0013-7227/00/$03.00/0
Endocrinology

Vol. 141, No. 10
Printed in U.S.A.

In Vivo Treatment with GDF-9 Stimulates Primordial and Primary Follicle Progression and Theca Cell Marker CYP17 in Ovaries of Immature Rats*

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ABSTRACT

Growth differentiation factor (GDF)-9 is a cystine knot-containing hormone of the transforming growth factor-β superfamily produced by the oocyte. In GDF-9 null mice, follicle development is arrested at the primary stage and GDF-9 treatment *in vitro* enhances preantral follicle growth. Immature female rats were treated with recombinant GDF-9 for 7 or 10 days. At 10 days, treatment with GDF-9 augmented ovarian weights, concomitant with an increase in the number of primary and small preantral follicles by 30 and 60%, respectively. Furthermore, the number of primordial follicles was decreased by 29%, but the number of large preantral follicles was not affected. In contrast, treatment with FSH increased the number of small and large preantral follicles by 36 and 177% but did not influence the number of primary and primordial follicles. Immunoblot analysis showed an increase of CYP17, a theca cell marker, in the ovarian homogenate after treatment with GDF-9 but not FSH. The present results indicate that *in vivo* treatment with GDF-9 enhances the progression of primordial and primary follicles into small preantral follicles. Thus, GDF-9 treatment could provide an alternative approach to stimulate early follicle development in addition to the widely used FSH that acts mainly on the development of more advanced follicles. (*Endocrinology* **141:** 3814–3820, 2000)

DEVELOPMENT of the mammalian ovary is characterized by the initial endowment of a fixed number of primordial follicles that is gradually depleted during reproductive life. The follicles develop through primordial, primary, and preantral stages before acquiring an antral cavity (1, 2). In rodents, primordial follicles are formed during the first few days of life. At postnatal day three, only very few naked oocytes remain and primordial follicles with a flattened layer of granulosa cells predominate in the ovary (3). Some follicles begin to grow as soon as they are formed but most enter a state of suspended animation (4). Primordial follicles are considered the resting pool, which is subsequently depleted during the reproductive life. Initiation of follicle growth from the primordial to primary stage is characterized both by changes of granulosa cell shape and an increase in oocyte size (5). The primary follicle has cuboidal-shaped granulosa cells that proliferate and lead to the formation of preantral follicles concomitant with an increase in oocyte size. Oocyte growth ceases at the onset of antral formation. Once follicles reach the small antral stage, most of them undergo atresia unless rescued by FSH (6–8). Under the influence of gonadotropins, the antrum is formed and selected antral follicles further increase in size until they reach the preovulatory stage.

One of the distinctive features of primary and preantral follicle development is the formation of a thecal cell layer. Theca cells are derived from the mesenchymal tissue surrounding the follicles. Some of these mesenchymal cells seem to be associated with follicles as early as the primordial stage (9). Primary follicles show a thin layer of theca cells which increase in number during follicle progression. Following stimulation by LH, theca cells secrete androgens to serve as substrates for the estrogen-producing granulosa cells (10, 11).

It is well accepted, that pituitary-derived gonadotropins stimulate follicle growth and maturation to reach the preovulatory stage (12). However, follicular growth cannot be completely accounted for by changes in circulating gonadotropins. Small follicles enter the growing pool even in hypophysectomized mice (13), whereas follicles can grow up to the small antral stage in hypogonadotrophic mice (14). In addition to endocrine hormones, folliculogenesis is controlled by intraovarian autoregulatory factors that could initiate and stimulate the growth of follicles independent of gonadotropins (2, 15).

Apart from paracrine factors of granulosa and theca cell origin, growth differentiation factor-9 (GDF-9) secreted by the oocyte has recently been shown to play a role in follicular development. GDF-9-deficient mice, similar to steel panda mutants of the kit ligand gene (16), display an arrest of follicle growth at the primary follicle stage (17). The ovaries of GDF-9 null animals lack several theca cell markers including CYP17 and c-kit (18). Although our recent studies showed that treatment with recombinant GDF-9 induces preantral follicle growth *in vitro* (19), the effect of *in vivo* treatment with

Received February 17, 2000.
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* This study was supported by NIH Grant HD-31398 and the NIH Specialized Cooperative Centers Program in Reproduction Research.

GDF-9 has not been investigated. In the present study, we demonstrate that treatment with recombinant GDF-9 *in vivo*, enhances ovarian weight and primordial as well as primary follicle progression up to the small preantral stage in immature rats. In addition, ovarian CYP17, a marker for the theca interna cells (20), is also increased in these animals.

Materials and Methods

Reagents and hormones

Recombinant GDF-9 was generated in mammalian cell lines and characterized as previously described (19). Briefly, expression vectors for wild-type and epitope-tagged GDF-9 were constructed using pcDNA3.1 Zeo (Invitrogen, Carlsbad, CA). Human embryonic kidney 293T cells were transfected with the expression vectors, and clonal cell lines stably expressing wild-type and tagged GDF-9 were selected under 1 mg/ml of Zeocin (Invitrogen). Conditioned media were harvested after 4 days of serum-free culture. Quantitation of N-tagged GDF-9 was done following purification using a Nickel-column (Amersham Pharmacia Biotech, Stockholm, Sweden) and measurement of protein content using Micro BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Purified N-tagged GDF-9 was then used as a standard for the quantitation of wild-type GDF-9 in the conditioned media of 293T cells by immunoblots using specific GDF-9 antibodies. Recombinant human FSH (Org 32489E; 10 IU/μg) was a gift from Organon NV (Oss, The Netherlands).

Animal model

Neonatal Sprague Dawley female rats were obtained from Simonsen Laboratories (Gilroy, CA). Groups of ten rats each were housed together with a lactating mother for the duration of the experiments. Animals were housed under controlled humidity, temperature and light regimen and fed *ad libitum* on a standard rat chow. After hormonal treatments, animals were anesthetized and killed using CO_2. Animal care was consistent with institutional and NIH guidelines.

To assess the role of GDF-9 on follicular development, female rats at 5 days of age were injected ip twice daily with 10 μg recombinant rat GDF-9 in 200 μl conditioned media or with FSH (10 IU). Both the GDF-9 and the FSH dose corresponded to approximately 100-fold of the concentrations needed for optimal *in vitro* bioactivity (21). Animals at 5 days of age were used because their ovaries contain mainly primary and primordial follicles, the progression of which were found to be altered in GDF-9 null mice (17).

Control animals were injected with PBS (200 μl). Rats were injected for either 7 or 10 days. The body weight of the animals was recorded daily as an index of animal growth. All animals were killed 12 h after the last injection. The ovaries were collected in L-15 Leibovitz medium (Life Technologies, Inc., Gaithersburg, MD) and cleaned from surrounding tissues. Each ovary was weighed individually using a scale sensitive for μg ranges (Mettler balance, Mettler Instrument Corp. Hightstown, NJ). One of the ovaries of each animal was fixed in Karnowsky solution (2.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer). The remaining ovary was frozen and stored at −80 C.

Histological evaluation

Ovaries fixed for at least 24 h were dehydrated, embedded in paraffin, and serially sectioned at 6 μm intervals. The sections were mounted on glass slides and stained with Mayers hematoxylin and eosin. Follicles were counted using the dissector and fractionator principles (22, 23). One-fifth of the sections of each ovary was chosen for analysis. Follicle stages were determined in a manner similar to the classification used by Flaws *et al.* (24). Only follicles with a visible nucleolus in the oocyte were considered, thus avoiding the counting of atretic follicles. Primordial and primary follicles were counted at a magnification of 400×, whereas preantral follicles were counted at a magnification of 200×.

Immunoblot analysis

Frozen rat ovaries collected after *in vivo* treatment with either GDF-9, FSH or saline were thawed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% SDS, 5 mM EGTA, 0.5 mM MgCl2, 0.5 mM MnCl2, and 0.2 mM phenylmethyl-sulfonylfluoride) at 150 μl per ovary and homogenized. The samples were fractionated using SDS-PAGE in 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were then incubated with a rabbit polyclonal antibody to porcine CYP-17 (1:8000 dilution, obtained from Dr. Anita Payne, Stanford University School of Medicine, Stanford, CA), followed by incubation with horseradish peroxidase-conjugated sheep antirabbit IgG and immunofluorescent imaging with the ECL System (Amersham Pharmacia Biotech).

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections from each treatment group as previously described (25) using the Vectastain ABC elite kit from Vector Laboratories, Inc. (Burlingame, CA). Briefly, tissue sections were deparaffinized in xylene and rehydrated in graduated ethanol washes. Endogenous peroxidase activity was blocked with hydrogen peroxide in absolute methanol. Nonspecific binding was blocked with 20% goat serum. Sections were incubated with the rabbit polyclonal antibody to CYP-17 at 1:1000 dilution for 2 h at room temperature. After washing, the sections were incubated for 30 min with second antibody conjugated to horseradish peroxidase, and diamino/benzoate staining was performed. The sections were washed, counterstained lightly with hematoxylin, and dehydrated before mounting with Permount (Fisher Scientific, Fair Lawn, NJ). Negative control sections were treated identically except that nonimmune serum was substituted for the first antibody. Photomicroscopy was performed at 200× magnification using a Nikon optiphot system.

Statistical analysis

Differences in ovarian weight and in the number of follicles between treatment groups were evaluated by ANOVA. Significant differences were assigned at $P < 0.05$.

Results

In vivo treatment with GDF-9 increases ovarian weight in immature rats

Immature rats at 5 days of age were treated with either GDF-9, FSH, or saline for 7 and 10 days. The body weight of the rats did not differ between the treatment groups and was 28 ± 1 g and 37 ± 1 g after 7 and 10 days of treatment, respectively. As shown in Fig. 1, ovarian weight was increased by 32 and 29% above controls in rats treated with GDF-9 after 7 and 10 days of treatment, respectively. Likewise, treatment with FSH induced 45 and 74% increases in ovarian weight above controls after 7 and 10 days of treatment, respectively.

In vivo treatment of immature rats with GDF-9 decreases the number of primordial follicles but increases the number of primary and small preantral follicles

Ovarian sections of animals treated with either GDF-9, FSH, or saline were analyzed. Follicles with an intact oocyte surrounded by a single layer of flattened granulosa cells were scored as primordial (Prd., Fig. 2A, *left panel*). Follicles were scored as primary if they consisted of an intact enlarged oocyte and a single layer of cuboidal granulosa cells (Prim., Fig. 2A, *left panel*). Intermediate follicles with a single granulosa layer that consisted of both flattened and cuboidal cells were considered to be in transition from primordial to primary follicles (Int., Fig. 2A, *left panel*) and also scored as primary in the present study. As described before (26), pre-

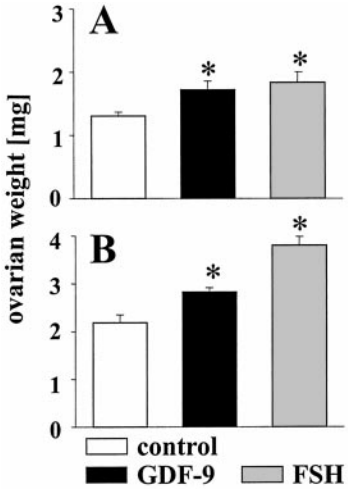

FIG. 1. *In vivo* treatment with GDF-9 and FSH increases ovarian weight in rats. Immature rats at 5 days of age were treated with either GDF-9 (10 μg), FSH (10 IU), or saline twice daily. A, Treatment for 7 days. B, Treatment for 10 days. Both GDF-9 and FSH significantly increased ovarian weight above control after both treatment periods. The data are shown as mean ± SEM for individual ovaries of three rats in each treatment group. Significant differences ($P < 0.05$) as compared with the control group are indicated by *asterisks*.

antral follicles in rats cannot be classified accurately according to the number of granulosa cell layers as used by Pedersen and Peters (27) for mice. Therefore, we divided preantral follicles into two categories. Follicles with more than one but less than four layers of granulosa cells were scored as small preantral follicles (SPA, Fig. 2A, *middle panel*), whereas follicles with four or more granulosa cell layers were scored as large preantral follicles (LPA, Fig. 2A, *right panel*).

After 7 days of treatment with GDF-9, the number of primordial follicles was significantly reduced compared with the control group (Fig. 2B). Furthermore, treatment with GDF-9 increased the number of primary follicles by 23% above control levels. In addition, the number of small preantral follicles was increased by 75% above control levels. However, no effect of GDF-9 on the number of large preantral follicles was observed ($P > 0.05$). In contrast to GDF-9, treatment with FSH had no effect on the number of primordial and primary follicles but increased the number of small and large preantral follicles by 60 and 92%, respectively.

After 10 days of treatment (Fig. 2C), the number of primordial follicles was reduced by 29% in animals treated with GDF-9. Furthermore, GDF-9 treatment significantly enhanced the number of both primary (30%) and small preantral follicles (60%), whereas the number of large preantral follicles was not significantly affected ($P > 0.05$). Again, treatment with FSH predominantly affected the number of large preantral follicles as reflected by an increase of 177%, whereas the number of small preantral follicles was increased only by 36%.

The differential effects between GDF-9 and FSH treatments on follicle progression can also be seen in representative ovarian sections of the three groups analyzed (Fig. 3). While the ovaries in control animals contained multiple primordial and primary follicles in the ovarian lobe region (Fig. 3A), the GDF-9-treated group showed few primordial follicles but many primary and small preantral follicles (Fig. 3B). In contrast, ovaries from the FSH-treated group contained mainly large preantral follicles. These morphological obser-

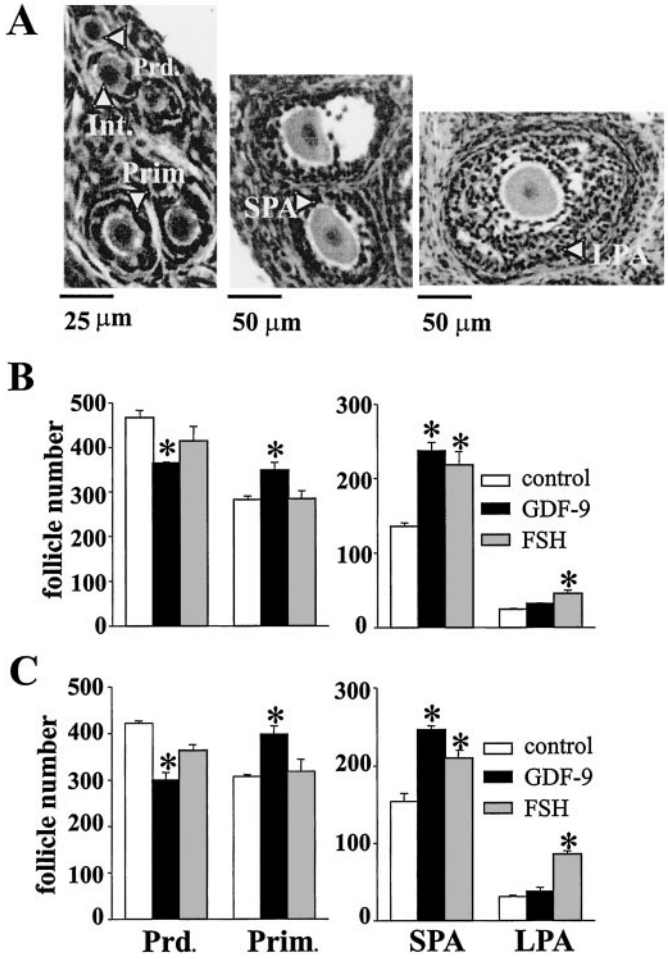

FIG. 2. Effect of treatment with GDF-9 and FSH on follicle progression after 7 days of treatment. A, Morphological analysis of follicles at specific stages of development. Follicle classification: *Left panel*: Prd, primordial follicles with an oocyte surrounded by flattened granulosa cells. Prim, Primary follicles with cuboidal-shaped granulosa cells. Int, Intermediate follicles with some cuboidal and some flattened granulosa cells. Preantral follicles with more than one layer of granulosa cells were divided into small preantral (less than 4 layers of granulosa cells) (SPA, *middle panel*) and large preantral (4 or more layers of granulosa cells) (LPA, *right panel*). B, Immature rats at 5 days of age were treated for 7 days with either GDF-9, FSH, or saline. The number of follicles at different stages were counted in one-fifth of the sections per ovary. As compared with the control group, GDF-9 treatment increased the number of primary follicles and decreased the number of primordial follicles (*left panel*). Furthermore, GDF-9 treatment significantly increased the number of small preantral follicles (*right panel*). In contrast, FSH increased the number of small and large preantral follicles without affecting the number of primordial and primary follicles. The data are shown as mean ± SEM for three rats in each treatment group. C, Immature rats at 5 days of age were treated with either GDF-9, FSH, or saline for 10 days. The total number of follicles at different stages were counted in one-fifth of the sections of each ovary as shown in Fig. 2A. GDF-9 increased the number of primary (Prim., *left panel*) and small preantral follicles (SPA, *right panel*). In contrast, FSH treatment increased the number of both small and large preantral follicles. The data are shown as mean ± SEM for three rats in each treatment group.

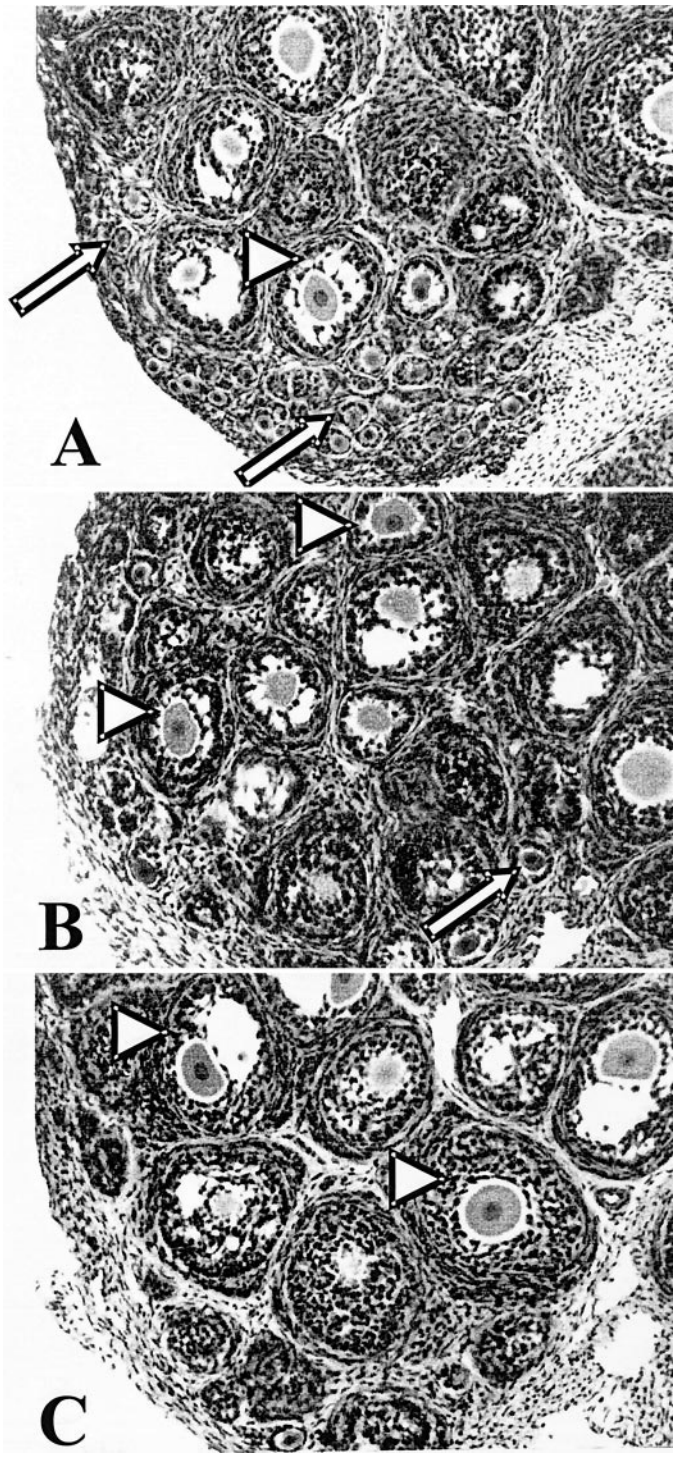

FIG. 3. Ovarian sections from immature rats after treatment with GDF-9 or FSH for 10 days. Representative ovarian sections for each treatment group are shown. Each panel displays a partial view of the largest cross section of the ovary at the same magnification (200×). In all three panels, the lobe region adjacent to the ovarian hilum is shown. A, In ovaries from control rats, a larger portion of this region is filled with primordial and primary follicles (*arrows*). Toward the inner area, some small preantral follicles can be seen (*arrowhead*). B, In ovaries treated with GDF-9, few primordial follicles are found, whereas primary follicles (*arrow*) and small preantral follicles (*arrowheads*) are present in this region. C, Treatment with FSH induced mainly large preantral follicles (*arrowheads*) in the ovary.

vations are consistent with the hypothesis that GDF-9 mainly promotes the development of primordial and primary follicles, whereas FSH preferentially enhances preantral follicle progression.

In vivo treatment of immature rats with GDF-9 for 10 days increases the ovarian CYP17 content and CYP17 staining in theca cells

Ovaries collected from animals treated for 10 days with either GDF-9, FSH, or saline were homogenized and used for immunoblot analysis of ovarian CYP17 content. As shown in Fig. 4, treatment with GDF-9 increased the amount of CYP17 per ovary approximately 3-fold above controls. In contrast, treatment with FSH did not affect CYP17 levels in the ovary.

Increases in the amount of CYP17 were confirmed by staining of ovarian sections with specific CYP17 antibodies after 10 days of treatment (Fig. 5). Theca cells of preantral follicles in ovaries from GDF-9-treated, as well as FSH-treated, animals showed distinctive CYP17 staining (Fig. 5, B and C). In contrast, theca cells in ovarian sections from control (Fig. 5A) did not show a marked staining.

Discussion

The present study showed that GDF-9 treatment *in vivo* enhances ovarian weight, primordial and primary follicle progression, as well as the ovarian content of CYP17, a marker specific for theca cells. Treatment with GDF-9 increased the number of primary and small preantral follicles, concomitant with a decrease in the number of primordial follicles. In addition, the number of large preantral follicles was only minimally affected. In contrast, treatment with FSH markedly increased the number of small preantral and large preantral follicles but had minimal effects on primordial and primary follicle stages.

Our results indicate that primordial follicles are responsive to GDF-9 and that the oocyte factor, when produced in a given follicle, could influence the initiation of primordial

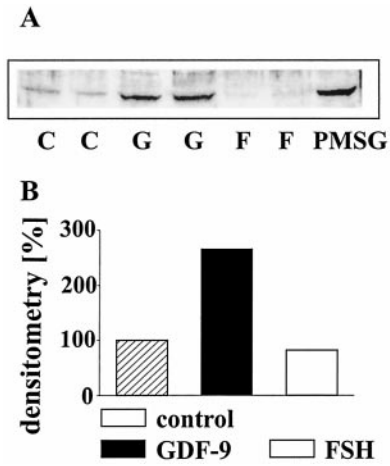

FIG. 4. Treatment with GDF-9, but not FSH, increases immunoreactive CYP17 antigen in the ovary. A, Ovaries of each treatment group were used for immunoblot analysis (C: control; G: GDF-9; F: FSH). Ovaries from PMSG-treated immature rats served as a positive control (PMSG). B, Densitometry analysis of three separate experiments for each treatment group.

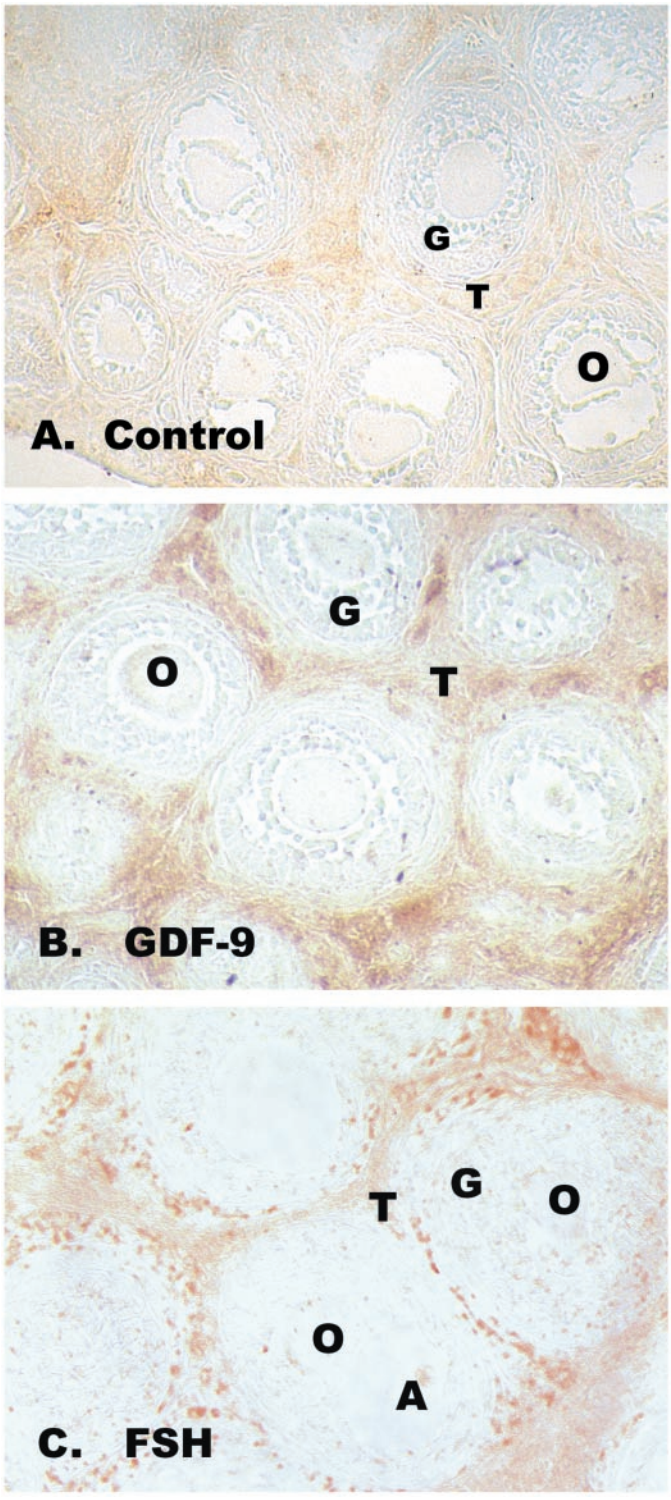

FIG. 5. Immunolocalization of CYP17 to theca cells of GDF-9-treated rats. Ovaries were treated for 10 days with saline (A), GDF-9 (B), or FSH (C). Shown are representative sections of each treatment group displaying multiple preantral follicles at a magnification of 200×.

follicle growth. Because the expression of GDF-9 messenger RNA (mRNA) and protein was confined to primary and larger follicular stages in rats (19, 28), mice (17, 29), and human (30), the present findings suggest that once GDF-9 is produced by the oocyte of a given primordial follicle, this follicle could start to grow. Of interest, in ovine and bovine ovaries, GDF-9 mRNA was found in primordial follicles as well (31). Because follicles can progress to the primary stage in GDF-9 null mice (17), it is possible that GDF-9 is not absolutely required for the transition from primordial to primary follicles. Furthermore, the enhancement of primary follicle progression could lead to an increase in the number of primordial follicles entering the growing pool (32). Therefore, one cannot exclude the possibility that the effect observed here of GDF-9 on primordial follicles is secondary to its stimulation of primary follicle progression to small preantral follicles. The observed stimulatory effect of GDF-9 on the increase in number of small preantral follicles could be due to the enhanced initiation of primordial follicle growth and/or the transition of the primary to the small preantral follicle stage. A predominant role of GDF-9 on the progression of primary follicles to the preantral stage is consistent with the arrest of follicles at the primary stage in GDF-9 null mice.

Based on antibody neutralization experiments, the initiation of primordial follicle growth also has been shown to be regulated by the granulosa cell-derived kit ligand acting on the c-kit receptors present in the oocyte (33). Furthermore, kit ligand, similar to GDF-9, was shown to induce primordial follicle growth in ovarian explants *in vitro* (34). Therefore, early follicle progression could be coordinated through the combined actions of oocyte-derived GDF-9 and granulosa cell-derived kit ligand. This hypothesis is underscored by kit ligand stimulation of oocyte development and the observation that kit ligand is up-regulated in GDF-9-deficient animals (18, 35).

In addition to the involvement of GDF-9 and kit ligand in early follicular development, the differentiation processes occurring during early follicle growth have been shown to be initiated by neurotransmitters contained in ovarian nerves (36, 37). Furthermore, a recent study indicated that androgens promote the initiation of primordial follicle growth in primates (38). However, GDF-9 is the only characterized factor of oocyte origin shown to influence the initiation of follicle growth.

The regulatory role of oocyte factors in the control of antral follicle development has been extensively studied (39–41). A recent study showed that oocytes influence kit ligand expression in preantral follicles (42). The present results show that the oocyte, via secretion of GDF-9, not only affects preantral and antral follicular stages (21), but also influences the initiation of follicular growth at the primordial stage, and the progression into primary and preantral follicles.

In addition to follicular growth, GDF-9 enhances the ovarian CYP17 content and CYP17 staining in theca cells. CYP17 is present in steroidogenic theca interna cells and is found in immature rat ovaries (43). Few theca cells are present during the initiation of growth of primordial follicles (9) and primary follicles contain one layer of theca cells. These cells proliferate and the number of theca cell layers increases with follicular progression, likely in response to intraovarian factors (44). In our study, the amount of CYP17 is increased after GDF-9 treatment. The increase in ovarian CYP17 content could be due to either a higher abundance of preantral fol-

licles and therefore more follicles with more layers of theca cells and/or to a specific increase in CYP17 levels per cell. Consistent with these findings, GDF-9-deficient animals show reduced CYP17 expression (18). To date, the localization of GDF-9 receptors is not known. Therefore, the observed effect of GDF-9 on CYP17 expression could be either mediated through direct action on theca cells or indirectly through granulosa cells. Future studies on GDF-9-responsive promoter elements in the CYP17 gene are of interest.

In contrast to GDF-9, FSH treatment did not affect the number of primary follicles but induced more small and large preantral follicles. This is consistent with earlier studies showing that FSH stimulates preantral follicular growth (25) and that a decrease of circulating FSH levels retards folliculogenesis in immature rats (45). Furthermore, it is known that both ovarian FSH receptors and circulating FSH are present in immature rats (46–48). Therefore, although FSH is not absolutely required for follicular development at this stage as shown by studies using hypophysectomized and hypogonadotrophic animals (49, 50), the rate of follicle development is augmented by FSH.

In conclusion, treatment with GDF-9 was shown to induce primordial follicle growth and enhance the transition from the primordial and primary to the small preantral follicular stage. To date, GDF-9 is the only factor secreted by the oocyte shown to influence these specific stages of follicular development *in vivo*. Current ovarian stimulation protocols for infertility treatment mainly influence antral follicle growth using gonadotropins (51). In poor responders to gonadotropin stimulation (52) and in cases of premature ovarian failure, enhanced ovulation rates are difficult to achieve with current infertility treatment protocols. Oocyte donation is the only option for these patients desiring pregnancy (53). Because ovaries from most women with premature ovarian failure contain primordial follicles (54), treatment with GDF-9 to stimulate both primordial and primary follicle development represents an alternative approach. Although kit ligand also stimulates these follicular stages, it has been used to promote the proliferation of pluripotent progenitor cells in the hematopoietic system in patients undergoing chemotherapy (55). Due to its wide-ranging actions, the potential use of kit ligand for infertility treatment is likely to be complicated. Because the GDF-9 null mice, unlike kit ligand mutants, lack severe defects in tissues other than the ovary, the tissue-specific role of GDF-9 in follicle growth makes this oocyte hormone a potential candidate for future infertility treatments.

Acknowledgments

We thank Caren Spencer for editorial assistance, Lisa Raskin (Department of Comparative Medicine, Stanford University, CA) for the imbedding and sectioning of ovarian tissues, Dr. Anita H. Payne (School of Medicine, Stanford University, CA) for CYP17 antibody and Organon NV (The Netherlands) for recombinant FSH.

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