significant difference between the two experimental groups (P < 0.01; Fig. 2, C and D). 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 (Fig. 2Bd). No significant difference in the change in follicular volume was observed between the CTL MO + CTL and the GDF-9 MO + GDF-9 group (P > 0.05; Fig. 2, C and D).

FSH (10 ng/ml) stimulated preantral follicle growth (P < 0.05, CTL MO + FSH vs. CTL MO + CTL; Fig. 2D). In the presence of FSH, GDF-9 MO down-regulation also suppressed follicular growth (P < 0.01, GDF-9 MO + FSH vs. CTL MO + FSH).

Effect of intraoocyte injection of GDF-9 MO on steroidogenesis in preantral follicles in vitro

Although FSH augmented progesterone production in preantral follicles (P < 0.01, CTL MO + FSH vs. CTL MO + CTL), neither the addition of GDF-9 nor GDF-9 down-regulation significantly influence progesterone biosynthesis irrespective of the presence of FSH (Fig. 3A). There was no significant difference in the progesterone concentration between the CTL MO + CTL, CTL MO + GDF-9, CTL MO + GDF-9 MO + GDF-9 group (P > 0.05).

Treatment with GDF-9-stimulated testosterone production in the follicles injected with CTL MO [550.3 ± 108.5 pg/ml (CTL MO + GDF-9) vs. 268.6 ± 78.0 pg/ml (CTL MO + CTL), P < 0.05; 3B]. GDF-9 MO markedly decreased androgen biosynthesis [14.1 ± 5.7 pg/ml (GDF-9 MO + CTL), P < 0.05 vs. CTL MO + CTL], a response attenuated by exogenous GDF-9 [475.9 ± 110.5 pg/ml (GDF-9 MO + GDF-9), P < 0.01 vs. GDF-9 MO + CTL]. The amount of testosterone in GDF-9 MO + GDF-9 was not different from those in CTL MO + CTL (P > 0.05; Fig. 3B). FSH did not affect testosterone level in the CTL...
MO-injected follicles (P > 0.05, CTL MO + FSH vs. CTL MO + CTL) but significantly suppressed GDF-9 MO-induced testosterone down-regulation (P < 0.05, GDF-9 MO + FSH vs. GDF-9 MO + CTL). GDF-9 appeared to be more effective in stimulating androgen production than FSH in the CTL MO-injected follicles [550.3 ± 108.5 pg/ml (CTL MO + GDF-9) vs. 293.3 ± 67.5 pg/ml (CTL MO + FSH), P < 0.05], although no significant difference was observed between the GDF-9 MO + GDF-9 and the GDF-9 MO + FSH group.

Although treatment with GDF-9 and FSH did not alter estradiol production in the follicles injected with CTL MO (Fig. 3C), GDF-9 MO markedly decreased estradiol production [<10 pg/ml (GDF-9 MO + CTL) vs. 20.1 ± 7.3 pg/ml (CTL MO + CTL)]. In the GDF-9 MO-injected follicles, addition of GDF-9 into the culture media increased estradiol production [<10 pg/ml (GDF-9 MO + CTL) vs. 84.2 ± 29.4 pg/ml (GDF-9 MO + GDF-9)], FSH also augmented estradiol production in the GDF-9 MO-injected follicles irrespective of the presence of GDF-9 (P < 0.01).

Based on the results of Figs. 1 and 3, GDF-9 appeared to preferentially stimulate the production of testosterone rather than estradiol in the follicles during preantral to early antral transition.

**Androgen action is involved in GDF-9-induced preantral follicle growth in vitro**

To determine whether and how androgen modulates GDF-9-induced preantral follicles in vitro, the CTL MO- and GDF-9 MO-injected follicles were cultured with different concentrations of DHT (A; 0–10 µM), a nonaromatizable androgen, or estradiol (E2; B; 0–10 µM). The percentage change of follicular volume on d 4 of culture is defined as the volume difference between d 4 and d 0 (the day of microinjection) expressed as a percentage of the volume at d 0. Results represent the means ± SEM of a total of 20 follicles from four or five independent experiments. Bars with different superscripts are significantly different at P < 0.05. Note that the addition of DHT, not estradiol, to the culture media prevented the growth arrest induced by GDF-9 antisense on d 4.
In the GDF-9 MO-injected follicles, DHT also enhanced preantral follicle growth in a concentration-dependent manner (Fig. 4A). Although GDF-9 down-regulation suppressed follicular growth ($P < 0.01$), the addition of 1 and 10 nM of DHT to the culture media prevented this response ($P < 0.01$). In contrast, treatment with estradiol (0–10 μM) did not alter the growth of CTL-MO or GDF-9-MO injected follicles (Fig. 4B).

Furthermore, to examine the role of androgen action in GDF-9-induced preantral follicle growth, the CTL MO- and GDF-9 MO-injected follicles were cultured with or without GDF-9 and with different concentrations of flutamide (0–10 μM), a specific AR antagonist. Although exogenous GDF-9 prevented the follicular growth arrest caused by GDF-9 MO ($P < 0.01$), addition of flutamide (10 μM) to the culture media suppressed this response ($P < 0.01$; Fig. 5). Flutamide did not alter the growth of the CTL MO-injected follicles ($P > 0.05$). These results demonstrated that the effects of androgen on the GDF-9-induced preantral follicle growth were not due to aromatization to estradiol, and were inhibited by an antagonist to the androgen receptor.

**GDF-9 is essential for the expression of follicular CYP17A1 mRNA in vitro**

To determine by what means GDF-9 regulates follicular androgen action during this stage, real-time quantitative PCR analysis of CYP17A1 (mainly expressed in thecal cells) and AR (mainly expressed in granulosa cells) was performed on total RNAs from the MO-injected follicles. GDF-9, but not FSH, augmented CYP17A1 mRNA levels in the follicles injected with CTL-MO ($P < 0.05$, CTL MO + GDF-9 vs. CTL MO + CTL), whereas GDF-9 MO markedly decreased this response ($P < 0.05$, GDF-9 MO + CTL vs. CTL MO + CTL; Fig. 6A). The down-regulation of CYP17A1 transcript by GDF-9 MO was prevented by exogenous GDF-9 ($P < 0.05$, GDF-9 MO + GDF-9 vs. GDF-9 MO + CTL), and CYP17A1 mRNA abundance in GDF-9 MO + GDF-9 was not different from those in CTL MO + CTL ($P > 0.05$). FSH also augmented CYP17A1 mRNA levels in the GDF-9 MO-injected follicles ($P < 0.05$, GDF-9 MO + FSH vs. GDF-9 MO + CTL). Addition of GDF-9 or GDF-9 down-regulation did not affect AR mRNA expression in the preantral follicles (Fig. 6B). No significant difference in AR mRNA abundance was observed between the CTL MO + CTL, CTL MO + GDF-9, GDF-9 MO + CTL, and GDF-9 MO + GDF-9 group ($P > 0.05$).

**Discussion**

Although oocyte-somatic cell communication is important in early follicular development, our knowledge of the precise role of the oocyte-derived factor GDF-9 during the preantral-early antral transition is incomplete. In the present study, we have shown for the first time that: 1) intraoocyte injection of GDF-9 MO antisense suppressed rat preantral follicle growth in vitro, whereas GDF-9 enhanced follicular development; 2) GDF-9 augmented testosterone production in preantral follicles; 3) GDF-9 MO suppressed androgen production and CYP17A1 mRNA expression in cultured follicles, a response attenuated by exogenous...
GDF-9; 4) the nonaromatizable androgen DHT rescued the follicular growth arrest by GDF-9 down-regulation; and 5) the specific AR antagonist flutamide suppressed GDF-9-induced preantral follicle growth in vitro. These results suggest that GDF-9 controls ovarian follicular development from the preantral stage to early antral stage by up-regulating follicular androgen biosynthesis.

The exact role of GDF-9 on follicular differentiation during preantral-early antral transition is not clear (19). Nevertheless, it is possible that GDF-9 stimulates thecal cell recruitment, proliferation, and differentiation and induces the formation of thecal cell layer during this early stage of the follicular development. Ovaries from GDF-9 null mice exhibit a developmental block at the primary follicle stage, which is characterized by failed thecal layer formation in early follicles (22). GDF-9 is believed to be more important for the differentiation than the recruitment of thecal cell because the double-mutant (GDF-9 and inhibin-α) mouse exhibits morphological thecal cells surrounding the preantral follicles without detectable selective thecal markers, CYP17A1 and LH receptor (23). GDF-9 treatment increases androgen production in cultured rat theca-interstitial cells (18) and promotes murine ovarian expression of the specific thecal cell marker CYP17A1 (22). A recent study also indicated that GDF-9 increases thecal cell number and DNA synthesis in thecal cells of small bovine follicles (19). In the present study, we demonstrated that GDF-9 augments androgen production and CYP17A1 mRNA expression in the preantral follicles, whereas GDF-9 down-regulation suppressed this response, indicating that GDF-9 is involved in the thecal cell differentiation during preantral-early antral transition. Whether the increased follicular CYP17A1 content is a result of increased thecal cell number and/or CYP17 levels per cell remains to be elucidated.

Ovarian androgens, primarily androstenedione and testosterone, are produced by thecal cells and act via receptors (ARs) localized to granulosa cells, stromal cells, and oocytes (14). Inactivation of AR in female mice results in premature ovarian failure, indicating that normal folliculogenesis requires AR-mediated androgen action (17). AR expression is highest in granulosa cells of rat small preantral and early antral follicles (24), raising the possibility that androgens are important paracrine regulators of follicular growth during preantral to early antral transition. Although androgens have long been implicated as an inhibitor of antral follicular development (25, 26), recent evidence suggests that the effect of androgens on follicular growth is dependent on the stage of follicular development and that androgens also have a growth promoting role in early folliculogenesis. Administration of testosterone or DHT to adult rhesus monkeys significantly increased the number of preantral and small antral follicles as well as granulosa and thecal cell proliferation (27). In vitro studies have shown that androgens (e.g. testosterone, DHT, androstenedione) stimulate preantral follicle growth and granulosa cell mitosis in mice (28), the transition of primary follicle to secondary follicle in cattle (29), and follicular survival in humans (30). An AR antagonist, but not an aromatase inhibitor, inhibited this growth response, indicating that the conversion of androgens to estrogens was not responsible for the follicle growth (31). DHT has also been shown to enhance porcine granulosa cell proliferation by facilitating the action of GDF-9 in vitro (32). In the present study, the nonaromatizable androgen DHT, but not estradiol, rescued the follicular growth arrest by GDF-9 down-regulation. The specific AR antagonist flutamide suppressed GDF-9-induced preantral follicle growth in vitro. These findings suggest that androgens exert a direct stimulatory action on the follicular development, especially during the preantral-early antral stage transition.

Androgens enhance the FSH action in the follicles by increasing FSH receptor expression, FSH-induced granulosa cell aromatase activity and proliferation, and follicular growth (33). GDF-9 augments FSH-induced preantral follicle growth (4, 17), whereas GDF-9 down-regulation suppressed FSH-stimulated follicular development. Although GDF-9 is required for the expression of FSH receptor in rat preantral follicles (4), whether these growth responses are modulated through thecal androgen actions awaits further investigation.

Tetsuka et al. (24) reported that a gradient of AR immunostaining existed in large follicles of the rat ovary, with cumulus cells and antral granulosa cells strongly expressing more AR protein than do peripheral layers. Nevertheless, the present result suggests that GDF-9 is not the oocyte-secreted factor that influences AR expression in follicles because GDF-9 did not alter AR mRNA levels in the cultured preantral follicles.

Previous study showed that GDF-9 stimulates basal estradiol synthesis in rat undifferentiated granulosa cells but suppresses FSH-induced progesterone and estradiol production (12). Because granulosa cells often undergo luteinization in culture (34) and excess FSH induces premature granulosa cell differentiation in vitro (35), it is possible that some of the granulosa cell responses in vitro might be more related to a potential role for GDF-9 in inhibiting premature luteinization rather than to its effect on normal follicular function (13, 18). Nevertheless, the present studies indicate that GDF-9 stimulates preantral follicle production of estradiol, but not progesterone, although whether GDF-9 enhances the expression of aromatase in granulosa cells and/or increases the synthesis of androgen substrate for aromatization remains to be elucidated. In the GDF-9 MO-injected follicles, FSH stimulated progesterone and estradiol production in the preantral follicles irrespective of the presence of GDF-9, which might be related to the FSH-induced expression of steroi
dogenic acute regulatory (36), CYP11A1 (36), and CYP19A1 (37) genes in granulosa cells. Solovyeva et al. (18) reported that GDF-9 enhanced forskolin-stimulated androstenedione production in rat theca-interstitial cells, whereas Spicer et al. reported that GDF-9 inhibits LH and IGF1-induced steroidogenesis by bovine thecal cells (19). Whether thecal cells also undergo luteinization in vitro and GDF-9 modulates this process, is not known.

Although the present results suggest that GDF-9 controls preantral follicle growth by up-regulating thecal CYP17A1 expression and androgen biosynthesis, whether the observed effect of GDF-9 is mediated through a direct action on thecal cells or indirectly on granulosa cells remains to be investigated. 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 (38), resulting in the phosphorylation
and activation of Smo- and Mad-related protein (Smad)-2 and Smad3 in granulosa cells (38–40). In rodents, activin-like receptor kinase-5 mRNA/protein and Smad2/3 proteins are expressed in the oocyte, granulosa, and thecal cells of both preantral and antral follicles (41), whereas BMP receptor type II mRNA expression is observed only in granulosa cells (42). These results suggest that thecal cells are not capable of responding to GDF-9 and that GDF-9 indirectly modulates theca-cell function through a granulosal factor(s). Nevertheless, one could not exclude the possibility that additional type I and type II receptors for GDF-9 might be present in thecal cells because in vitro studies demonstrated direct actions of GDF-9 on theca-cell androgen synthesis in rats (18) and cattle (19).

Intracellular Smad signaling molecules might also play roles in controlling CYP17A1 expression and steroid production in various types of cells. There are eight Smad proteins in total, Smad1–8. Typically, Smad2/3 are activated by members of the TGF-β/activin subfamilies (e.g. TGF-β, activin, and GDF-9), and Smad1/5/8 are activated by members of the BMP subfamilies (e.g. BMP-4, -6, -7, and -15). BMP-4, -6, and -7 suppressed basal and LH-induced CYP17A1 mRNA expression and androgen production in bovine thecal cells (43). Müllerian-inhibiting substance, which also interacts through the Smad1/5/8 pathway, inhibits the CAMP-induced expression of CYP17A1 mRNA in Leydig cells (44). In contrast, Smad2/3 signaling activated by activin increases CYP17A1 expression and steroid production in bovine thecal cells (43).

Growth differentiation factor-9 (GDF-9) is required for ovarian follicle maturation but not male fertility. Nat Genet 26:216–220

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