Effects of Fibroblast Growth Factor 9 (FGF9) on Steroidogenesis and Gene Expression and Control of *FGF9* mRNA in Bovine Granulosa Cells

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Gene expression of fibroblast growth factor-9 (FGF9) is decreased in granulosa cells (GC) of cystic follicles compared with normal dominant follicles in cattle. The objectives of this study were to investigate the effects of FGF9 on GC steroidogenesis, gene expression, and cell proliferation and to determine the hormonal control of GC FGF9 production. GC were collected from small (1–5 mm) and large (8–22 mm) bovine follicles and treated *in vitro* with various hormones in serum-free medium for 24 or 48 h. In small- and large-follicle GC, FGF9 inhibited ($P < 0.05$) IGF-I-, dibutyryl cAMP-, and forskolin-induced progesterone and estradiol production. In contrast, FGF9 increased ($P < 0.05$) GC numbers induced by IGF-I and 10% fetal calf serum. FGF9 inhibited ($P < 0.05$) *FSHR* and *CYP11A1* mRNA abundance in small- and large-follicle GC but had no effect ($P > 0.10$) on *CYP19A1* or *StAR* mRNA. In the presence of a 3β-hydroxysteroid dehydrogenase inhibitor, trilostane, FGF9 also decreased ($P < 0.05$) pregnenolone production. IGF-I inhibited ($P < 0.05$) whereas estradiol and FSH had no effect ($P > 0.10$) on *FGF9* mRNA abundance. TNFα and wingless-type mouse mammary tumor virus integration site family member-3A decreased ($P < 0.05$) whereas T_4 and sonic hedgehog increased ($P < 0.05$) *FGF9* mRNA abundance in control and IGF-I-treated GC. Thus, GC *FGF9* gene expression is hormonally regulated, and FGF9 may act as an autocrine regulator of ovarian function by slowing follicular differentiation via inhibiting IGF-I action, gonadotropin receptors, the cAMP signaling cascade, and steroid synthesis while stimulating GC proliferation in cattle. **(*Endocrinology* 153: 4491–4501, 2012)**

The vertebrate fibroblast growth factor (FGF) family consists of 23 members that are involved in a wide variety of biological processes including embryonic development, cell proliferation, tissue repair, angiogenesis, and cancer metastasis as well as organogenesis of the central nervous system, lungs, and limbs (1–4). Two prototypic members of the FGF family (acidic and basic FGF, now called FGF1 and FGF2, respectively) were isolated approximately 30 yr ago by heparin-affinity chromatography (for review see Ref. 3). FGF share significant homology in their central core amino acid sequences that allows for dual binding of low-affinity heparin sulfate proteoglycan binding sites and high-affinity transmembrane tyrosine kinase receptors (4–8), through which members of this family signal.

Using microarray technology, *FGF9* mRNA abundance was found to be down-regulated in cystic follicles compared with noncystic follicles of cattle (9), indicating that FGF9 may play a role in follicular development and cyst formation. In another microarray study, gene expression of one of the FGF receptors (FGFR) for FGF9, *FGFR2IIIc* (10), was found to be up-regulated by IGF-I in porcine granulosa cells (GC) (11), suggesting that FGF9 may also be involved in porcine follicular growth. In rats, *FGF9* mRNA and protein is localized in granulosa and theca cells, receptors exist for FGF9 including FGFR2 and FGFR3, and FGF9 stimulated progesterone (P4) production (12). However, an effect of FGF9 on GC estradiol (E2) production has not been reported for any species. We hypothesized that FGF9 may also regulate aromatase activity of GC.

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Abbreviations: ANG, Angiogenin; dbcAMP, dibutyryl cAMP; E2, estradiol; FCS, fetal calf serum; FGF, fibroblast growth factor; FGFR, FGF receptor; GC, granulosa cells; hCG, human chorionic gonadotropin; LG, large follicles; P4, progesterone; PGE2, prostaglandin E2; SHH, Sonic hedgehog; SM, small follicles; WNT3A, wingless-type mouse mammary tumor virus integration site family member 3A.

In tissues other than the ovary, FGF9 is widely expressed in embryos and fetuses (13, 14) and has a wide array of functions including male sex determination (15), lung development (13), and glial cell growth (16). FGF9, a 208-amino-acid protein, was first purified in 1993 (17) from supernatants of cultured human glioma cells and is highly conserved with greater than 93% homology among mouse, rat, and human, suggesting a role of vital importance (17).

Because GC produce FGF2 (18–20) and FGF9 (9, 12) and respond to both ligands (12, 21–24), both FGF2 and FGF9 may be acting as autocrine regulators of GC function. However, little information exists regarding hormonal regulation of *FGF9* gene expression in GC, and no studies have investigated the effects of FGF9 on ovarian function in mono-ovular species such as humans and cattle. We also hypothesized that GC *FGF9* gene expression is controlled by reproductive hormones such as E2, FSH, LH, and IGF-I. Thus, our objectives were to determine the effects of FGF9 on steroidogenesis, proliferation, and gene expression of bovine GC as well as to determine whether granulosa *FGF9* gene expression is hormonally regulated.

Materials and Methods

Reagents and hormones

The reagents used in cell culture were Ham's F-12 (F12), DMEM, gentamicin, sodium bicarbonate, trypan blue, deoxyribonuclease, collagenase, and fetal calf serum (FCS) (Sigma-Aldrich Chemical Co., St. Louis, MO). The hormones used in cell culture were purified ovine FSH (FSH activity, 15× NIH-FSH-S1 U/mg) and ovine LH (LH activity, 2.3× NIH-LH-S1 U/mg); recombinant human IGF-I, FGF9, angiogenin (ANG), wingless-type mouse mammary tumor virus integration site family member 3A (WNT3A), and TNFα and recombinant mouse Sonic hedgehog (SHH) (amino terminus peptide; R&D Systems, Minneapolis, MN; all carrier-free); testosterone (Steraloids, Wilton, NH); and E2, cortisol, prostaglandin E2 (PGE2), forskolin, T_4, trilostane, and dibutyryl cAMP (dbcAMP) (Sigma-Aldrich).

Cell culture

Ovaries from nonpregnant beef heifers were collected from a local abattoir, follicular fluid was aspirated from small follicles (SM) (1–5 mm) and large follicles (LG) (8–22 mm) to isolate GC as previously described (25, 26). GC were resuspended in serum-free medium (1:1 DMEM/F12 containing 2.0 mM glutamine, 0.12 mM gentamicin, 38.5 mM sodium bicarbonate) containing collagenase and deoxyribonuclease (Sigma-Aldrich) at 1.25 and 0.5 mg/ml, respectively, to prevent clumping. Viability of SMGC and LGGC was determined by trypan blue exclusion method and averaged 75.7 and 50.3%, respectively, and is within the range of viabilities previously reported for these cell types collected from abattoir tissues (24–28). Approximately 2.0×10^5 viable cells were plated on 24-well Falcon multiwell plates (no. 3047; Becton Dickinson, Lincoln Park, NJ) in 1 ml medium containing 10% FCS and cultured in an environment of 5% CO_2 and 95% air at 38.5 C for the first 48 h with a medium change at 24 h. Cells were then washed twice with 0.5 ml serum-free medium followed by addition of different hormonal treatments applied in serum-free medium for 24 or 48 h depending on the experiment.

RNA extraction and quantitative RT-PCR

At the end of the treatment period, medium was either aspirated or collected from each well depending on the experiment, and cells from two replicate wells were lysed in 0.5 ml TRI reagent solution (Life Technologies, Inc., Grand Island, NY) as previously described (25, 26). RNA samples were solubilized in diethylpyrocarbonate-treated water (Life Technologies), quantitated at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), and stored at −80 C.

Cholesterol side-chain cleavage enzyme (*CYP11A1*), FSH receptor (*FSHR*), steroidogenic acute regulatory protein (*StAR*), aromatase (*CYP19A1*), and *FGF9* primers and probes for quantitative RT-PCR were designed using Primer Express software (Foster City, CA) as previously reported (9, 25, 26). The bovine *CYP11A1* and *CYP19A1* primer and probe sequences and information are described by Lagaly *et al.* (25). The information and sequences for bovine *FGF9*, *FSHR*, and *StAR* primers and probes are described by Grado-Ahuir *et al.* (9), Spicer and Aad (26), and Spicer *et al.* (28), respectively. A "highly similar sequences" BLAST query search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted for each primer and probe to ensure specificity of the designed primers and probes. This was also done to assure that they were not designed from any homologous regions coding for other genes. Relative quantification of target gene mRNA were expressed using the comparative threshold cycle method as previously described (26, 27). Furthermore, RT-PCR products were run on agarose gels to verify the length and size of the expected target genes.

RIA, ELISA, and cell counting

P4 and E2 RIA were conducted as previously described (25, 26). Intraassay coefficients of variation averaged 6.3 ± 1.0% for the P4 RIA and 8.3 ± 1.5% for the E2 RIA.

A pregnenolone ELISA (ALPCO Diagnostics, Salem, NH) was used to quantify concentrations of pregnenolone in culture medium. Intraassay coefficient of variation averaged 6.6 ± 1.8%.

To determine cell numbers, culture medium was aspirated from wells, and cells were washed, trypsinized, and counted on a Coulter counter (Z2 Coulter particle count and size analyzer; Beckman Coulter, Hialeah, FL) as previously described (25, 26).

Experimental design

Experiment 1 was designed to test the effect of FGF9, FSH, and IGF-I on steroidogenesis of bovine SMGC. Cells were cultured for 48 h in 10% FCS medium and then washed twice with 0.5 ml serum-free medium, and six treatments were applied for an additional 48 h as follows (all treatments included 30 ng/ml FSH and 500 ng/ml testosterone as an estrogen precursor): 0, 3, 10, or 30 ng/ml FGF9 with or without 30 ng/ml IGF-I. Doses of FSH and IGF-I were selected based on previous studies (25, 27). FSH was added to all treatments because IGF-I alone has little or no effect on steroid production (25, 27). Medium was changed

at 24 h. After 48 h treatment, medium was collected for steroid RIA and cells were counted.

Experiment 2 was designed to test the effect of FGF9 on SMGC cell proliferation. Cells were cultured for 48 h in 10% FCS medium and then treated for an additional 24 or 48 h with either 0 or 30 ng/ml FGF9 in the presence of 10% FCS. Separate cultures were terminated at 0, 24, and 48 h, and cells were counted. The dose of FGF9 was selected based on results from experiment 1.

Experiment 3 was designed to test the effect of FGF9 on db-cAMP-, forskolin-, and IGF-I-induced steroid production in bovine SMGC and LGGC. Cells were cultured as described in experiment 1 (except LGGC were allowed 96 h of growth in 10% FCS medium rather than 48 h due to low confluencies) with 12 treatments applied for 48 h with a medium change at 24 h as follows (all treatments included 500 ng/ml testosterone and 30 ng/ml FSH): control, FGF9 (30 ng/ml), dbcAMP (1 mg/ml), db-cAMP plus FGF9, forskolin (4.1 μg/ml), and forskolin plus FGF9 (all with or without 30 ng/ml IGF-I). Doses of dbcAMP and forskolin were based on previous studies (24, 29). FSH was added to all treatments because IGF-I alone has little or no effect on steroid production (25, 27). After the second 24-h treatment period, medium was collected for steroid RIA and cells were counted.

Experiment 4 was designed to test the effect of FGF9 on *CYP11A1*, *CYP19A1*, *StAR*, and *FSHR* mRNA abundance in SMGC and LGGC. Cells were cultured as previously described for experiment 1 except that the following treatments were only applied for 24 h (all treatments included 100 ng/ml IGF-I and 500 ng/ml testosterone): FSH (30 ng/ml) and FSH plus FGF9 (10 ng/ml). After 24 h treatment, cells were lysed for RNA extraction as described earlier. FGF9 at 10 ng/ml was selected because it inhibited E2 and P4 production to control levels without IGF-I in experiment 3, and IGF-I at 100 ng/ml was selected because our previous studies show that 100 ng/ml IGF-I stimulates steroidogenic enzyme gene expression within 24 h (25).

Experiment 5 was designed to further elucidate the mechanisms by which FGF9 inhibited progestin production by SMGC. The effect of FGF9 (30 ng/ml) on production of pregnenolone (the immediate precursor of progesterone) in the presence of trilostane (150 μM), an inhibitor of 3β-hydroxysteroid dehydrogenase activity, was evaluated. Cells were cultured as previously described for experiment 1 except that SMGC were cultured in phenol red-free medium (so as not to interfere in the ELISA). As in experiment 3, SMGC were treated with 30 ng/ml IGF-I and FSH. The dose of trilostane was selected based on a previous study with swine GC (30). After the second 24-h treatment period, medium was collected for pregnenolone ELISA and cells were counted.

Experiment 6 was designed to test effects of LH and T_4 on *FGF9* mRNA abundance in SMGC treated with FSH and IGF-I. T_4 was tested because of its effects on steroidogenesis (31) and the ability of thyroid hormones to induce *FGFR* mRNA in chondrocytes (32). Cells were cultured as described in experiment 1 with treatments applied for 48 h with a medium change at 24 h as follows: LH (0 or 30 ng/ml) and/or T_4 (0 or 100 ng/ml) in the presence of IGF-I (30 ng/ml) plus FSH (30 ng/ml). Doses of LH and T_4 were based on previous studies (27, 31). After the second 24-h treatment period, cells were lysed for RNA extraction as described earlier.

Experiment 7 was designed to test effects of IGF-I, cortisol, PGE2, SHH, WNT3A, and ANG on *FGF9* mRNA abundance in SMGC. Cortisol and PGE2 were tested because of their reported stimulatory effect on *FGF9* mRNA in nonovarian human tissues (33, 34), and SHH, WNT3A and ANG were tested because of their recent implication in cystic follicle development (9) and ovarian IGF-I stimulation (11). Cells were cultured as described above with treatments applied for 24 h as follows (all treatments include 30 ng/ml IGF-I and no FSH): control, cortisol (300 ng/ml), PGE2 (300 ng/ml), SHH (500 ng/ml), WNT3A (300 ng/ml), and ANG (300 ng/ml). A second experiment evaluated singular treatment of IGF-I (30 ng/ml), FSH (30 ng/ml), or E2 (300 ng/ml). Doses of cortisol, PGE2, SHH, WNT3A, ANG, and E2 were selected based on previous studies showing that these doses significantly alter GC function (35–38). After 24 h treatment, cells were lysed for RNA extraction as described earlier.

Experiment 8 was designed to test effects of IGF-I and TNFα on *FGF9* mRNA abundance in SMGC and LGGC. TNFα was evaluated because of its involvement in *FGF9* mRNA expression in fetal rat lung explants (39). Cells were cultured as described above with treatments applied for 24 h as follows: control, TNFα (30 ng/ml), IGF-I (30 ng/ml), and IGF-I plus TNFα. Doses of IGF-I and TNFα were selected based on previous studies showing that these doses significantly alter steroidogenesis (27, 40, 41). After 24 h treatment, cells were lysed for RNA extraction as described earlier.

Statistical analysis

For each experiment, three different pools were used as experimental replicates, and each treatment was replicated two or three times in each experiment. Each pool of LGGC was obtained from five to seven follicles. Each pool of SMGC was generated from a total volume of 6–8 ml follicular fluid (from 20–30 ovaries) per pool within each experimental replicate. Steroid production was expressed as nanograms or picograms per 10^5 cells per 24 h, and cell numbers determined at the end of the experiment were used for this calculation. For RNA experiments, medium was applied to four wells, and duplicate samples for each pool of cells were derived by combining RNA from two wells. Treatment effects on dependent variables (*e.g.* steroid production and *CYP19A1* mRNA abundance) were determined using ANOVA and the general linear models procedure of SAS for Windows (version 9.2; SAS Institute Inc., Cary, NC). Data from experiment 1 were analyzed as a 2×4 factorial ANOVA. Data from experiments 2, 4, and 7 were analyzed as a one-way ANOVA. Data from experiment 3 were analyzed as a $2 \times 2 \times 3$ factorial ANOVA. Data from experiments 5, 6, and 8 were analyzed as a 2×2 factorial ANOVA. Mean differences were determined by Fisher's protected least significant differences test (42) only if significant main effects in the ANOVA were detected. Data are presented as least square means ± SEM.

Results

Experiment 1: effect of FGF9 dose and IGF-I on SMGC steroidogenesis

In SMGC, FGF9 decreased ($P < 0.05$) IGF-I plus FSH-induced E2 production in a dose-dependent manner (Fig. 1A) such that 10 and 30 ng/ml completely inhibited the IGF-I-induced increase in E2 production with an esti-

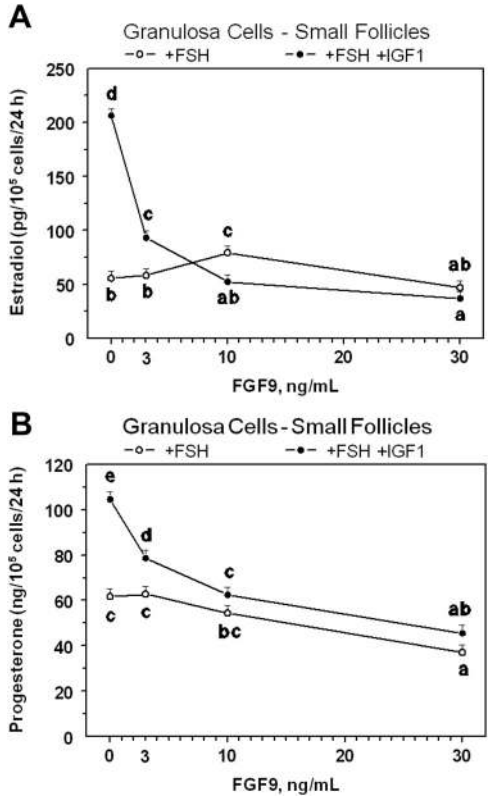

FIG. 1. Dose response of SMGC to FGF9 (experiment 1). A, Effect of FGF9 (0, 3, 10, or 30 ng/ml) on IGF-I-induced (0 or 30 ng/ml) E2 production by SMGC; B, effect of FGF9 (0, 3, 10, or 30 ng/ml) on IGF-I-induced (0 or 30 ng/ml) P4 production by SMGC. Within a *panel*, means without a common *letter* differ ($P < 0.05$). All cells were concomitantly treated with 30 ng/ml FSH.

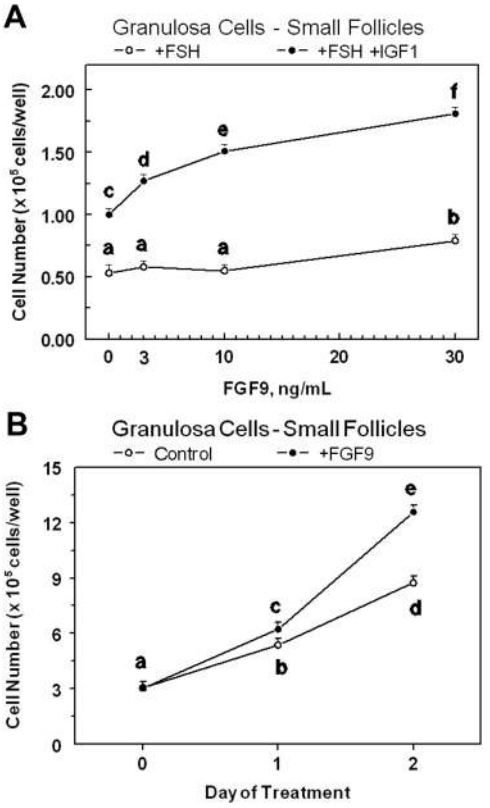

FIG. 2. Effect of FGF9 on granulosa cell proliferation. A, Stimulatory effect of FGF9 (0, 3, 10, or 30 ng/ml) on IGF-I-induced cell (0 or 30 ng/ml) numbers in SMGC (experiment 1). All cells were concomitantly treated with 30 ng/ml FSH. Cells were treated for 2 d with the indicated hormones. B, Stimulatory effect of FGF9 (30 ng/ml) on 10% FCS-stimulated proliferation of SMGC (experiment 2). Cells were treated for either 1 or 2 d with FGF9. Within a *panel*, means without a common *letter* differ ($P < 0.05$).

mated dose of FGF9 necessary to inhibit 50% of the maximum (*i.e.* IC_{50}, calculated from inhibition curves that were linearized using semi-log plots) of 2.2 ng/ml. In the absence of IGF-I, FGF9 (3 and 30 ng/ml) had no effect ($P > 0.10$) on E2 production but increased ($P < 0.05$) E2 production by 42% at 10 ng/ml (Fig. 1A). FGF9 decreased ($P < 0.05$) IGF-I plus FSH-induced P4 production in a dose-dependent manner (Fig. 1B) such that 10 and 30 ng/ml completely inhibited the IGF-I-induced increase in P4 production with an estimated IC_{50} of 5 ng/ml. In the absence of IGF-I, FGF9 at 30 ng/ml decreased ($P < 0.05$) P4 production but at 3 and 10 ng/ml had no effect ($P > 0.10$; Fig. 1B).

Only 30 ng/ml FGF9 stimulated ($P < 0.05$) SMGC numbers by 67% in the absence of IGF-I (Fig. 2A) and in a dose-dependent fashion in the presence of IGF-I with a maximum stimulation of 1.81-fold and an estimated dose of FGF9 necessary to stimulate 50% of the maximum (*i.e.* ED_{50}) of 6 ng/ml.

Experiment 2: effect of FGF9 on SMGC proliferation induced by 10% FCS

FGF9 (30 ng/ml) further enhanced ($P < 0.05$) SMGC proliferation stimulated by 10% FCS (Fig. 2B). Granulosa cells grew 2.9- and 4.1-fold between d 0 and 2 in control and FGF9-treated cultures, respectively ($P < 0.05$; Fig. 2B). Cell numbers in FGF9-treated cells were greater ($P < 0.05$) than controls on d 1 and 2 (Fig. 2B).

Experiment 3: effect of FGF9 on dbcAMP-, forskolin-, and IGF-I-induced steroid production in SMGC and LGGC

Both dbcAMP (1 mg/ml) and forskolin (4.1 μg/ml) alone increased ($P < 0.05$) E2 production above basal levels in SMGC (Fig. 3A). FGF9 alone had no effect ($P > 0.10$) on basal or forskolin- or dbcAMP-induced E2 production. However, FGF9 inhibited ($P < 0.05$) IGF-I-induced E2 production in IGF-I alone (Fig. 3A) as well as in dbcAMP plus IGF-I and forskolin plus IGF-I-treated SMGC by 91, 45, and 64%, respectively.

Treatments of dbcAMP and forskolin alone increased ($P < 0.05$) SMGC P4 production, and FGF9 had no effect ($P > 0.10$) on basal or dbcAMP- and forskolin-induced P4 production (Fig. 3B). However, IGF-I stimulated ($P < 0.05$) control and dbcAMP- and forskolin-induced P4

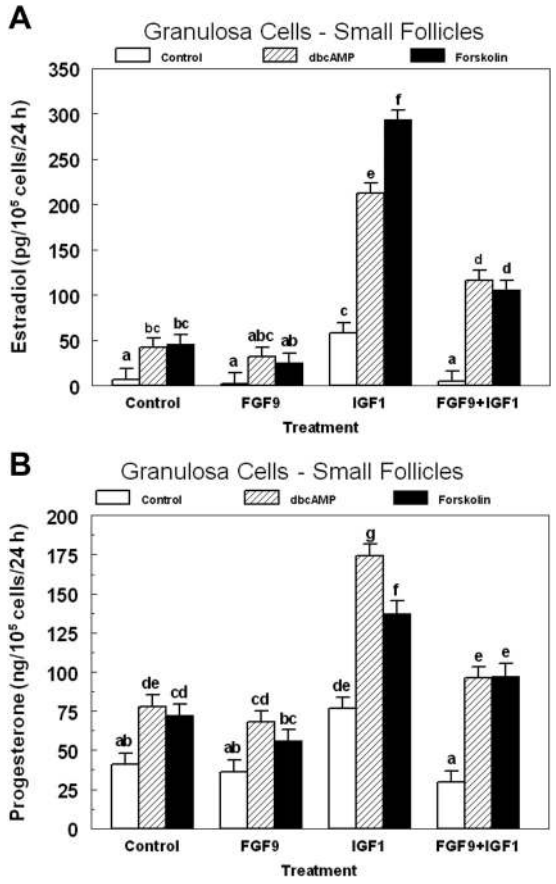

FIG. 3. Effects of FGF9 and IGF-I on steroid production of SMGC induced by dbcAMP and forskolin (experiment 3). A, Effect of FGF9 (0 or 30 ng/ml) on basal and IGF-I-induced (0 or 30 ng/ml) E2 production by SMGC treated concomitantly with either dbcAMP or forskolin; B, effect of FGF9 (0 or 30 ng/ml) on basal and IGF-I-induced (0 or 30 ng/ml) P4 production by SMGC treated concomitantly with either dbcAMP or forskolin. Within a *panel*, means without a common *letter* differ ($P < 0.05$). All cells were concomitantly treated with 30 ng/ml FSH.

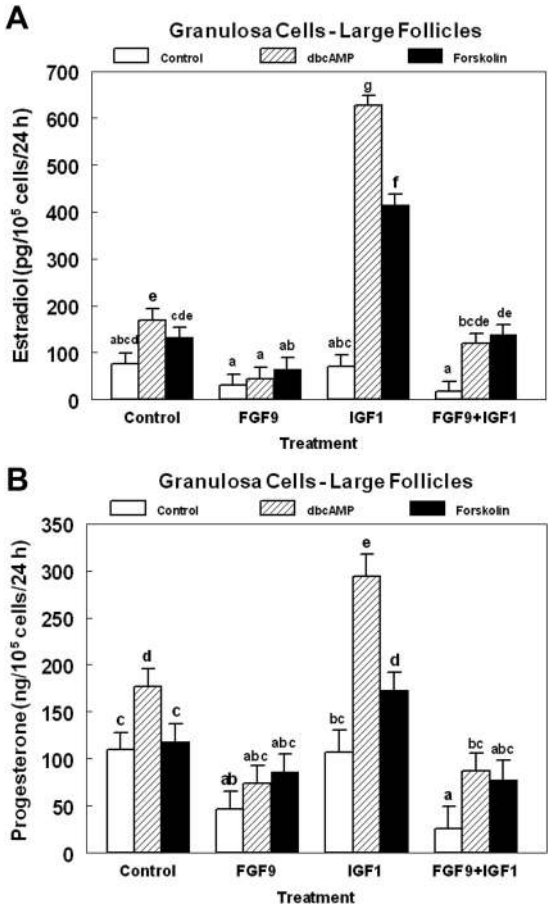

FIG. 4. Effects of FGF9 and IGF-I on steroid production of LGGC induced by dbcAMP and forskolin (experiment 3). A, Effect of FGF9 (0 or 30 ng/ml) on basal and IGF-I-induced (0 or 30 ng/ml) E2 production by LGGC treated concomitantly with either dbcAMP or forskolin; B, effect of FGF9 (0 or 30 ng/ml) on basal and IGF-I-induced (0 or 30 ng/ml) P4 production by LGGC treated concomitantly with either dbcAMP or forskolin. Within a *panel*, means without a common *letter* differ ($P < 0.05$). All cells were concomitantly treated with 30 ng/ml FSH.

production, and FGF9 attenuated this stimulation by 61, 55, and 29%, respectively.

Treatment of dbcAMP alone increased ($P < 0.05$) LGGC E2 production above basal levels, whereas forskolin and IGF-I were without effect (Fig. 4A). FGF9 alone did not affect ($P > 0.10$) basal E2 production but inhibited ($P < 0.05$) dbcAMP- and forskolin-induced E2 production. IGF-I stimulated ($P < 0.05$) dbcAMP- and forskolin-induced E2 production (Fig. 4A), and FGF9 attenuated ($P < 0.05$) this stimulation by 81 and 67%, respectively.

Treatment of dbcAMP alone in LGGC increased ($P < 0.05$) P4 production above controls (Fig. 4B), whereas treatment with forskolin alone and IGF-I alone had no effect ($P > 0.10$). FGF9 alone inhibited ($P < 0.05$) basal and dbcAMP-induced P4 production by LGGC, whereas IGF-I stimulated ($P < 0.05$) both dbcAMP- and forskolin-induced P4 production, and FGF9 inhibited this stimulation 55 and 76%, respectively (Fig. 4B). FGF9 also inhibited IGF-I-alone-induced P4 production by 70%.

Experiment 4: effect of FGF9 treatment on *CYP19A1*, *CYP11A1*, *StAR*, and *FSHR* mRNA in SMGC and LGGC

FGF9 (10 ng/ml) had no significant effect on *CYP19A1* mRNA (Fig. 5A) or *StAR* mRNA (Fig. 5C) abundance, decreased ($P < 0.05$) *CYP11A1* mRNA abundance by 68% (Fig. 5B), and decreased ($P < 0.05$) *FSHR* mRNA abundance by 24% in SMGC cotreated with FSH and IGF-I (Fig. 5D). In LGGC, FGF9 decreased ($P < 0.05$) *CYP11A1* mRNA abundance by 45% (Fig. 5B), had no significant effect on *CYP19A1* mRNA (Fig. 5A) or *StAR* mRNA (Fig. 5C) abundance, and decreased ($P < 0.05$) *FSHR* mRNA abundance by 32% (Fig. 5D).

Experiment 5: effect of FGF9 on pregnenolone production by SMGC

In SMGC stimulated with 30 ng/ml FSH and IGF-I, pregnenolone production increased dramatically ($P <$

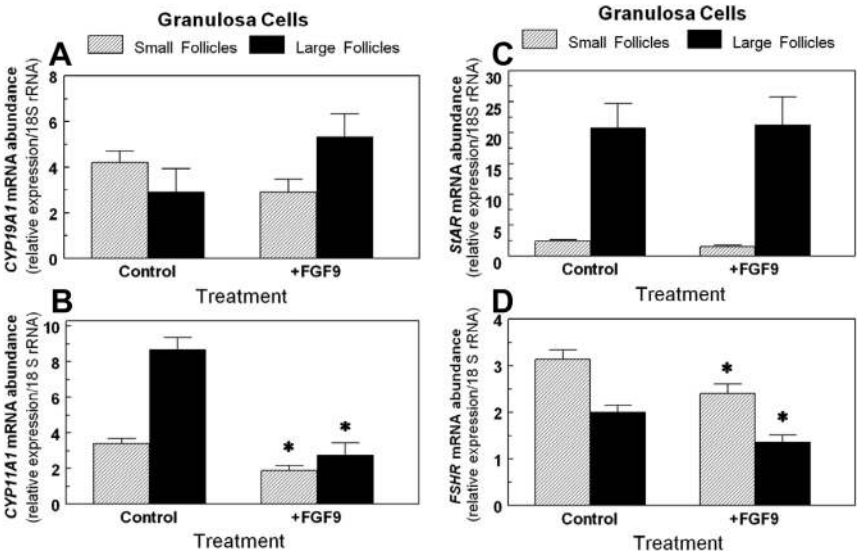

FIG. 5. Effect of FGF9 (10 ng/ml) on steroidogenic enzyme, *FSHR* and *StAR* gene expression in GC of small and large follicles (experiment 4). A, Lack of effect of FGF9 on *CYP19A1* (aromatase) mRNA abundance; B, inhibitory effect of FGF9 on *CYP11A1* (side-chain cleavage enzyme) mRNA abundance; C, lack of effect of FGF9 on *StAR* mRNA abundance in SMGC and LGGC; D, inhibitory effect of FGF9 on *FSHR* mRNA abundance in SMGC and LGGC. *, Within a *panel*, mean differs from its respective control ($P < 0.05$). GC from small and large follicles were cultured for 48 h in the presence of 10% FCS and then treated with FGF9 for 24 h. All cells were concomitantly treated with 100 ng/ml IGF-I and 30 ng/ml FSH. Values are means of three separate experiments (± SEM) and normalized to constitutively expressed *18S* rRNA.

0.001) in the presence of trilostane from 80.7 ± 6.7 pg/10^5 cells·24 h to 23.1 ± 1.2 ng/10^5 cells·24 h. Cotreatment with 30 ng/ml FGF9 reduced ($P < 0.001$) pregnenolone production by 55% (to 10.4 ± 1.2 ng/10^5 cells·24 h) in the presence of trilostane and by 50% (to 40.6 ± 6.7 pg/10^5 cells·24 h) in the absence of trilostane.

Experiment 6: effect of LH and T_4 on *FGF9* mRNA in SMGC

In the absence and presence of LH, T_4 increased ($P < 0.05$) *FGF9* mRNA abundance by 27 and 18%, respectively, compared with controls in SMGC (Fig. 6A). LH had no effect ($P > 0.10$) on *FGF9* mRNA abundance (Fig. 6A).

Experiment 7: effect of cortisol, PGE2, SHH, WNT3A, ANG, IGF-I, FSH, and E2 on *FGF9* mRNA in SMGC

Treatments of cortisol, PGE2, and ANG had no effect ($P > 0.10$) on *FGF9* mRNA abundance in SMGC (Fig. 6B). In contrast, 500 ng/ml SHH increased ($P < 0.05$) *FGF9* mRNA abundance, whereas 300 ng/ml WNT3A decreased ($P < 0.05$) *FGF9* mRNA abundance in SMGC (Fig. 6B). In another experiment, IGF-I (30 ng/ml) inhibited ($P < 0.05$; by 45%) but FSH (30 ng/ml) and E2 (300 ng/ml) did not significantly affect *FGF9* mRNA (data not shown).

Experiment 8: effect of IGF-I and TNFα on *FGF9* mRNA in SMGC and LGGC

Combined SMGC and LGGC analysis revealed that treatments of IGF-I (30 ng/ml) and/or TNFα (30 ng/ml) had no effect ($P > 0.10$) on *FGF9* mRNA abundance in SMGC (Fig. 7). In contrast, IGF-I, TNFα, and IGF-I plus TNFα decreased ($P < 0.05$) *FGF9* mRNA abundance in LGGC by 70, 44, and 92%, respectively (Fig. 7). When SMGC data were analyzed separately, the IGF-I effect was significant, decreasing *FGF9* mRNA by 39% ($P < 0.05$).

Discussion

The current study is the first to demonstrate FGF9 effects on steroidogenesis, cell proliferation, and gene expression in ovarian GC of a mono-ovular species. The presence of *FGF9* mRNA was first demonstrated in bovine ovaries by Grado-Ahuir and co-workers (9). In rat ovaries, *FGF9* has been localized to thecal and stromal cells as well as in the basement membrane of follicles (12). Other members of the FGF family have also been found in ovarian cells. FGF2 protein has been localized in granulosa and theca cells from cattle (43), rats (44), and chickens (45). *FGF7*, also known as keratinocyte growth factor (*KGF*), mRNA has been detected in bovine theca cells (46). *FGF8*, *FGF10*, and *FGF17* mRNA are expressed in both granulosa and theca cells of cattle (47–49), whereas *FGF18* mRNA is expressed exclusively in bovine theca cells (50). *FGF14* mRNA has been detected in human oocytes (51). FGF3, -4, -5, -6, -11, -12, -15, -16, -19 -20, -21, and -23 have yet to be identified in oocytes, GC, or theca cells. A commonality of these FGF mentioned here is that they are all capable of binding to multiple FGFR. For example, FGF9 binds to FGFR1IIIc, FGFR2IIIc, FGFR3IIIb, FGFR3IIIc, and FGFR4 (10).

Previously, effects of FGF9 on ovarian cell steroidogenesis have been minimally studied. Treatment of cultured rat GC with FGF9 increases P4 production in the absence and presence of FSH (12). FGF9 also stimulates mouse Leydig cell testosterone production in a dose- and time-dependent manner in the absence of human chorionic gonadotropin (hCG), but FGF9 had no effect in the presence of hCG (52). Our results show the opposite effect

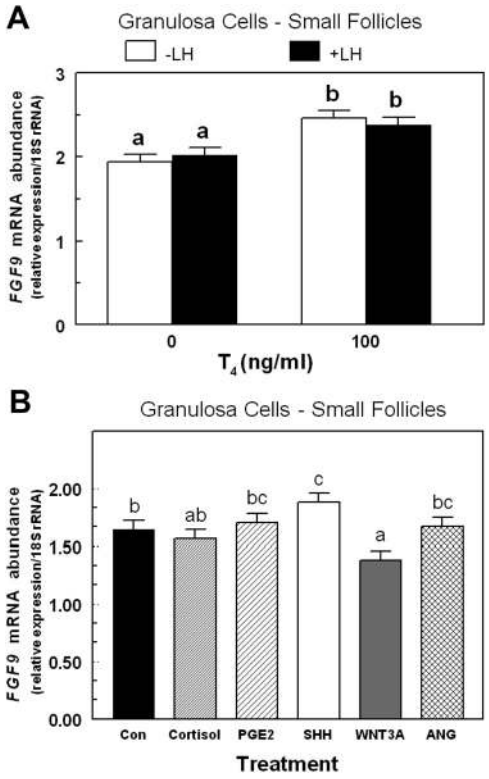

FIG. 6. Effects of various hormones on *FGF9* gene expression in GC of small follicles. A, Effect of 2 d treatment of LH (30 ng/ml) and/or T_4 (100 ng/ml) on *FGF9* mRNA abundance in SMGC (experiment 6). Cells were concomitantly treated with 30 ng/ml IGF-I and 30 ng/ml FSH. B, Effect of 24 h treatment of cortisol (300 ng/ml), PGE2 (300 ng/ml), SHH (500 ng/ml), WNT3A (300 ng/ml), and ANG (300 ng/ml) on *FGF9* mRNA abundance in IGF-I-treated [control (Con)] SMGC (experiment 7). Values are means of three separate experiments (± SEM) and normalized to constitutively expressed *18S* rRNA. Within a *panel*, means without a common *letter* differ ($P < 0.05$).

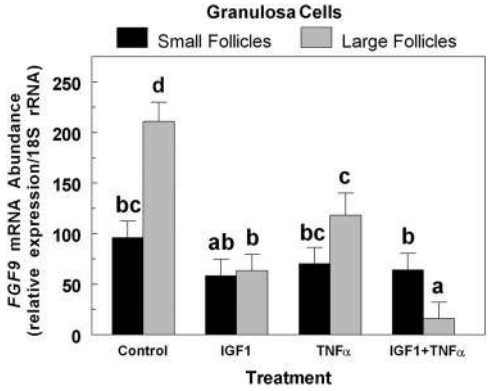

FIG. 7. Effects of TNFα and IGF-I on *FGF9* gene expression in GC of small and large follicles (experiment 8). Granulosa cells from small and large follicles were cultured for 48 h in the presence of 10% FCS and then treated with TNFα (0 or 30 ng/ml) or IGF-I (0 or 30 ng/ml) for 24 h. Cells were concomitantly treated with 30 ng/ml FSH. Values are means of three separate experiments (± SEM) and normalized to constitutively expressed *18S* rRNA. Within a *panel*, means without a common *letter* differ ($P < 0.05$).

of FGF9 on bovine GC steroidogenesis, decreasing both P4 and E2 production stimulated by IGF-I plus FSH, IGF-I plus FSH and dbcAMP, and IGF-I plus FSH and forskolin, and suggest that a species difference in FGF9 function may exist such that FGF9 is stimulatory in polyovular animals but inhibitory in mono-ovular animals. Interestingly, FGF8 strongly suppresses FSH-induced E2 production in rat GC (53), and FGF18 strongly suppresses FSH-induced E2 production in bovine GC (50). Similar inhibitory effects have been noted for FGF2 in rats (54), pigs (55), and cattle (24, 56). Because multiple receptors for FGF exist, a specific cell type and species response to a specific FGF is likely determined by the cadre of FGFR that are present in a particular cell type. Moreover, the IC_{50} for FGF9 (*i.e.* 2–5 ng/ml) reducing steroidogenesis suggests that FGF9 effects are likely mediated by high-affinity receptors because these values are in line with the dissociation constant (K_d) values obtained for FGF9 and basic FGF binding to their respective receptors (57, 58).

There are a total of eight FGF receptors, which includes their respective splice variants (10). *FGFR3IIIb* and *FGFR3IIIc* mRNA are present in bovine GC (46, 48, 59), whereas *FGFR4* mRNA has been localized in bovine theca cells (48). *FGFR1IIIc* and *FGFR2IIIc* mRNA are expressed in bovine corpora lutea (60) and in rat GC (12). Interestingly, *FGFR3IIIc* mRNA is expressed in both granulosa and theca cells of cattle (48), whereas mice do not express *FGFR3IIIc* in either cell type and express *FGFR4* mRNA only in GC (61). Thus, the presence or absence of a certain FGFR could dramatically impact the effect of a particular FGF on a given cell or tissue, and species differences in a specific FGF response may be due to the cadre of specific FGFR present on that cell type. Additional work will be required to verify this suggestion.

Consistent with a suppression in hormone-stimulated P4 production, we also found an inhibitory effect of FGF9 on *CYP11A1* and *FSHR* mRNA abundance in both SMGC and LGGC in cattle, further explaining a mechanism by which GC steroidogenesis may be inhibited by FGF9. A concomitant increase in GC steroidogenesis and *CYP11A1* mRNA expression was induced by FGF9 in rats (12), supporting the parallelism between steroid production and steroidogenic enzyme gene expression. A recent study in cattle revealed inhibitory effects of FGF18 on *StAR*, *CYP19A1*, *CYP11A1*, and *FSHR* mRNA expression in GC (50). FSH triggers GC proliferation, prevents atresia, induces synthesis of LH receptors, and increases *CYP11A1* and *CYP19A1* mRNA abundance in cattle (62). If FGF9 inhibits FSH effects on GC by down-regulating both *FSHR* and *CYP11A1*, it could dramatically alter production of P4. Additional evidence for an inhibitory effect of FGF9 on *CYP11A1* was obtained from the

present studies showing that FGF9 dramatically reduced production of pregnenolone (the immediate product of CYP11A1) in the presence of trilostane, an inhibitor of 3β-hydroxysteroid dehydrogenase. Collectively, these results indicate that FGF9 exerts inhibitory effects on the biosynthesis of pregnenolone as well as its product P4.

Why a decrease in E2 production in GC treated with FGF9 was not accompanied with a decrease in *CYP19A1* mRNA abundance in the present study remains to be determined. One possible explanation for this may be due to a single nucleotide deletion in the cAMP-response element-like sequence in the bovine *CYP19A1* gene that is necessary for induction of the reporter by cAMP in humans (63) and rats (64). In bovine GC with bovine *CYP19A1* ovary reporter DNA constructs, no increase of reporter gene activity in response to forskolin was noted over the empty vector (65). Given that FSH and LH stimulate GC production of cAMP (66, 67), an abundance of cAMP could stimulate E2 production within the smooth endoplasmic reticulum (68) but would not necessarily stimulate *CYP19A1* mRNA expression within the nucleus. Another explanation for lack of effect of FGF9 on *CYP19A1* mRNA expression despite the inhibition of E2 would be that FGF9 may be acting on aromatase protein in a posttranslational manner. In serum-starved human monocyte leukemia cells, IGF-I stabilizes aromatase protein via a posttranscriptional mechanism increasing aromatase by inhibiting autophagy, which is a degradation pathway for the disposal of cellular components (69). If FGF9 blocks the IGF-I-induced inhibition of aromatase autophagy, increased turnover of aromatase would occur and lead to less E2 produced. Additional research will be required to elucidate FGF9 effects on aromatase in the bovine. However, inhibition of E2 production (aromatase activity) by SMGC without a decrease in *CYP19A1* mRNA is not without precedence (70), and concentrations of E2 in follicular fluid of first-wave dominant follicles in cattle were not correlated with *CYP19A1* mRNA abundance (71). If FGF9 is inhibiting E2 production without having an effect on *CYP19A1* mRNA, it might act by destabilizing the aromatase protein and shortening its half-life and enzymatic activity. Thus, FGF9 may be acting to attenuate the IGF-I stabilization effect on aromatase after translation.

Our findings also show that bovine GC treated with dbcAMP or forskolin have an increase in E2 production in the presence of FSH and particularly in the presence of FSH plus IGF-I. It is known that E2 stimulates *CYP19A1* mRNA in bovine SMGC (72), which will have an autocrine positive feedback regulation on E2 production as the follicle grows. We found that IGF-I treatment of bovine LGGC decreased *FGF9* mRNA abundance. So it is plausible that as a follicle grows, and IGF-I stimulates more E2 production, FGF9's inhibition on steroidogenesis is attenuated as less FGF9 is produced. This is consistent with the occurrence of low *FGF9* mRNA abundance in GC of cystic follicles (9), which are characterized by an increase in E2 production. We also found that TNFα and WNT3A decreased whereas T_4 and SHH increased *FGF9* mRNA abundance in bovine GC, a finding not previously reported. Moreover, cortisol, PGE2, FSH, and E2 had no effect on bovine GC *FGF9* mRNA abundance in the present study. TNFα, T_4, WNT, and SHH have all been reported to affect ovarian cell function (31, 37, 38, 73, 74), and hormone regulation of mRNA abundance of FGF is not without precedence. Previous studies have reported that E2 induces *FGF9* mRNA in human endometrial stromal cells (75), cortisol increases *FGF9* mRNA in human fetal brain aggregates (33), and platelet-derived growth factor-BB decreases *FGF9* mRNA in human bladder smooth muscle cells (76). Other studies using ovarian cells indicate that P4 and T_4 increases *FGF2* mRNA abundance in rat GC (19, 20), and E2 also increases *FGF2* mRNA in endothelial cells derived from bovine corpora lutea (77). *In vivo* treatment of rats with diethylstilbestrol had no effect on ovarian *FGF9* mRNA, whereas equine CG or hCG decreased ovarian *FGF9* mRNA expression (12). More recently, Portela *et al.* (50) found that LH stimulated *FGF18* mRNA abundance in bovine theca cells, whereas in the present study, LH had no effect on *FGF9* mRNA in bovine SMGC. Thus, regulation of *FGF* gene expression is likely cell type and species dependent. Unfortunately, the concentration of FGF9 in follicular fluid is unknown, and additional research will be required to clarify the physiological significance of locally produced FGF9 within the ovary.

FGF9 influence on GC proliferation has not yet been published for any species. Previously, FGF9 has been shown to be an endometrial stromal growth factor in humans (75). Our data are the first to show a stimulatory effect of FGF9 on bovine GC proliferation. We found that FGF9 significantly stimulates bovine SMGC proliferation in a dose-dependent manner in the presence and absence of IGF-I as well as in the presence of 10% FCS. We found that FGF9 significantly stimulates bovine SMGC numbers in a dose-dependent manner in the presence of IGF-I with an ED_{50} of 6 ng/ml and further suggests that the FGF9 effect in GC is being mediated by high-affinity receptors. Similarly, FGF2 increases cell numbers in anchorage-independent cultures of bovine GC (78), and FGF2 protein has been localized in bovine primordial, primary, and preantral follicles (43, 79). Thus, FGF9 and FGF2 may act during all stages of follicular development by stimulating ovarian cell proliferation while inhibiting differentiation.

LGGC appeared to have a greater ability to produce FGF9 than did SMGC, which suggests that *FGF9* mRNA may be regulated during follicle growth. The inhibitory effects of TNFα and WNT3A on *FGF9* mRNA are particularly interesting and extend the roles of both TNFα and WNT3A in ovarian function. Previous studies in cattle show inhibitory effects of TNFα on ovarian follicular steroidogenesis and encompass granulosa and theca cells as well as LH, insulin, and IGF-I action (41, 73). Spicer (73) suggested that TNFα may play paracrine and endocrine roles, inhibiting ovarian function during luteal regression as well as during infection and disease. WNT3A is a member of the WNT signaling family, which are gonadotropin-regulated and act in conjunction with FSH to regulate GC steroidogenesis via β-catenin (74). Thus, the present study also implicates TNFα and WNT3A in regulating angiogenic factors within the ovary because FGF9 has been implicated in stimulating angiogenesis (80).

Our results show that FGF9 may act as an autocrine dedifferentiation factor regulating ovarian function in cattle via a down-regulation of hormone-stimulated steroidogenesis and steroidogenic enzyme gene expression and by concomitantly stimulating cell proliferation. FGF9 inhibition of steroid production is likely via attenuation of both gonadotropin receptor and steroidogenic enzyme gene expression. How endogenous FGF9 production by GC influence their response to exogenously added FGF9 will require further study. By understanding FGF9's mechanism of action, this may allow us to better understand both normal and abnormal ovarian function such as the development of follicular cysts.

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