Expression and effect of fibroblast growth factor 9 in bovine theca cells

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Abstract

Fibroblast growth factor 9 (FGF9) protein affects granulosa cell (GC) function but is mostly localized to theca cell (TC) and stromal cell of rat ovaries. The objectives of this study were to determine the 1) effects of FGF9 on TC steroidogenesis, gene expression, and cell proliferation; 2) mechanism of action of FGF9 on TCs; and 3) hormonal control of *FGF9* mRNA expression in TCs. Bovine ovaries were collected from a local slaughterhouse and TCs were collected from large (8–22 mm) follicles and treated with various hormones in serum-free medium for 24 or 48 h. FGF9 caused a dose-dependent inhibition ($P < 0.05$) of LH- and LH+IGF1-induced androstenedione and progesterone production. Also, FGF9 inhibited ($P < 0.05$) LH+IGF1-induced expression of *LHCGR*, *CYP11A1*, and *CYP17A1* mRNA (via real-time RT-PCR) in TCs. FGF9

had no effect ($P > 0.10$) on *STAR* mRNA abundance. Furthermore, FGF9 inhibited dibutyryl cAMP-induced progesterone and androstenedione production in LH+IGF1-treated TCs. By contrast, FGF9 increased ($P < 0.05$) the number of bovine TCs. Abundance of *FGF9* mRNA in GCs and TCs was several-fold greater ($P < 0.05$) in small (1–5 mm) vs large follicles. Tumor necrosis factor α and WNT5A increased ($P < 0.05$) abundance of *FGF9* mRNA in TCs. In summary, expression of *FGF9* mRNA in TCs is developmentally and hormonally regulated. FGF9 may act as an autocrine regulator of ovarian function in cattle by slowing TC differentiation via inhibiting LH+IGF1 action via decreasing gonadotropin receptors and the cAMP signaling cascade while stimulating proliferation of TCs.

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Introduction

The peptide hormone fibroblast growth factor 9 (FGF9) is downregulated in cystic follicles compared with non-cystic follicles of cattle (Grado-Ahuir *et al.* 2011), indicating that FGF9 may play a role in follicular development and cyst formation. In another microarray study, *FGFR2IIIc* mRNA, one of the receptors for FGF9 (Ornitz *et al.* 1996), was upregulated by insulin-like growth factor 1 (IGF1) in cultured porcine granulosa cells (GCs; Grado-Ahuir *et al.* 2009), suggesting that FGF9 may also be involved in porcine follicular growth. In rats, *Fgf9* mRNA and protein is localized in stromal cells and theca cells (TCs); receptors for FGF9 (i.e. FGFR2 and FGFR3) exist in GCs, TCs, and luteal cells; and FGF9 stimulates progesterone production by GCs (Drummond *et al.* 2007).

A plethora of studies detail the role of bone FGF2 (i.e. bFGF) in ovarian function (for reviews, see Gospodarowicz & Ferrara (1989) and Reynolds & Redmer (1998)). In cattle, binding sites for FGF2 have been identified in GCs (Neufeld *et al.* 1987). This finding is significant as both FGF2 and FGF9 share a similar receptor, FGFR2IIIc (Ornitz *et al.* 1996). In particular, FGF2 has been shown to have significant effects on GC proliferation, steroidogenesis, differentiation, and apoptosis (Gospodarowicz & Bialecki 1979, Tilly *et al.* 1992,

Anderson & Lee 1993, Vernon & Spicer 1994). FGF2 also affects bovine TCs by stimulating cell numbers, inhibiting androstenedione and progesterone production, and reducing the number of IGF1 binding sites (Spicer & Stewart 1996a).

To date, no studies have been done in cattle to investigate TC *FGF9* expression or the effects of FGF9 on TC function. Thus, our objectives were to determine the effects of FGF9 on steroidogenesis, proliferation, and gene expression of bovine TCs as well as to determine whether theca *FGF9* gene expression is hormonally regulated.

Materials and Methods

Reagents and hormones

The reagents and hormones used in cell culture were Ham's F-12 (F12), DMEM, gentamicin, sodium bicarbonate, trypan blue, protease, collagenase, hyaluronidase, DNase, forskolin, dibutyryl cAMP (dbcAMP), and FCS from Sigma-Aldrich Chemical Co.; LH (LH activity: $2.3 \times \text{NIH-LH-S1 U/mg}$) from National Hormone & Pituitary Program (Torrance, CA, USA); and recombinant human IGF1, FGF9, and wingless-type mouse mammary tumor virus integration site family member 3A (WNT3A) and recombinant bovine tumor

necrosis factor α (TNF α ; R&D Systems, Minneapolis, MN, USA; all carrier-free).

The reagents used for RIA were [¹²⁵I]iodo-progesterone (MP Biomedicals, Irvine, CA, USA), anti-progesterone rabbit antiserum (X-16) provided by Dr P Natashima Rao (Southwestern Foundation for Research Education, San Antonio, TX, USA), normal rabbit serum (Linco Research, Inc., St. Charles, MO, USA), and Androstenedione Double Antibody – ¹²⁵I RIA Kit (MP Biomedicals).

Cell culture

Ovaries from nonpregnant beef heifers were collected from a local abattoir and transported to the laboratory in 0.9% saline with 1% streptomycin/penicillin on ice. TCs were collected from large follicles (8–22 mm) that appeared healthy having good vascularity and moderately transparent follicular fluid. Large follicles were bisected longitudinally with a scalpel after the follicular fluid had been aspirated and the GCs scraped free from the theca interna via blunt dissection. The theca interna tissue was removed via microdissection and enzymatically digested for 1 h at 37 °C on a rocking platform as described previously (Stewart *et al.* 1995, Spicer & Chamberlain 1998, Spicer *et al.* 2008). Non-digested thecal tissue was removed via filtration through a sterile syringe filter holder with a mesh screen of 149 μ m mesh (Gelman Sciences, Ann Arbor, MI, USA). TCs were then centrifuged at 50 g for 7 min, washed twice in medium (1:1 DMEM and F12 containing 2.0 mM glutamine, 0.12 mM gentamicin, and 38.5 mM sodium bicarbonate), and resuspended in serum-free medium containing collagenase and DNase (Sigma–Aldrich Chemical Co.) at 1.25 and 0.5 mg/ml respectively to prevent clumping of cells before plating. The purity of TCs prepared this way was >90% (Roberts & Skinner 1990, Spicer & Stewart 1996b, Spicer *et al.* 2008).

Viability of TCs was determined by trypan blue exclusion method and averaged 96.9%. Approximately 2.0×10^5 viable cells were plated on 24-well Falcon multiwell plates (no. 3047; Becton Dickinson, Lincoln Park, NJ, USA) in 1 ml medium containing 10% FCS for the first 48 h. Cells were cultured in an environment of 5% CO₂ and 95% air at 38.5 °C with a medium change at 24 h. Cells were then washed twice with 0.5 ml serum-free medium followed by the addition of different hormonal treatments applied in serum-free medium for 24 or 48 h depending on the experiment.

RNA extraction and RT-PCR for mRNA quantification

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), and RNA was isolated from cell lysates as described previously (Spicer & Aad 2007, Lagaly *et al.* 2008, Spicer *et al.* 2008). Each treatment containing four wells generated two replicate samples of RNA. RNA was quantitated at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop

Technologies, Inc., Wilmington, DE, USA), diluted in diethylpyrocarbonate-treated water (Life Technologies), and stored at –80 °C.

CYP11A1, *CYP17A1*, *STAR*, and *LHCGR* primers and probes for quantitative RT-PCR were designed using Primer Express Software (Foster City, CA, USA) as previously reported (Lagaly *et al.* 2008). The bovine *CYP11A1* and *FGF9* primer and probe sequences and information are described by Lagaly *et al.* (2008) and Grado-Ahuir *et al.* (2011) respectively. The bovine *CYP17A1*, *STAR*, and *LHCGR* primer and probe sequences are described by Spicer *et al.* (2008). A ‘highly similar sequences’ BLAST query search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was conducted for each primer and probe to ensure the 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. For all RT-PCR runs, a no template control and a no reverse transcriptase control were included to ensure the lack of contaminants in the master mix and the absence of any genomic DNA contamination respectively. Furthermore, the RT-PCR products were run on agarose gels to verify the length and size of the expected target genes, and the same RT-PCR cDNA samples were sequenced to verify the amplified product. Target gene expression was normalized to constitutively expressed 18S rRNA and relative quantity of target gene mRNAs was expressed as $2^{-\Delta\Delta C_t}$ using the relative comparative threshold cycle method as described previously (Lagaly *et al.* 2008, Grado-Ahuir *et al.* 2011).

RIA and cell counting

Progesterone RIA was conducted as described previously (Baraño & Hammond 1985, Spicer & Enright 1991). Androstenedione RIA was conducted using an Androstenedione Double Antibody – ¹²⁵I RIA Kit (MP Biomedicals) as described previously (Stewart *et al.* 1995, Spicer *et al.* 2008). Intra-assay coefficients of variation averaged 6% and 8% respectively for the progesterone and androstenedione assays. Assay sensitivity, defined as 95% of total binding, averaged 0.8 ng/ml and 21 pg/ml for the progesterone and androstenedione RIA respectively.

Cell numbers were determined using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Hialeah, FL, USA) as described previously (Langhout *et al.* 1991, Lagaly *et al.* 2008). Briefly, wells were washed twice with 0.5 ml saline (0.9%) before trypsin (0.25% solution; 0.5 ml) was added and allowed to incubate for 20 min at room temperature. Wells were then scraped, aspirated, washed an additional time, and diluted 1:10 in saline.

Experimental design

Experiment 1 Experiment 1 was designed to test the effect of IGF1 and the dose-dependent effect of FGF9 on steroidogenesis of bovine large-follicle TCs. Cells were

cultured in 10% FCS for 48 h and treatments were given for an additional 48 h as follows: 0, 3, 10, or 30 ng/ml FGF9 with or without 30 ng/ml IGF1. Medium was changed every 24 h. After 48 h of treatment, medium was collected for progesterone and androstenedione determinations and cells were counted. All treatments included 30 ng/ml LH because progesterone and androstenedione production is not induced by IGF1 in the absence of LH (Stewart *et al.* 1995), and preliminary experiments showed that FGF9 had no significant effect on steroid production by TCs in the absence of LH.

Experiment 2 Experiment 2 was designed to test the effect of FGF9 on LH-, IGF1-, dbcAMP-, and forskolin-induced steroidogenesis of large-follicle TCs. Cells were cultured as described in Experiment 1 and treatments were applied for 48 h as follows (all treatments included 30 ng/ml LH): control, FGF9 (30 ng/ml), dbcAMP (1 mg/ml), dbcAMP plus FGF9, forskolin (4·1 µg/ml), and forskolin plus FGF9 (all with or without 30 ng/ml of IGF1). Doses of dbcAMP and forskolin were based on previous studies (McArdle *et al.* 1991, Vernon & Spicer 1994). After 48 h of treatment, medium was collected for progesterone and androstenedione determinations and cells were counted.

Experiment 3 Experiment 3 was designed to test the effect of FGF9 on *CYP11A1*, *CYP17A1*, *STAR*, and *LHCGR* mRNA abundance in large-follicle bovine TCs. Cells were cultured as described previously for Experiment 1 except that treatments were only applied for 24 h. For the effect of FGF9 on *CYP11A1*, *CYP17A1*, *STAR*, and *LHCGR* mRNA, two treatments were used (all treatments included 30 ng/ml IGF1): LH (30 ng/ml; control) and LH plus FGF9 (30 ng/ml). Following 24 h of incubation, medium was aspirated and cells were lysed for RNA extraction as described earlier.

Experiment 4 Experiment 4 was designed to determine the effect of follicle size and cell type on *FGF9* mRNA abundance. GCs and TCs from small (GCs, 1–5 mm; TCs, 3–6 mm) and large (>8 mm) follicles were collected into six pools for each size and cell type, each containing cells from follicles of at least five individual animals, RNA extracted, and real-time RT-PCR conducted as described earlier.

Experiment 5 Experiment 5 was designed to test the effects of WNT3A, TNFα, and IGF1 plus LH on *FGF9* mRNA abundance in cultured TCs. TNFα was evaluated because of its involvement in *Fgf9* mRNA expression in fetal rat lung explants (Land & Darakhshan 2004). WNT3A was tested because of its recent implication in cystic follicle development (Grado-Ahuir *et al.* 2011) and ovarian IGF1 stimulation (Grado-Ahuir *et al.* 2009). Cells were cultured as described earlier with treatments applied for 24 h as follows: control, TNFα (30 ng/ml), and WNT3A (100 ng/ml) with or without IGF1 (30 ng/ml) plus LH (30 ng/ml). Doses of IGF1, LH, WNT3A, and TNFα were selected based on previous studies showing that these doses significantly alter

steroidogenesis (Spicer 1998, Spicer & Chamberlain 1998, Castañon *et al.* 2012). After 24 h of treatment, cells were lysed for RNA extraction as described earlier.

Statistical analysis

For each experiment, three different pools were used as experimental replicates. Each pool of TCs was obtained from five to seven follicles from four to six animals. Steroid production was expressed as nanogram or picogram/10⁵ 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. The treatment effects of the dependent variables (e.g. steroid production and *CYP11A1* mRNA abundance) were determined using ANOVA and the general linear models procedure of SAS for Windows (version 9.2, SAS Institute, Inc., Cary, NY, USA). Data from Experiment 1 were analyzed as a 2×4 factorial ANOVA. Data from Experiment 2 were analyzed as a 2×2×3 factorial ANOVA. Data from Experiment 3 were analyzed as a one-way ANOVA. Data from Experiment 4 were analyzed as a 2×2 factorial ANOVA. Data from Experiment 5 were analyzed as a 2×3 factorial ANOVA. Mean differences were determined by Fisher's protected least significant differences test (Ott 1977) if significant main effects in the ANOVA were detected. Data were presented as the least square mean ± S.E.M.

Results

Experiment 1: effect of IGF1 and the dose-dependent effect of FGF9 on cell numbers and steroidogenesis of TCs

FGF9 alone (30 ng/ml) stimulated ($P<0\cdot05$) TC numbers by 1·4-fold, but FGF9 at 3 and 10 ng/ml was without effect ($P>0\cdot10$). Similarly, in the presence of IGF1, only FGF9 at 30 ng/ml stimulated ($P<0\cdot05$) cell numbers by 1·5-fold (Fig. 1). The estimated effective dose (ED₅₀) of FGF9 stimulating 50% of the maximal cell number response (calculated from stimulation curves that were linearized using a semi-log plot) averaged 12 ng/ml.

FGF9 decreased ($P<0\cdot05$) IGF1 (30 ng/ml) plus LH-induced progesterone production in a dose-dependent manner such that 10 and 30 ng/ml completely inhibited ($P<0\cdot05$) the IGF1-induced increase in progesterone production (Fig. 2A). The estimated inhibitory concentration of FGF9 inhibiting 50% (IC₅₀) of the IGF1-induced maximal cell number response (calculated from inhibition curves that were linearized using a semi-log plot) averaged 9 ng/ml. In the absence of IGF1, but in the presence of LH, FGF9 at 10 and 30 ng/ml inhibited ($P<0\cdot05$) progesterone production by 15 and 59% respectively (Fig. 2A).

Similarly, FGF9 decreased ($P<0\cdot05$) IGF1 plus LH-induced androstenedione production in a dose-dependent manner such that 3, 10, and 30 ng/ml inhibited IGF1-induced

Figure 1 Stimulatory effect of FGF9 (0, 3, 10, or 30 ng/ml) on IGF1-induced cell (0 or 30 ng/ml) numbers in large-follicle TCs (Experiment 1). All cells were concomitantly treated with 30 ng/ml LH. ^{a,b,c,d,e}Within a panel, means without a common letter differ ($P < 0.05$).

androstenedione production by 12, 41, and 79% respectively, with an IC_{50} of 9.4 ng/ml (Fig. 2B). Under basal conditions (i.e. LH alone without IGF1), FGF9 at 30 ng/ml (but not 3 or 10 ng/ml) decreased ($P < 0.05$) androstenedione production by 37% (Fig. 2B).

Experiment 2: effect of FGF9 on LH-, IGF1-, dbcAMP, and forskolin-induced steroidogenesis of TCs

Treatment of dbcAMP alone and forskolin alone increased ($P < 0.05$) progesterone production, and the addition of FGF9 (30 ng/ml) attenuated ($P < 0.05$) basal and dbcAMP-induced progesterone production without affecting forskolin-induced progesterone production of TCs (Fig. 3A). IGF1 (30 ng/ml) treatment increased ($P < 0.05$) progesterone production in control and dbcAMP- and forskolin-treated TCs. FGF9 attenuated ($P < 0.05$) IGF1- and IGF1 plus dbcAMP-induced progesterone production in TCs but had no effect on IGF1 plus forskolin-induced progesterone production (Fig. 3A).

Forskolin alone and FGF9 alone decreased ($P < 0.05$) androstenedione production; however, FGF9 had no effect ($P > 0.10$) on androstenedione production in dbcAMP- or forskolin-treated TCs (Fig. 3B). IGF1 increased ($P < 0.05$) androstenedione production in control and dbcAMP- and forskolin-treated TCs (Fig. 3B). FGF9 attenuated ($P < 0.05$) the stimulation in IGF1 alone- and IGF plus dbcAMP-treated TCs but had no effect ($P > 0.10$) on androstenedione production in IGF1 plus forskolin-treated TCs (Fig. 3B).

Experiment 3: effect of FGF9 on LHCGR, STAR, CYP11A1, and CYP17A1 mRNA abundance in TCs

In LH plus IGF1-treated TCs, FGF9 (30 ng/ml) decreased ($P < 0.05$) abundance of *LHCGR* (Fig. 4A), *CYP11A1*

(Fig. 4C), and *CYP17A1* mRNA (Fig. 4D) by 97, 77, and 97% respectively. By contrast, *STAR* mRNA abundance was not affected ($P > 0.20$) by FGF9 (Fig. 4B).

Experiment 4: the effect of follicle size and cell type on FGF9 mRNA abundance

Real-time RT-PCR revealed that TCs from small follicles had 38.5-fold greater ($P < 0.01$) abundance of *FGF9* mRNA than TCs from large follicles (Fig. 5). Similarly, GCs from small follicles had 14.8-fold greater ($P < 0.01$) abundance of *FGF9* mRNA than GCs from large follicles (Fig. 5). Abundance of *FGF9* mRNA in GCs of small and large follicles was two- and six-fold greater ($P < 0.05$) respectively than the abundance of *FGF9* mRNA in TCs.

Figure 2 Inhibitory effect of FGF9 (0, 3, 10, or 30 ng/ml) on IGF1-induced (0 or 30 ng/ml) progesterone (Panel A) and androstenedione (Panel B) production by large-follicle TCs (Experiment 1). All cells were concomitantly treated with 30 ng/ml LH. ^{a,b,c,d,e,f}Within a panel, means without a common letter differ ($P < 0.05$).

Figure 3 Effects of FGF9 and IGF1 on steroid production of large-follicle TCs induced by dibutyl cAMP (dbcAMP) and forskolin (Experiment 2). (Panel A) Effect of FGF9 (0 or 30 ng/ml) on basal and IGF1-induced (0 or 30 ng/ml) progesterone production by large-follicle TCs treated concomitantly with either dbcAMP or forskolin. (Panel B) Effect of FGF9 (0 or 30 ng/ml) on basal and IGF1-induced (0 or 30 ng/ml) androstenedione production by large-follicle TCs treated concomitantly with either dbcAMP or forskolin. ^{a,b,c,d,e,f,g} Within a panel, means without a common letter differ ($P < 0.05$). All cells were concomitantly treated with 30 ng/ml LH.

Experiment 5: effect of $TNF\alpha$ and WNT3A on FGF9 mRNA in cultured TCs

Analysis revealed that treatment of WNT3A (100 ng/ml) and $TNF\alpha$ (30 ng/ml) each increased ($P < 0.05$) FGF9 mRNA abundance by 1.9- and 1.5-fold respectively in untreated TCs (Fig. 6). IGF1 plus LH increased ($P < 0.05$) FGF9 mRNA abundance by 47%; however, $TNF\alpha$ was without effect ($P > 0.10$) when co-treated with IGF1 and LH (Fig. 6). In comparison, WNT3A increased ($P < 0.05$) FGF9 mRNA abundance above IGF1 plus LH-treated controls, but levels

did not differ ($P > 0.10$) from WNT3A-treated TCs in the absence of IGF1 and LH (Fig. 6).

Discussion

This study is the first to demonstrate FGF9 effects on steroidogenesis and gene expression in ovarian TCs. Because FGF9 mRNA is present in bovine GCs and TCs, FGF9 may act as an autocrine and/or paracrine regulator of TC function in addition to the paracrine role of FGF9 proposed for rat GCs by Drummond *et al.* (2007). In the rat ovary, FGF9 protein has been localized to the TCs and stromal cells, as well as in the basement membrane of follicles (Drummond *et al.* 2007). Other FGF family proteins, such as FGF2, have been localized in GCs and TCs of cattle (van Wezel *et al.* 1995). FGF7, also known as keratinocyte growth factor, mRNA has been detected in bovine TCs (Parrott & Skinner 1998). FGF8, -10, and -17 mRNA are expressed in bovine GCs and TCs (Buratini *et al.* 2005, 2007, Machado *et al.* 2009), and FGF18 mRNA is localized primarily in bovine TCs (Portela *et al.* 2010). FGF14 mRNA has been detected in human oocytes (Assou *et al.* 2006). Adding to the complexity of the ovarian FGF system, these FGFs are capable of binding to multiple FGFRs, and FGF9 in particular binds to FGFR1IIIc, FGFR2IIIc, FGFR3IIIb, FGFR3IIIc, and FGFR4 (Ornitz *et al.* 1996).

The effects of FGF9 on ovarian cell steroidogenesis have been minimally studied. For the first time in any species, we observed that FGF9 significantly inhibits both progesterone

Figure 4 Effect of FGF9 (30 ng/ml) on *LHCR*, *STAR*, and steroidogenic enzyme gene expression in TCs of large follicles (Experiment 3). (Panel A) Inhibitory effect of FGF9 on *LHCR* mRNA abundance in large-follicle TCs; (Panel B) lack of effect of FGF9 on *STAR* mRNA abundance in large-follicle theca cells. (Panel C) Inhibitory effect of FGF9 on *CYP17A1* mRNA abundance. (Panel D) Inhibitory effect of FGF9 on *CYP17A1* mRNA abundance. All cells were concomitantly treated with 30 ng/ml IGF1 and LH. Values are means of three separate experiments ($\pm$ S.E.M.) and normalized to constitutively expressed 18S rRNA. *Within a panel, mean differs from its respective control ($P < 0.05$).

Figure 5 Effect of the size of follicle on the abundance of GCs and TCs *FGF9* mRNA in bovine follicles (Experiment 4). GCs and TCs from small (1–5 mm) and large (8–22 mm) follicles were collected, RNA isolated, and quantitative RT-PCR used to quantify mRNA levels. Values are normalized to constitutively expressed *18S* rRNA. ^{a,b,c}Means ($n=6$) without a common superscript differ ($P<0.05$).

and androstenedione production by TCs. In this study, the IC_{50} for FGF9 (i.e. 9–14 ng/ml) reducing steroidogenesis suggests that FGF9 effects are being mediated by high-affinity receptors because these values are in line with the K_d values obtained for FGF9 and FGF2 binding to their respective receptors in other tissues (Neufeld & Gospodarowicz 1985, Hecht *et al.* 1995). In rat GCs, FGF9 increases progesterone production (Drummond *et al.* 2007), and in mouse Leydig cells, FGF9 stimulates testosterone production (Lin *et al.* 2010).

Because FGF9 and FGF2 are capable of binding to some of the same FGFRs (e.g. FGFR4), it is reasonable that FGF2 may have a similar effect to FGF9. Indeed, FGFR4 has been localized in bovine TCs (Buratini *et al.* 2005). However, inhibitory effects have been noted for FGF2 in ovarian cells of both cattle and rats. *In vitro*, FGF2 inhibits hormone-induced androstenedione production by bovine (Spicer & Stewart 1996a) and by chicken (Li & Johnson 1993) TCs. Also, FGF2 inhibited forskolin-induced 17α -hydroxyprogesterone production by human TCs (McAllister 1995). Similarly, FGF2 strongly inhibited hCG-stimulated androgen biosynthesis in cultured whole ovarian dispersates from immature rats (Hurwitz *et al.* 1990). In immature rat Leydig cells, co-treatment with FGF2 caused a dose-dependent decrease in LH-stimulated testosterone production (Fauser *et al.* 1988). This inhibition of testosterone by FGF2 caused a 12-fold increase in progesterone production while also inhibiting the conversion of exogenously added androgen precursors, suggesting that FGF2 might inhibit CYP17A1 activity (Fauser *et al.* 1988), the latter of which was observed in this study for FGF9. Because there are a total of eight FGF receptors (Ornitz *et al.* 1996), a specific cell type and species response to a specific FGF is likely determined by the cadre of FGFR(s) that are present in that cell type. For example, cattle express *FGFR3IIIc*

mRNA in both GCs and TCs (Buratini *et al.* 2005) while mice do not express *FGFR3IIIc* mRNA in either cell type and only expresses *FGFR4* mRNA in GCs (Puschbeck *et al.* 1997). *FGFR3IIIb* and *FGFR3IIIc* mRNA are present in bovine GCs (Parrott & Skinner 1998, Berisha *et al.* 2000, 2004, Buratini *et al.* 2005), while *FGFR4* mRNA has been localized in bovine TCs (Buratini *et al.* 2005). In addition to species differences, expression of FGFR subtypes may change in a specific cell type during differentiation. For example, both FGF1 and FGF2 have a stimulatory effect on basal testosterone production by fetal/neonatal Leydig cells, whereas FGF1 inhibited LH-stimulated testosterone production by adult rat Leydig cells, suggesting that Leydig cells may acquire more *FGFR2IIIb* or *FGFR3IIIb* (the only FGFRs that FGF2 does not bind to) as they develop (Laslett *et al.* 1995, 1997).

Our observations that FGF9 inhibited LH and LH plus IGF1-induced progesterone production as well as dbcAMP-induced progesterone production provide direct evidence for a site of action of FGF9 that is distal to cAMP generation. The inability of FGF9 to inhibit forskolin-induced progesterone production is paradoxical because forskolin should be inducing cAMP production via stimulation of adenylate cyclase and, thus, its action also should have been inhibited by FGF9. Possible explanations for this paradox include 1) forskolin may activate other G-protein receptors that upregulate steroidogenic genes independent of cAMP (Dowless *et al.* 2005), 2) FGF9 may inhibit forskolin-sensitive G proteins that inhibit adenylate cyclase activity (Maneuf & Brotchie 1997), and/or 3) forskolin may activate G proteins that induce a dual second messenger molecule and signaling pathway that converges at a site distal to cAMP action (Paul *et al.* 2010). Why dbcAMP and forskolin were able to significantly increase androstenedione production only in FGF9-treated TCs will require further elucidation but

Figure 6 Effect of TNFα (30 ng/ml) and WNT3A (100 ng/ml) on *FGF9* gene expression in TCs of large follicles (Experiment 5). Values are means of three separate experiments ($\pm$ S.E.M.) and normalized to constitutively expressed *18S* rRNA. ^{a,b,c}Within a panel, mean differs from its respective control ($P<0.05$).

suggests that there may be multiple intracellular signaling pathways that are modulated by FGF9. The relationships among adenylate cyclase, cAMP, and steroidogenesis in TCs are complex and further studies will be needed to develop a coherent framework with which these apparently contradictory findings can be reconciled.

In accordance with FGF9's inhibitory effect on bovine TC steroidogenesis, we observed that FGF9 attenuated IGF1 plus LH-induced *CYP11A1*, *CYP17A1*, and *LHCGR* mRNA expression. However, abundance of *STAR* mRNA, the important cholesterol transport protein (Manna *et al.* 2009), was not influenced by FGF9 in this study. This provides an explanation for the mechanism by which FGF9 acts on TC steroidogenesis. No studies to date have been done investigating FGF9 effects on bovine TC gene expression; however, one study using immature rat Leydig cells found an inhibitory effect of FGF2 on LH-stimulated androgen production along with an inhibition on mRNA abundance of *Cyp11a1* and *Cyp17a1* (Xiao *et al.* 2010). Also consistent with an inhibitory effect of FGF9 on *LHCGR* mRNA in this study, FGF2 inhibited the FSH-induced *Lhcgr* mRNA expression and/or cAMP production in cultured rat (Oury & Darbon 1988) and pig (Biswas *et al.* 1988) GCs. Thus, it seems that FGF9 and FGF2 (both of which can bind to similar FGFRs) have similar inhibitory effects on androgen production and steroidogenic enzyme expression in TCs of both polyovular and monovular mammals. By contrast, FGF9 appears to stimulate basal and FSH-induced progesterone production by stimulating synthesis of *Star* and *Cyp11a1* mRNA in rat GCs (Drummond *et al.* 2007).

FGF9 influence on TC proliferation has not yet been published for any species. Previously, FGF9 was shown to be an endometrial stromal growth factor in humans (Tsai *et al.* 2002). We found that FGF9 significantly stimulated bovine TC numbers in a dose-dependent manner in the presence of IGF1 with an ED₅₀ of 12 ng/ml and further suggest that the FGF9 effect in TCs is being mediated by high-affinity receptors. In the absence of IGF1, FGF9 was a much less potent stimulator of cell numbers, suggesting that IGF1 may increase the sensitivity of TCs to FGF9. Whether IGF1 increases FGF9 sensitivity by increasing FGF9 receptors in bovine TCs is unknown, but studies using rat skeletal muscle satellite cells indicate that IGF1 can induce *Fgfr1* mRNA (Sheehan & Allen 1999). Spicer & Stewart (1996a) noted that treatment of FGF2 increased bovine TC numbers but decreased IGF1 receptor numbers, providing evidence for a FGF-IGF interaction within bovine TCs. Both FGF1 and FGF2 stimulate proliferation of GCs of cattle (Hoshi *et al.* 1995, Rodgers *et al.* 1996) and proliferation of GC and TC of chickens (Roberts & Ellis 1999), suggesting that multiple FGFs may interact with GC and TC to regulate ovarian function in vertebrates.

TCs and GCs from small follicles appeared to have a greater ability to produce FGF9 than did GCs and TCs from large follicles, suggesting that *FGF9* mRNA may be regulated during follicle growth. Similarly, a previous study in rats

indicated that whole ovarian *Fgf9* mRNA levels decreased during follicle growth: from day 4 to 12 of life and after gonadotropin treatment *in vivo* (Drummond *et al.* 2007). Because GCs, stroma cells, and TCs are included in whole ovarian samples, these previous results are difficult to interpret in terms of which ovarian cell type may be contributing to the change in *Fgf9* gene expression. In this study, *FGF9* mRNA abundance was greater in GCs than TCs, and a combined treatment of LH and IGF1 increased abundance of *FGF9* mRNA in cultured TCs, but to an amount less than that of WNT3A. The stimulatory effects of TNF α and WNT3A on *FGF9* mRNA abundance in TCs of this study are particularly interesting and extend the roles of both TNF α and WNT3A in ovarian function. Other studies in cattle show inhibitory effects of TNF α on ovarian follicular steroidogenesis and include GC and TC as well as LH, insulin, and IGF1 action (Spicer & Alpizar 1994, Spicer 1998). WNT3A is one of 19 members of the WNT signaling family, some of which are gonadotropin-regulated and act in conjunction with FSH to regulate GC steroidogenesis via β -catenin (Hernandez Gifford *et al.* 2009, Lapointe & Boerboom 2011, Castañon *et al.* 2012). Collectively, this study implicates LH, IGF1, TNF α , and WNT3A in a role that may involve regulation of the pro-angiogenic factor, FGF9 (Behr *et al.* 2010), within the ovary.

In conclusion, results of this study indicate that FGF9 may regulate ovarian TC function in cattle via a downregulation of steroidogenesis via inhibition of cAMP signal transduction and steroidogenic enzyme gene expression and by stimulating cell proliferation. FGF9 inhibition of steroid production is likely via attenuation of both gonadotropin receptor and steroidogenic enzyme gene expression. Understanding ovarian FGF9 production and FGF9's mechanism of action as a dedifferentiation factor may allow us to decipher regulation of both normal and abnormal ovarian functions such as the occurrence of follicular cysts.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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