Changes in fibroblast growth factor receptors-1c, -2c, -3c, and -4 mRNA in granulosa and theca cells during ovarian follicular growth in dairy cattle

L. F. Schütz[*,1], A. M. Hemple*, B. C. Morrell*, N. B. Schreiber[*], J. N. Gilliam[†], C. Cortinovis[‡], M. L. Totty[*], F. Caloni[‡], P. Y. Aad[**], and L. J. Spicer[*,2]

[*]Department of Animal and Food Sciences, Oklahoma State University, Stillwater, 74078,

[†]Department of Veterinary Clinical Sciences, Oklahoma State University, Stillwater, 74078,

[‡]Università degli Studi di Milano, Department of Veterinary Medicine, Milan, Italy,

[**]Department of Natural and Applied Sciences, Notre Dame University – Louaizeh, Lebanon

[1]Current address: Department of Agriculture, Veterinary & Rangeland Sciences, University of Nevada, Reno, NV, USA

[2]Corresponding author: leon.spicer@okstate.edu

Running head: FGFR mRNA changes during follicular growth

ABSTRACT

The various fibroblast growth factors (FGF) regulate their function via binding to four main FGF receptor (FGFR) subtypes and their splice variants, *FGFR1b, FGF1c*, *FGFR2b, FGFR2c* and *FGFR3c* and *FGFR4*, but which of these FGFR are expressed in the granulosa (GC) and theca cells (TC), the two main cell layers of ovarian follicles, or change during follicular development is unknown. We hypothesized that *FGFR1c*, *FGFR2c* and *FGFR3c* (but not *FGFR4*) gene expression in GC (but not TC) would change with follicular development. Hence, the objective of this study was to determine if abundance of *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* mRNA change according to follicular size, steroidogenic status, and days post-ovulation during growth of first-wave dominant follicles in Holstein cattle exhibiting regular estrous cycles. Estrous cycles of non-lactating dairy cattle were synchronized, and ovaries were collected on either day 3 to 4 (n = 8) or day 5 to 6 (n = 8) post-ovulation for GC and TC RNA extraction from small (1 to 5 mm), medium (5.1 to 8 mm) or large (8.1 to 18 mm) follicles for real-time PCR analysis. In GC, *FGFR1c* and *FGFR2c* mRNA relative abundance was greater in estrogen (E2)-inactive (i.e., concentrations of E2 < progesterone, P4) follicles of all sizes than in GC from large E2-active follicles (i.e., E2 > P4), whereas *FGFR3c* and *FGFR4* mRNA abundance did not significantly differ among follicle types or days post-estrus. In TC, medium E2-inactive follicles had greater *FGFR1c* and *FGFR4* mRNA abundance than large E2-active and E2–inactive follicles on day 5 to 6 post-ovulation whereas *FGFR2c* and *FGFR3c* mRNA abundance did not significantly differ among follicle types or day post-estrus. In vitro experiments revealed that androstenedione increased abundance of *FGFR1c*, *FGFR2c* and *FGFR4* mRNA in GC whereas estradiol decreased *FGFR2c* mRNA abundance. Neither androstenedione nor estradiol affected abundance of the various *FGFR* mRNAs in cultured TC. Taken together, the findings that *FGFR1c* and

FGFR2c mRNA abundance was less in GC of E2-active follicles and *FGFR1c* and *FGFR4* mRNA was greater in TC of medium inactive follicles at late than at early growing phase of the first dominant follicle support an anti-differentiation role for FGF and their FGFR as well as support the idea that steroid-induced changes in FGF and their receptors may regulate selection of dominant follicles in cattle.

Key Words: Fibroblast growth factor receptors (FGFR), follicle growth, theca cell, granulosa cell, cattle.

1. Introduction

Ovarian folliculogenesis is a tightly regulated process where the somatic cells of the follicle, granulosa (GC) and theca (TC) cells, communicate in a coordinated way with the oocyte for both follicular and oocyte growth and maturation [1, 2, 3]. Several fibroblast growth factors (FGF) have been implicated as important regulators of ovarian function, playing autocrine, paracrine, and endocrine roles in the regulation of development of ovarian follicles [for reviews, see 4, 5]. These polypeptides belong to a family of 22 members in mammals [6, 7], and, to date, ten members have been detected in the ovary: FGF1, 2, 7-10, 16-18, 22 [4, 5, 8]. In cattle, these FGF play diverse roles in ovarian function and in order for FGF to exert their actions in the ovary, they need to bind to high affinity receptors (FGFR). The FGFR is a single chain transmembrane tyrosine kinase with two or three immunoglobulin-like domains and a heparin-binding domain in the extracellular ligand-binding portion [9, 10, 11]. There are four distinct genes encoding for FGFR (*FGFR1-FGFR4*) in vertebrates and mRNA alternative splicing occurs in the immunoglobulin-like domains III of the *FGFR1*, *FGFR2*, and *FGFR3* genes (but not of

78 *FGFR4*), generating diversity of sequence and resulting in various isoforms [11, 12, 13].
79 According to ligand binding specificity, the preferred receptors for FGF produced in the ovary
80 are: FGFR1c for FGF1 and FGF2; FGFR3c for FGF1, FGF2, FGF8, FGF9, FGF16, FGF17, and
81 FGF18 [6, 11]. In addition, FGFR2c is the second preferred receptor for FGF9 and FGF16; and
82 FGFR4 is the second preferred receptor for FGF8, FGF17, and FGF18 [6, 11]. Because much
83 work has been done showing effects of FGF1, FGF2, FGF8 and FGF9 on bovine ovarian cells,
84 the preferred receptors for these ligands were selected to be measured in the present study (i.e.,
85 FGFR1c, 2c, 3c and 4).

86 In cattle, FGFR1c and FGFR2b have been detected in GC [14, 15] and oocytes [16, 17],
87 FGFR1b has been detected in GC, TC, and oocytes [17, 18], FGFR2c and FGFR3c have been
88 detected in both GC and TC [14, 19], and FGFR4 has been detected exclusively in TC [19] but
89 only two studies have evaluated two of these receptors (i.e., FGFR1b and FGFR2b) in ovarian
90 follicles of beef cattle during the first follicular wave [18, 20]. In addition, some FGFR change
91 according to follicular fate in beef cattle: *FGFR1b* and *FGFR2b* mRNA abundance is greater in
92 GC of presumed subordinate follicles than presumed dominant follicles [14, 20]; *FGFR3c*
93 mRNA abundance is greater in GC (but not in TC) of small healthy follicles and in response to
94 FSH [19]; *FGFR4* mRNA abundance is greater in small than in large follicles [19]. Some of
95 these changes in FGFR are associated with changes in follicular fluid **(FFL)** estradiol **(E2)** levels
96 [14, 20], but whether steroids directly regulate these changes in FGFR in bovine follicles is
97 unknown. Moreover, how endogenous production of FGFR1c, FGFR2c, FGFR3c and FGFR4 by
98 ovarian follicular cells change during selection of dominant follicles in cattle is unknown. Hence,
99 the objective of this study was to determine if mRNA abundance of *FGFR1c*, *FGFR2c*,
100 *FGFR3c*, and *FGFR4* in GC and TC changes during growth of first-wave dominant follicles in

cattle exhibiting regular estrous cycles, and to determine if steroids regulate expression of the various *FGFR* in GC and TC of cattle.

2. Materials and methods

2.1. In vivo experimental design

Samples analyzed for this study were obtained from an experiment previously performed using non-lactating Holstein cows (n = 18) from Oklahoma State University herd [21]. All cows were non-lactating (5.4 ± 0.7 yr of age) and identified to be culled for nonreproductive reasons from the Oklahoma State University herd. Briefly, estrous cycles were synchronized using two injections (i.m.) of prostaglandin $F_{2\alpha}$ (Lutalyse®, 25 mg) with an interval of 11 d, after which, follicle development was monitored daily via ultrasonography using an Aloka 500V with a 7.5 MHz probe. Following ovulation, cows were assigned to be ovariectomized either at 3 d to 4 d (early growing phase of the first dominant follicle; n = 9 cows) or 5 d to 6 d post-ovulation (late growing phase of the first dominant follicle; n = 9 cows) as previously described [21]. From the 18 cows used in the synchronization program, two failed (one from 3 d to 4 d and one from 5 d to 6 d post-ovulation groups) to ovulate and were excluded from this experiment. After each ovariectomy, ovaries were put on ice, and transported to the laboratory where diameters of all follicles ≥ 5 mm (surface diameter) in diameter were recorded, the numbers of all follicles ≥ 1 mm in diameter on the ovarian surface were determined, and ovarian tissue and fluid collected as previously described [21]. The animal experimentation described in this report was approved by the Oklahoma State University Institutional Animal Care and Use Committee (Protocol No. AG106).

For GC sample collection, follicles were categorized by surface diameter as small (1 to 5 mm), medium (5.1 to 8 mm) or large (8.1 to 18 mm); TC samples were collected from only medium and large follicles. The FFL from medium and large follicles was aspirated individually and centrifuged to obtain GC, and FFL from small follicles was pooled within each ovary and then centrifuged to obtain GC as previously described [21]. After centrifugation, FFL was aspirated and stored at -20 °C for measurement of E2 and progesterone (P4) via RIA. After collection of FFL, each medium and large follicle was bisected *in situ*, the inner wall was scraped, rinsed with Ham's F-12 to remove any remaining GC, and these GC were combined with GC collected from FFL as previously described [21]. GC collected from small follicles were kept separate for each ovary. GC were lysed in 0.5 mL of TRIzol® reagent solution (Life Technologies, Inc., Grand Island, NY) and stored frozen at -80 °C until RNA extraction (see description below). TC were dissected from the bisected follicles and placed in 0.75 mL of TRIzol Reagent and homogenized for 2 to 3 min on ice using the Omni TH tissue homogenizer (Omni International Inc., Marietta, GA) with Omni Tip™ disposable generator probes as previously described [22].

2.2. In vitro experiments

The *in vivo* results suggested that E2 may be inhibitory to *FGFR* gene expression. To test this hypothesis, we designed experiments to test the effects of E2 and androstenedione (A4) on *FGFR* mRNA abundance in GC and TC. The dose (i.e., 300 ng/mL) of E2 and A4 was selected to represent concentrations of these steroids found in dominant follicles [23, 24] and based on previous studies [25-27]. Ovaries from nonpregnant cyclic (i.e., corpora lutea present) beef cattle were collected from a local slaughterhouse, and based on surface diameter, GCs were collected from small (1 to 5 mm) follicles via aspiration of FFL as previously described [21, 27,

28]. To isolate TC, large (8 to 20 mm) follicles were bisected with a scalpel after aspiration of FFL, GC were separated from theca interna via blunt dissection, theca interna tissue removed from the follicle wall, enzymatically digested, and non-digested thecal tissue was removed via filtration as previously described [25, 28, 29]. TC were centrifuged at 50 × g for 8 min, the pellets were washed twice in serum-free medium (1:1 DMEM and F12 with 38.5 mM sodium bicarbonate, 0.12 mM gentamicin, and 2.0 mM glutamine; Sigma-Aldrich Chemical Company, St. Louis, MO) and then re-suspended in serum-free medium containing collagenase and DNase to prevent clumping as previously described [8].

Viability of GC and TC used for cell culture was determined by trypan blue exclusion method using a hemacytometer, and averaged 42.2 ± 2.4% and 95.6 ± 0.8%, respectively. On average, 3.5×10^5 viable cells/well were plated on 24-well Falcon multi-well plates (No. 3047; Becton Dickinson, Lincoln Park, NJ) with 1 mL of medium/well and cultured (at 38.5°C with 5% CO_2 and 95% air) in 10% fetal calf serum (Equitech-Bio, Inc.; Kerrville, TX) for the first 48 h with medium changed every 24 h. Cells were washed twice with serum-free medium (0.5 mL) and three treatments were applied in serum-free medium (1 mL/well) as follows: control, E2 (300 ng/mL) or A4 (300 ng/mL) (Sigma-Aldrich Chemical Co.). After 24 h of treatment, cells were lysed in TRIzol reagent (Life Technologies, Inc.) and extracted for RNA. Each experiment was replicated three times. This culture system was developed to yield hormonally responsive non-luteinized GC and TC [27-29]. In this system, aromatase activity of GC remains responsive to FSH, insulin and IGF-I and increases between d 3 and 4 of culture [28, 30], and the TC remain responsive to LH and IGF1 in terms of *CYP17A1* mRNA and A4 production [29, 30]. Contemparary GC cultures in the present study responded to IGF1, with E2 secretion averaging 8 ± 1 pg/mL in FSH-treated controls vs 146 ± 18 pg/mL in IGF1 plus FSH-treated cultures. Also,

contemparary TC cultures in the present study responded to IGF1, with androstenedione secretion averaging 324 ± 32 pg/mL in LH-treated controls vs. 578 ± 122 pg/mL in IGF1 plus LH-treated cultures.

2.3. Extraction of RNA and quantitative PCR

Ovarian follicular cells, GC and TC, were lysed in TRIzol® reagent solution (Life Technologies, Inc., Grand Island, NY) as described elsewhere [21, 25]. 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.

Primers and probes for *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* (supplied as 5' FAM reporter dye and a 3' TAMRA quencher dye; TaqMan TAMARA; Applied Biosystems Inc., Foster City, CA) for quantitative PCR (Table 1) were designed using Primer Express software (Foster City, CA). Relative mRNA abundance of target genes was quantified using fluorescent quantitative single-step RT-PCR using a CFX96 Real-Time System in 96-well plates (Bio-Rad, Hercules, CA). On each 96-well plate, samples were placed in duplicate wells to determine an average threshold cycle (Ct) value of both target gene and housekeeping gene. Thus, for the *in vivo* study, 24 samples balanced across d 3 and d 6 groups were included on each plate. Quality control for PCR was conducted as previously described [25]; intra-assay CV for real-time PCR averaged 0.75%. In addition, 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 used to verify the amplified sequence. Target gene expression was normalized to constitutively expressed *18S* ribosomal RNA (*18S* rRNA; supplied as a VIC probe; TaqMan Ribosomal RNA Control

191 Reagent, Applied Biosystems Inc.). The relative quantity of target gene mRNAs was expressed
192 as $2^{-\Delta\Delta Ct}$ using the relative comparative threshold cycle (Ct) method as previously described [32].
193 The housekeeping gene, *18S* rRNA (accession no. X03205.1) was selected because previous
194 studies show it to be a stable gene over a variety of treatments [25, 33, 34].

195 *2.4. P4 and E2 RIA*

196 Concentrations of P4 and E2 in FFL were determined by RIA as previously described
197 [21, 23]. All samples were run in one assay for each of the steroid RIA. The intra-assay CV for
198 P4 and E2 RIA was 11.6 % and 10.6 %, respectively. The inter-assay CV for P4 and E2 RIA
199 average 23% and 14%, respectively, and recoveries of mass are >99% [23, 24].

200 *2.5. Statistical analyses*

201 Data were analyzed using the general linear models procedure of the Statistical Analysis
202 System (SAS) for Windows (version 9.4, SAS Institute Inc., Cary, NC) and are presented as the
203 least squares means (± SEM) of measurements. For Exp. 1, main factors were days post-
204 ovulation (early, 3 d to 4 d, and late, 5 d to 6 d, growing phase of the first dominant follicle),
205 follicle status based on size (small, medium, or large in the case of GC, and medium or large in
206 the case of TC) and follicle estrogenic status (E2 active: E2>P4 concentrations or E2 inactive:
207 E2<P4 concentrations), and their various interactions. Some cows had two E2-active follicles on
208 3 d to 4 d whereas some cows had no E2-active follicles on 5 d to 6 d. Also, if FFL samples were
209 lost during collection, then E2-status could not be determined and gene expression data was not
210 included in the analysis. For analysis of E2 concentrations in the subset of FFL samples, main
211 factors were: days post-ovulation (early, 3 d to 4 d, and late, 5 d to 6 d, growing phase of the first
212 dominant follicle), follicle group based on size (small E2-inactive, large E2-active or large E2-
213 inactive), and their various interaction. To evaluate the relationships among variables measured

in follicles > 5 mm in diameter (i.e., those collected individually), Pierson correlation coefficients were generated using CORR procedure of SAS. Because of the wide range and heterogeneous variances of the variables measured, log-transformed variables were correlated among each other. In vitro experiments (Exp. 2 and 3) were replicated three times (biological replicate) and within each experiment each treatment was duplicated, and data were analyzed via one-way ANOVA. To correct for heterogeneity of variance, target genes abundance was analyzed after transformation to natural log (x + 1). Mean differences were assessed using Fisher's protected least significant differences test [35] only if significant main effects (in ANOVA) were detected. Significance was declared at $P < 0.05$.

3. Results

3.1. In vivo Exp. 1

3.1.1. Follicle size, E2, and P4 concentrations in FFL

Follicle size and steroid concentrations in FFL have been reported for this study [21]. Briefly, diameter of large dominant E2-active, large subordinate E2-inactive, and medium E2-inactive follicles averaged 12.9 ± 0.5, 9.48 ± 0.36, and 6.37 ± 0.23 mm, respectively. Concentrations of E2 in FFL of large dominant E2-active, large subordinate E2-inactive, medium E2-inactive, and small E2-inactive follicles averaged 186.5 ± 29.5, 8.45 ± 3.7, 2.3 ± 0.8, and 2.0 ± 0.2 ng/mL, respectively. Concentrations of P4 in FFL did not differ ($P > 0.10$) among follicle groups and ranged between 61 ± 7 and 236 ± 42 ng/mL.

3.1.2. GC FGFR1c mRNA

234 Abundance of *FGFR1c* mRNA, the main receptor for FGF1 and FGF2, was significantly
235 affected by follicle group, but not by days post-ovulation or their interaction. Specifically,
236 *FGFR1c* mRNA abundance was 4.3-, 6.1-, and 4.2-fold greater ($P < 0.01$) in large, medium, and
237 small E2-inactive (E2/P4 ratio < 1), respectively, than in large E2-active (E2/P4 ratio > 1)
238 follicles, and was 1.4–fold greater ($P < 0.05$) in medium E2-inactive than in large and small E2-
239 inactive follicles (Fig. 1A). No other significant differences were detected among follicles of
240 different sizes and steroidogenic status.

241 *3.1.3. GC FGFR2c mRNA*

242 Abundance of *FGFR2c* mRNA, the second main receptor for FGF9 and FGF16, was
243 significantly affected by follicle group, but not by days post-ovulation or their interaction (Fig.
244 1A). Specifically, *FGFR2c* mRNA abundance was 7.5-, 10.4-, and 4.9-fold greater ($P < 0.01$) in
245 large, medium, and small E2-inactive follicles, respectively, than in large E2-active follicles. No
246 other significant differences were detected among follicles of different sizes and steroidogenic
247 status (Fig. 1A).

248 *3.1.4. GC FGFR3c and FGFR4 mRNA*

249 Abundance of *FGFR3c* mRNA in GC was not different ($P > 0.10$) among follicles of
250 different sizes and steroidogenic status or days post-ovulation (Fig. 1B). Abundance of *FGFR4*
251 mRNA in GC was not different ($P > 0.10$) among follicles of different sizes and steroidogenic
252 status or days post-ovulation (Fig. 1B).

253 *3.1.5. TC FGFR1c mRNA*

Abundance of *FGFR1c* mRNA in TC was significantly affected ($P < 0.05$) by follicle group, days post-ovulation, and their interaction. Specifically, *FGFR1c* mRNA abundance was 2.7- and 1.7-fold greater ($P < 0.05$) in medium E2-inactive than in large E2-active and small E2–inactive follicles, respectively, on 5 d to 6 d post-ovulation (Fig. 2A). Moreover, *FGFR1c* mRNA abundance was 2–fold greater in medium E2-inactive at late than at early growing phase of first dominant follicle. No significant differences in *FGFR1c* mRNA abundance were detected between large E2-active, large E2-inactive and medium E2-inactive follicles on 3 d to 4 d post-ovulation (Fig. 2A).

3.1.6. TC FGFR2c mRNA

Abundance of *FGFR2c* mRNA in TC was not different ($P > 0.10$) among follicles of different sizes and steroidogenic status or days post-ovulation. Relative abundance of *FGFR2c* mRNA averaged 1.0, 0.5, and 0.8 $\pm$ 0.3 for large E2-active, large E2-inactive, and medium E2-inactive follicles, respectively, on 3 d to 4 d post-ovulation, and averaged 0.6, 0.5 and 1.1 $\pm$ 0.3 for large E2-active, large E2-inactive, and medium E2-inactive follicles, respectively, on 5 d to 6 d post-ovulation.

3.1.7. TC FGFR3c and FGFR4 mRNA

Abundance of *FGFR3c* mRNA in TC was not different ($P > 0.10$) among follicles of different sizes and steroidogenic status or days post-ovulation. Relative abundance of *FGFR3c* mRNA averaged 1.0, 0.6, and 0.9 $\pm$ 0.3 for large E2-active, large E2-inactive, and medium E2-inactive follicles, respectively, on 3 d to 4 d post-ovulation, and averaged 0.9, 0.5 and 1.3 $\pm$ 0.3 for large E2-active, large E2-inactive, and medium E2-inactive follicles, respectively, on 5 d to 6 d post-ovulation.

Abundance of *FGFR4* tended ($P < 0.09$) to be affected by the follicle group x days post-ovulation interaction such that *FGFR4* mRNA abundance was 1.4–fold greater ($P < 0.05$) in medium E2-inactive at 5 d to 6 d post-ovulation than at 3 d to 4 d post-ovulation (Fig. 2B). In addition, *FGFR4* mRNA abundance was 5.2-fold greater ($P < 0.05$) in medium E2-inactive than in large E2-active follicles at late growing phase of first dominant follicle. No other significant differences were detected between follicles of different sizes at early or late growing phases of first dominant follicle.

3.1.8. Relative abundance of the various FGFR isoforms in GC and TC

Based on the average Ct values of each of the *FGFR* in freshly isolated GC and TC (Table 2), *FGFR1c* mRNA was the most abundant *FGFR* in both GC and TC. Specifically, abundance of GC *FGFR1c* mRNA was 30-, 42- and 315-fold greater than *FGFR2c*, *FGFR3c* and *FGFR4* mRNA abundance, respectively. In TC, *FGFR1c* mRNA abundance was 64-, 158- and 64-fold greater than *FGFR2c*, *FGFR3c* and *FGFR4* mRNA abundance, respectively.

3.1.9. Correlations

Negative correlations existed between follicular size and GC *FGFR1c* (r = -0.30), *FGFR2c* (r = - 0.32), and *FGFR4* (-0.36, $P < 0.01$, n = 108) mRNA abundance. In TC, a negative correlation existed between follicular size and *FGFR4* mRNA abundance (r = -0.29, $P < 0.01$, n = 82). No significant correlations were observed between follicular size and *FGFR3c* mRNA abundance in GC or between follicular size and *FGFR1c*, *FGFR2c*, or *FGFR3c* mRNA abundance in TC.

296 Negative correlations existed between FFL E2 concentrations and GC *FGFR1c* (r = -
297 0.71), *FGFR2c* (r = -0.70), *FGFR3c* (r = -0.42) and *FGFR4* (r = -0.42, $P < 0.01$, n = 108) mRNA
298 abundance. In TC, negative correlations existed between FFL E2 concentrations and *FGFR1c* (r
299 = -0.30) and *FGFR4* (r = -0.30, $P < 0.05$, n = 82) mRNA abundance, whereas no significant
300 correlations were observed between FFL concentrations of E2 and TC *FGFR2c* or *FGFR3c*
301 mRNA abundance.

302 Positive correlations were detected between FFL concentrations of P4 and GC *FGFR1c* (r
303 = 0.32, $P < 0.01$), *FGFR2c* (r = 0.22, $P < 0.05$), and *FGFR3c* (r = 0.35, $P < 0.01$, n = 108)
304 mRNA abundance. In TC, levels of P4 in FFL were also positively correlated with abundance of
305 *FGFR1c* (r = 0.35, $P < 0.01$) and *FGFR4* (r = 0.24, $P < 0.05$, n = 82) mRNA. No significant
306 correlations were detected between FFL concentrations of P4 and GC *FGFR4* mRNA abundance
307 or TC *FGFR2c* and *FGFR3c* mRNA abundance.

308 Negative correlations were detected between FFL E2/P4 ratio and GC *FGFR1c* (r = -
309 0.51), *FGFR2c* (r = -0.59), *FGFR3c* (r = -0.29) and *FGFR4* (r= -0.32, $P < 0.01$, n = 108) mRNA
310 abundance. In TC, *FGFR1c* mRNA abundance was negatively correlated with FFL E2/P4 ratio (r
311 = -0.30, $P < 0.01$, n = 82). No significant correlations were detected between FFL E2/P4 ratio
312 and GC *FGFR4* mRNA abundance or between FFL E2/P4 ratio and TC *FGFR2c*, *FGFR3c*, or
313 *FGFR4* mRNA abundance.

314 *3.2. In vitro Exp. 2 and 3*

315 *3.2.1. Effects of steroids on FGFR mRNA abundance in GC (Exp. 2)*

316 Treatment of small-follicle GC with E2 had no effect (P > 0.10) on *FGFR1c*, *FGFR2c*
317 and *FGFR3c* mRNA abundance (Fig. 3A). However, A4 increased ($P < 0.05$) *FGFR1c, FGFR2c*

and *FGFR4* mRNA abundance by 2-fold, while A4 treatment had no effect ($P > 0.10$) on *FGFR3c* mRNA abundance in small-follicle GC (Fig. 3A). Based on the average Ct values of each of the FGFR in cultured GC (Table 2), abundance of *FGFR3c* mRNA was 1.5-, 84- and 4390-fold greater than *FGFR1c*, *FGFR2c* and *FGFR4* mRNA abundance, respectively. Statistical analysis of the *18S* Ct revealed no significant effect of treatment.

3.2.2. Effects of steroids on FGFR mRNA abundance in TC (Exp. 3)

Treatment of large-follicle TC with E2 or A4 had no effect ($P > 0.10$) *FGFR1c*, *FGFR2c*, *FGFR3c* or *FGFR4* mRNA abundance in large-follicle TC (Fig. 3B). Based on the average Ct values of each of the FGFR in cultured TC (Table 2), abundance of *FGFR1c* mRNA was 128-, 1024- and 1352-fold greater than *FGFR2c*, *FGFR3c* and *FGFR4* mRNA abundance, respectively. Statistical analysis of the *18S* Ct revealed no significant effect of treatment.

4. Discussion

Evidence for a role of FGF in the mammalian ovary was first reported in the seventies [36] when acidic FGF (FGF1) was found to stimulate proliferation of bovine GC and luteal cells. To date, ten members of the FGF family have been shown to regulate ovarian follicular function via altering GC and TC proliferation and steroidogenesis [for reviews, see 4, 5, 13]. The diversity of roles played by FGF is influenced by the nature of the ligands and the four families of high affinity FGFR and cofactors that regulate the FGF signaling complex [12, 13, 37, 38]. The present study determined whether FGFR1c, FGFR2c, FGFR3c, and FGFR4 change according to follicular size, steroidogenic status, and days post-ovulation during growth of first-wave dominant follicles tracked via ultrasonography in dairy cattle. The present study also

revealed for the first time that the most abundant of these 4 isoforms of FGFR was *FGFR1c* and *FGFR3c* for GC and *FGFR1c* and *FGFR2c* for TC, and that *FGFR4* was a scarce mRNA particularly in GC. Consistent with the latter observation, Buratini et al. [19] was unable to detect *FGFR4* mRNA in GC of cattle.

In the present study, *FGFR1c* and *FGFR2c* mRNA abundance was greater in GC from E2-inactive follicles of all sizes (i.e., subordinate follicles) than in GC from large E2-active follicles (i.e., dominant follicles) whereas GC *FGFR3c* and *FGFR4* mRNA abundance did not differ among follicle types during the first follicular wave in dairy cattle. In beef cattle, *FGFR2b* but not *FGFR1b* mRNA abundance was greater in GC of subordinate vs. dominant follicles [18, 20]. Previous studies using abattoir-collected bovine ovaries found that mRNA for all four FGFR subtypes were detected in bovine cumulus cells [2], and determined that abundance of *FGFR3c* mRNA in GC significantly increased with increasing follicle size and with increasing E2 levels in FFL [19] whereas *FGFR2c* mRNA in GC did not change and *FGFR2b* mRNA in GC increased with increasing E2 levels in FFL [14]. Reasons for some of the differences between the present and previous studies is unknown but may be due to breed differences or due to abattoir vs. synchronized-estrus collected ovaries. Nonetheless, previous studies [14, 18, 20] consistently reported increased *FGFR2b* mRNA abundance in dominant and E2-active follicles.

Also in the present study, TC from medium E2-inactive follicles had greater *FGFR1c* and *FGFR4* mRNA abundance than TC from large E2-active follicles while TC *FGFR2c* and *FGFR3c* mRNA abundance did not differ among follicle types. Similarly, previous studies using abattoir-collected beef cattle ovaries determined that abundance of *FGFR4* mRNA in TC decreased with increasing follicle size but did not change with changes in E2 levels in FFL, whereas *FGFR3c* mRNA in TC did not change with either follicle size or E2 levels [19]. Berisha

et al. [14] reported that abundance of *FGFR2b* and *FGFR2c* mRNA in TC did not change with increasing E2 levels in FFL, whereas Castilho et al., [18] reported that abundance of *FGFR2b* mRNA in TC was greater in subordinate vs. dominant follicles. Therefore, collective evidence indicates that *FGFR* gene expression in TC is less regulated than *FGFR* gene expression in GC of cattle. In further support of this conclusion, abundance of *FGFR1c*, *FGFR2c*, *FGFR3c* and *FGFR4* mRNA in GC was negatively correlated with FFL E2 concentrations and E2/P4 ratio whereas only *FGFR1c* mRNA abundance in TC was negatively correlated with FFL E2 concentrations and E2/P4 ratio in the present study. These results also suggest that E2 may be inhibitory to *FGFR* gene expression. To test this hypothesis we evaluated the effect of E2 and A4 on *FGFR* gene expression in GC and TC and found that A4 stimulated *FGFR1c*, *FGFR2c* and *FGFR4* mRNA in GC but had no effect on expression of any of the *FGFR* genes in TC. In vitro treatment with E2 decreased only *FGFR2c* mRNA in GC, supporting the hypothesis that E2 may be directly driving the decrease in *FGFR2c* mRNA in E2-active follicles, a conclusion supported by the high negative correlation between GC *FGFR2c* mRNA abundance and FFL E2 concentrations (i.e., r = -0.70) in the present study. Similarly, in vivo, E2 inhibits *FGFR2* mRNA in mouse mammary glands [39].

Because androgens are needed to produce estrogens, and the novel observation that A4 increased *FGFR1c* and *FGFR2c* mRNA in GC suggests that elevated androgens (i.e., when aromatase is low) may act to slow differentiation by promoting increases in receptors for the known anti-differentiation factors, FGF2 and FGF9 [41]. A follicle's estrogenic status can be used to assess the health of follicles, and thus, large E2-active follicles are considered as those selected to escape atresia and become dominant [42, 43]. The present results indicate that FGFR1c and FGFR2c are produced in greater amounts in GC from subordinate than dominant

follicles, implying a pro-atretic or an anti-differentiation role for these receptors. The fact that relative abundance of mRNA for *FGFR1c* and *FGFR2c* in GC and *FGFR1c* in TC are negatively correlated with size and E2/P4 ratio reinforces this idea. The present study also supports the idea that E2 and A4 may be regulating some of the changes in GC *FGFR* mRNA abundance. The greater expression of GC *FGFR1c* and *FGFR2c* in E2-inactive follicles parallel changes in *FGF9* mRNA reported from this same study [44]. Interestingly, both E2 and androgens also induce *FGF9* mRNA expression in bovine GC [44]. In porcine GC, FGF9 suppresses whereas IGF1 induces *FGFR2c* mRNA expression [45], but whether FGF9 or IGF1 is directly inducing *FGFR1c* or *FGFR2c* expression in bovine GC will require further study. Previous studies have reported that FSH increases abundance of *FGFR1c*, *FGFR2c* and *FGFR3c* mRNA in bovine GC [17, 19], and thus, additional work should be conducted to evaluate if other hormones or growth factors regulate expression of the various *FGFR* in bovine GC and TC.

Abundance of GC *FGFR4* mRNA was not different among follicles of different steroidogenic status and sizes, but it was greater in TC from medium E2-inactive follicles at 5 d to 6 d post-ovulation than at 3 d to 4 d post-ovulation. In addition, *FGFR4* mRNA abundance in TC was negatively correlated with size and with FFL E2 concentrations and positively correlated with FFL P4 concentrations. Since transcripts for *FGFR4* only changed in TC, but not in GC across days, it is likely to suppose that the action of the ligands that bind to FGFR4 (e.g., FGF8) may be more functionally important in TC than in GC. This is in agreement with previous observations [19] where *FGFR4* mRNA was only detected in TC, but not in GC or oocytes from bovine antral follicles. Although we detected *FGFR4* mRNA in GC and its expression was up-regulated by A4, it was the least expressed *FGFR* in GC. The fact that *FGFR4* mRNA is increasing in medium-sized E2-inactive follicles during follicular selection and dominance is an

410 indication that this receptor could be playing a role in preventing differentiation of this size class
411 of antral follicles. Similarly, Buratini et al. [19] found that transcripts for *FGFR4* were greater in
412 TC from small than from large antral follicles of cattle. However, the hormones or growth
413 factors that are regulating these changes in TC *FGFR4* mRNA will require further study, as E2
414 and A4 had no effect on *FGFR4* mRNA abundance in TC of the present study.

415 Interestingly, several FGF that preferentially bind to FGFR1c, FGFR2c, FGFR3c, and
416 FGFR4 appear to be critical regulators of large follicle differentiation and atresia. For example,
417 FGF2 (preferentially binding to FGFR1c and FGFR3c), FGF9 (preferentially binding to FGFR3c
418 followed by FGFR2c), and FGF17 and FGF18 (preferentially binding to FGFR3c followed by
419 FGFR4) inhibit steroidogenic enzyme activity and FSH-stimulated E2 production by GC in cattle
420 [16, 41, 46, 47]. Because E2 is important for GC survival, oocyte maturation and differentiation
421 of dominant follicles [3, 48, 49], FGF suppression of E2 production may be playing a role in
422 inducing atresia or preventing differentiation of GC in cattle. The fact that some of the ligands
423 that preferentially bind to FGFR3c, including FGF9, FGF17, and FGF18, have a greater mRNA
424 abundance in subordinate or atretic antral follicles than in dominant follicles in cattle [8, 16, 44]
425 reinforces this idea. Furthermore, of the ligands mentioned above, FGF18 induced regression of
426 the dominant follicle when injected in vivo and increased cleaved caspase-3 in GC in vitro [50],
427 which is a major downstream effector of apoptosis and serves a marker for GC apoptosis [51],
428 confirming a role for FGF18 in the induction of atresia in bovine antral follicles.

429 It is noteworthy that some members of the FGF family that preferentially bind to
430 FGFR1c, FGFR2c and/or FGFR3c are mitogenic factors of ovarian follicle somatic cells of
431 cattle. Specifically, FGF1 [36, 52, 53] stimulates GC proliferation whereas FGF2 [54, 55] and
432 FGF9 [8, 48] stimulate both GC and TC proliferation. In addition, FGF9 appears to be

stimulating GC and TC proliferation via induction of expression of genes related to cell proliferation such as MAPK/ERK and CCND1 in TC [56]. Hence, FGFR1c, FGFR2c and FGFR3c and their ligands appear to be playing a positive role in development and selection of bovine antral follicles via stimulation of mitosis. However, additional research is needed to further elucidate the physiological regulation of the various FGFRs during growth and atresia of ovarian follicles in cattle.

5. Conclusions

In summary, GC of large, medium and small E2-inactive follicles had greater abundance of *FGFR1c* and *FGFR2c* mRNA than in large E2-active follicles, and *FGFR1c* and *FGFR4* mRNA abundance was greater in TC of medium E2-inactive follicles at the late than early growing phase of first dominant follicle. Furthermore, *FGFR1c* and *FGFR4* mRNA relative abundance was greater in TC of medium E2-inactive follicles than large E2-active and E2-inactive follicles at the late than early growing phase of first dominant follicle. In vitro evidence indicated that E2 may be directly inhibiting GC *FGFR2c* mRNA expression and the stimulatory effect of A4 on GC *FGFR1c, FGFR2c* and *FGFR4* indicates that changes in FFL androgen levels may be driving changes in GC *FGFR* gene expression as well. However, the hormones or factors that regulate changes in TC *FGFR1c* and *FGFR4* will require further study. Also, future research will be required to verify the protein expression levels of these various FGFR proteins in bovine GC and TC. Taken together, the previous and present findings suggest a role for FGF and their receptors as anti-differentiation factors of follicular GC and TC in a mono-ovulatory species such as cattle.

Acknowledgements

The authors thank Lingna Zhang, Jeff Williams and John Evans for laboratory assistance; David Jones, Jeff Davis and other members of the OSU Dairy Cattle Center for care and management of the cows. This work supported in part by: the NICHD, National Institutes of Health, through Agreement R15-HD-066302 (to L.J.S), and the Oklahoma State University Agricultural Experiment Station (OKL02970 to L.J.S.) and The Endowment of Howard M. & Adene R. Harrington Chair in Animal Science (Project 21-58500 to L.J.S.).

References

[1] Eppig JJ. Intercommunication between mammalian oocytes and companion somatic-cells. Bioessays 1991; 13:569-574.

[2] Zhang K, Hansen PJ, Ealy AD. Fibroblast growth factor 10 enhances bovine oocyte maturation and developmental competence in vitro. Reproduction 2010;140(6):815-26. doi: 10.1530/REP-10-0190.

[3] Fortune, JE., Rivera GM, Yang MY. Follicular development: The role of the follicular microenvironment in selection of the dominant follicle. Anim Reprod Sci 2004; 82-83:109-126.

[4] Chaves RN, de Matos MHT, Buratini J, de Figueiredo JR. The fibroblast growth factor family: Involvement in the regulation of folliculogenesis. Reprod Fertil Dev 2012; 24:905-915.

[5] Price CA. Mechanisms of fibroblast growth factor signaling in the ovarian follicle. J Endocrinol 2016; 228:R31-43.

[6] Ornitz DM, Itoh N. Fibroblast growth factors. Genome Biol 2001; 2:REVIEWS3005.

[7] Itoh N, Ornitz DM. Fibroblast growth factors: From molecular evolution to roles in development, metabolism and disease. J Biochem 2011; 149:121-130.

[8] Schreiber NB, Totty ML, Spicer LJ. Expression and effect of fibroblast growth factor 9 in bovine theca cells. J Endocrinol 2012; 215:167-175.

[9] Dell KR., Williams LT. A novel form of fibroblast growth factor receptor 2. Alternative splicing of the third immunoglobulin-like domain confers ligand binding specificity. J Biol Chem 1992; 267:21225-21229.

[10] Plotnikov AN, Hubbard SR, Schlessinger J, Mohammadi M. Crystal structures of two fgf-fgfr complexes reveal the determinants of ligand-receptor specificity. Cell 2000; 101:413-424.

[11] Ornitz DM, Itoh N. The fibroblast growth factor signaling pathway. Wiley Interdiscip Rev. Dev. Biol. 2015; 4:215-266.

[12] Itoh N, Ornitz DM. Evolution of the FGF and FGFR gene families. Trends Genet 2004; 20:563-569.

[13] Li XW, Wang C, Xiao J, McKeehan WL, Wang F. Fibroblast growth factors, old kids on the new block. Semin Cell Dev Biol 2016; 53:155-167.

[14] Berisha B, Sinowatz F, Schams D. Expression and localization of fibroblast growth factor (FGF) family members during the final growth of bovine ovarian follicles. Mol Reprod Dev 2004; 67:162-171.

[15] Buratini J, Pinto MGL, Castilho AC, Amorim RL, Giometti IC, Portela VM, Nicola ES, Price CA. Expression and function of fibroblast growth factor 10 and its receptor, fibroblast growth factor receptor 2B, in bovine follicles. Biol Reprod 2007; 77:743-750.

[16] Machado MF, Portela VM, Price CA, Costa IB, Ripamonte P, Amorim RL, Buratini J. Regulation and action of fibroblast growth factor 17 in bovine follicles. J Endocrinol 2009; 202:347-353.

497 [17] Zhang K, Ealy AD. Disruption of fibroblast growth factor receptor signaling in bovine cumulus-
498 oocyte complexes during in vitro maturation reduces subsequent embryonic development. Domest
499 Anim Endocrinol 2012; 42:230-238.

500 [18] Castilho ACS., Price CA, Dalanezi F, Ereno RL, Machado MF, Barros CM, Gasperin BG, Goncalves
501 PBD, Buratini J. Evidence that fibroblast growth factor 10 plays a role in follicle selection in cattle.
502 Reprod. Fertil. Dev 2017; 29:234-243.

503 [19] Buratini J, Teixeira AB, Costa IB, Glapinski VF, Pinto MGL, Giometti IC, Barros CM, Cao M,
504 Nicola ES, Price CA. Expression of fibroblast growth factor-8 and regulation of cognate receptors,
505 fibroblast growth factor receptor-3c and-4, in bovine antral follicles. Reproduction 2005; 130:343-
506 350.

507 [20] Gasperin BG, Ferreira F, Rovani MT, Santos JT, Buratini J, Price CA, Goncalves PB. FGF10
508 inhibits dominant follicle growth and estradiol secretion in vivo in cattle. Reproduction 2012;
509 143:815-823.

510 [21] Dentis JL, Schreiber NB, Gilliam JN, Schutz LF, Spicer LJ. Changes in brain ribonuclease (BRB)
511 mRNA in granulosa cells (GC) of dominant versus subordinate ovarian follicles of cattle and the
512 regulation of *BRB* gene expression in bovine GC. Domest Anim Endocrinol 2016; 55:32-40.

513 [22] Aad PY, Echternkamp SE, Sypherd DD, Schreiber NB, Spicer LJ. The hedgehog system in ovarian
514 follicles of cattle selected for twin ovulations and births: Evidence of a link between the IGF and
515 hedgehog systems. Biol Reprod 2012; 87:79.

516 [23] Stewart, R. E., L. J. Spicer, T. D. Hamilton, B. E. Keefer, L. J. Dawson, G. L. Morgan, and S. E.
517 Echternkamp. 1996. Levels of insulin-like growth factor (IGF) binding proteins, luteinizing
518 hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in
519 cattle exhibiting regular estrous cycles. Endocrinology 137:2842-2850.

[24] Spicer LJ, Enright WJ. Concentrations of insulin-like growth factor I and steroids in follicular fluid of preovulatory bovine ovarian follicles: effect of daily injections of a growth hormone-releasing factor analog and(or) thyrotropin-releasing hormone. J Anim Sci 1991;69(3):1133-1139. doi: 10.2527/1991.6931133x.

[25] Zhang L, Schütz LF, Robinson CL, Totty ML, Spicer LJ. Evidence that gene expression of ovarian follicular tight junction proteins is regulated in vivo and in vitro in cattle. J Anim Sci 2017; 95:1313-1324.

[26] Spicer LJ. Effects of estradiol on bovine thecal cell function in vitro: dependence on insulin and gonadotropins. J Dairy Sci 2005; 88:2412-2421.

[27] Langhout DJ, Spicer LJ, Geisert RD. Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. J Anim Sci 1991; 69 3321-3334.

[28] Spicer LJ, Chamberlain CS. Influence of cortisol on insulin- and insulin-like growth factor 1 (IGF-1)-induced steroid production and on IGF-1 receptors in cultured bovine granulosa cells and thecal cells. Endocrine 1998; 9:153-161.

[29] Stewart RE, Spicer LJ, Hamilton TD, Keefer BE. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. J Anim Sci 1995; 73:3719-3731.

[30] Spicer LJ, Alpizar E, Echternkamp SE. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production in vitro. J Anim Sci 1993; 71:1232-1241.

[31] Spicer LJ, Aad PY, Allen DT, Mazerbourg S, Payne AH, Hsueh AJ. Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: influence of

follicle size on responses to GDF9. Biol Reprod 2008; 78:243-53.

[32] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-408.

[33] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 2000; 46:69-81.

[34] Voge JL, Aad PY, Santiago CA, Goad DW, Malayer JR, Allen DT, Spicer LJ. Effect of insulin-like growth factors (IGF), FSH, and leptin on IGF-binding-protein mRNA expression in bovine granulosa and theca cells: quantitative detection by real-time PCR. Peptides 2004; 25:2195-2203.

[35] Ott L. 1977. An introduction to statistical methods and data analysis Duxbury Press, North Scituate, MA.

[36] Gospodarowicz D, Ill CR, Birdwell CR. Effects of fibroblast and epidermal growth-factors on ovarian cell-proliferation in vitro .1. Characterization of response of granulosa-cells to FGF and EGF. Endocrinology 1977; 100:1108-1120.

[37] Givol D, Yayon A. Complexity of FGF receptors: Genetic basis for structural diversity and functional specificity. FASEB J 1992; 6:3362-3369.

[38] Dailey L, Ambrosetti D, Mansukhani A, Basilico C. Mechanisms underlying differential responses to FGF signaling. Cytokine & Growth Factor Rev 2005; 16:233-247.

[39] Imagawa W, Pedchenko VK. In vivo inhibition of keratinocyte growth factor receptor expression by estrogen and antagonism by progesterone in the mouse mammary gland. J Endocrinol 2001; 171:319-327.

[40] Ireland JJ, Roche JF. Development of antral follicles in cattle after prostaglandin-induced luteolysis: Changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. Endocrinology 1982; 111:2077-2086.

568 [41] Schreiber NB, Spicer LJ. Effects of fibroblast growth factor 9 (FGF9) on steroidogenesis and gene
569 expression and control of FGF9 mRNA in bovine granulosa cells. Endocrinology 2012;
570 153:4491-4501.
571 [42] Mihm M, Evans AC. Mechanisms for dominant follicle selection in monovulatory species: a
572 comparison of morphological, endocrine and intraovarian events in cows, mares and women.
573 Reprod Domest Anim 2008; 43 Suppl 2:48-56.

574 [43] Ginther OJ. 2016. The theory of follicle selection in cattle. Domest Anim Endocrinol. 57:85-99.

575 [44] Schütz LF, Schreiber NB, Gilliam JN, Cortinovis C, Totty ML, Caloni F, Evans JR, Spicer LJ.
576 Changes in fibroblast growth factor 9 mRNA in granulosa and theca cells during ovarian follicular
577 growth in dairy cattle. J Dairy Sci 2016; 99:9143-9151.

578 [45] Evans JR, Schreiber NB, Williams JA, Spicer LJ. Effects of fibroblast growth factor 9 on
579 steroidogenesis and control of FGFR2IIIc mRNA in porcine granulosa cells. J Anim Sci
580 2014; 92:511-519.
581 [46] Vernon RK, Spicer LJ. Effects of basic fibroblast growth factor and heparin on follicle-stimulating
582 hormone-induced steroidogenesis by bovine granulosa cells. J Anim Sci 1994; 72:2696-2702.

583 [47] Portela VM, Machado M, Buratini J, Zamberlam G, Amorim RL, Goncalves P, Price CA.
584 Expression and function of fibroblast growth factor 18 in the ovarian follicle in cattle. Biol. Reprod
585 2010; 83:339-346.

586 [48] Spicer, L.J., and S.E. Echternkamp. The ovarian insulin and insulin-like growth factor system
587 with an emphasis on domestic animals. Dom. Anim. Endocrinol.12:223-245, 1995.
588 [49] Knecht M, Brodie AM, Catt KJ. Aromatase inhibitors prevent granulosa cell differentiation: An
589 obligatory role for estrogens in luteinizing hormone receptor expression. Endocrinology 1985;
590 117:1156-1161.

[50] Portela VM, Dirandeh E, Guerrero-Netro HM, Zamberlam G, Barreta MH, Goetten AF, Price CA. The role of fibroblast growth factor-18 in follicular atresia in cattle. Biol Reprod 2015; 92:14.

[51] Feranil JB, Isobe N, Nakao T. Apoptosis in the antral follicles of swamp buffalo and cattle ovary: Tunel and caspase-3 histochemistry. Reprod Domest Anim 2005; 40:111-116.

[52] Hoshi H, Konno S, Kikuchi M, Sendai Y, Satoh T. Fibroblast growth factor stimulates the gene expression and production of tissue inhibitor of metalloproteinase-1 in bovine granulosa cells. In Vitro Cell Dev Biol Anim 1995; 31:559-63.

[53] Schams D, Kosmann M, Berisha B, Amselgruber WM, Miyamoto A. Stimulatory and synergistic effects of luteinising hormone and insulin like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulosa cells. Exp Clin Endocrinol Diabetes 2001; 109:155-162.

[54] Gospodarowicz D, Cheng J, Lui GM, Baird A, Esch F, Bohlen P. Corpus luteum angiogenic factor is related to fibroblast growth factor. Endocrinology 1985; 117:2383-2391.

[55] Spicer LJ, Stewart RE. Interactions among basic fibroblast growth factor, epidermal growth factor, insulin, and insulin-like growth factor-I (IGF-I) on cell numbers and steroidogenesis of bovine thecal cells: Role of IGF-I receptors. Biol Reprod 1996; 54:255-263.

[56] Totty ML, Morrell BC, Spicer LJ. Fibroblast growth factor 9 (FGF9) regulation of cyclin D1 and cyclin-dependent kinase-4 in ovarian granulosa and theca cells of cattle. Mol Cell Endocrinol 2017; 440:25-33.

610 **Table 1**. Sequences and characteristics for primers (forward and reverse) and probes for real-time PCR amplification of target genes.

Target Gene[1]	Oligo[2]	Sequence	Accession	Tm[3] (°C)	Concentration (nM)
FGFR1c	FWD	AGGTGAACGGGAGTAAGATTGG	XM_010820329.3	56.5	200
	REV	GTGCAGCACCTCCATCTCTTT		57.6	200
	Probe	TCTTGAAGACGGCCGGAGTTAACACCA		63.3	100
FGFR2c	FWD	GTTCCAATGCGGAAGTGCTG	XM_010820096.3	57.1	200
	REV	GTTTTGGCAGGACAGTGAGC		56.8	200
	Probe	AGGCGGATGCTGGCGAGTATATTTGTAAGG		63.9	100
FGFR3c	FWD	TAACACCACCGACAAGGAGC	NM_174318.3	57.2	200
	REV	CCACGCAGAGTGATGGGAAA		57.6	200
	Probe	TGCGCAATGTCACCTTTGAGGACG		62.0	100
FGFR4	FWD	CACTGCCCCCCAGAGCTATAC	XM_005209123.3	59.5	200
	REV	AGGACCTTGTCCAGTGCCTCTA		59.6	200
	Probe	AGCACCCTCTCAGAGGCCCACTTTCA		65.3	100

[1]Target genes: = fibroblast growth factor receptor (FGFR) 1c, 2c, 3c and 4.

[2]FWD = forward; REV = reverse.

[3]Tm = melting temperature.

615

Table 2. Average Ct values from quantitative PCR analysis of *fibroblast growth factor receptor (FGFR) 1c*, *2c*, *3c* and *4* mRNA in granulosa cells (GC) and theca cells (TC) of Exp. 1-3.

620

Exp.	*FGFR* subtype	GC *FGFR* mRNA Ct averages[1]	TC *FGFR* mRNA Ct averages
1	*FGFR1c*	26.3	24.6
1	*FGFR2c*	31.2	30.6
1	*FGFR3c*	31.7	31.9
1	*FGFR4*	34.6	30.6
2	*FGFR1c*	23.3	--
2	*FGFR2c*	29.1	--
2	*FGFR3c*	22.7	--
2	*FGFR4*	34.8	--
3	*FGFR1c*	--	24.0
3	*FGFR2c*	--	30.8
3	*FGFR3c*	--	34.1
3	*FGFR4*	--	34.4

[1]Ct = Threshold cycle value.

625

Fig. 1. Effects of follicular size (Lg = Large; Md = Medium; Sm = Small) and E2 status (EA = estrogen active; EI = estrogen inactive) on *FGF1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* mRNA relative abundance in bovine granulosa cells averaged across days 3 to 4 and days 5 to 6. Panel A: Effects of follicular size and E2 status on *FGF1c* and *FGFR2c* mRNA in bovine granulosa cells; n = 16, 33, 64, and 29 and for Lg-EA, Lg-EI, Md-EI and Sm-EI, respectively. Panel B: Lack of effect of follicular size and E2 status on *FGFR3c* and *FGFR4* mRNA in bovine granulosa cells; n = 16, 33, 62, and 28 for Lg-EA, Lg-EI, Md-EI and Sm-EI, respectively. For each FGFR, values are expressed as a ratio (fold $\pm$ SEM) of the Lg-EA values. [abc]Within a panel and FGFR subtype, means without a common letter differ ($P < 0.05$).

Fig. 2. Effects of follicular size (Lg = Large; Md = Medium; Sm = Small), E2 status (EA = estrogen active; EI = estrogen inactive) and day post-ovulation (day 3 to 4 or day 5 to 6) on *FGF1c* (Panel A) and *FGFR4* (Panel B) mRNA relative abundance in bovine theca cells. Panel A: Effects of follicular size and E2 status on *FGF1c* mRNA in bovine theca cells; n = 9, 10, and 27 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 3 to 4; n = 4, 11, and 22 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 5 to 6. Panel B: Effects of follicular size and E2 status on *FGF4* mRNA in bovine theca cells; n = 9, 11, and 25 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 3 to 4; n = 4, 11, and 22 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 5 to 6. For each FGFR, values are expressed as a ratio (fold $\pm$ SEM) of the Lg-EA values. [ab]Within a panel, means without a common letter differ ($P < 0.05$).

Fig. 3: In vitro effects of E2 and A4 on abundance of *FGFR1c*, *FGFR2c*, *FGFR3c* and *FGFR4* mRNA in bovine granulosa cells (Exp. 3) and theca cells (Exp. 4). Granulsoa cells and theca cells were isolated and cultured in 10% FCS and then treated in serum-free medium with

300 ng/mL of either E2 or A4 for 24 h. Values (± SEM; n = 6) are expressed as a ratio (fold ± SEM) of the controls. *Within a Panel and FGFR subtype, mean differs from control ($P < 0.05$).

Figure 1

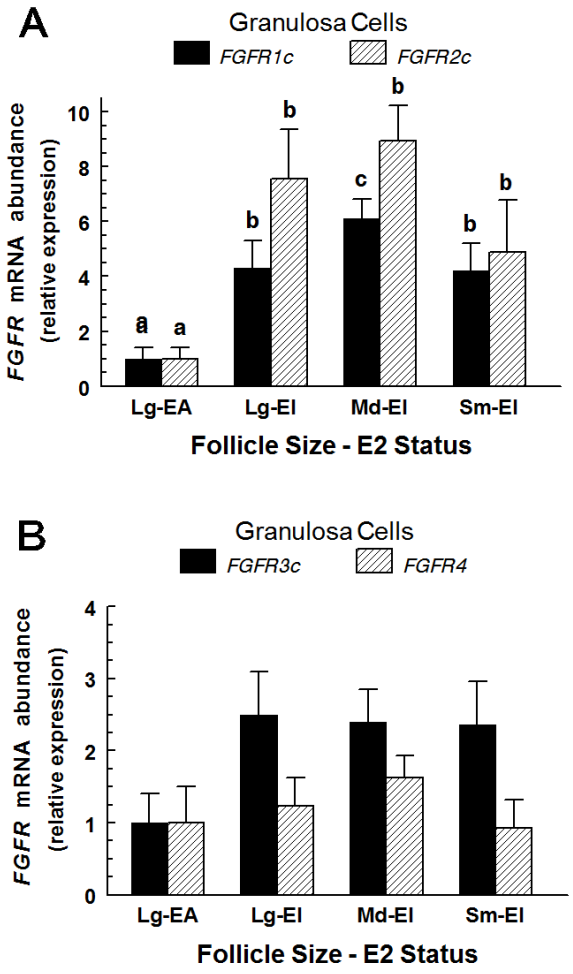
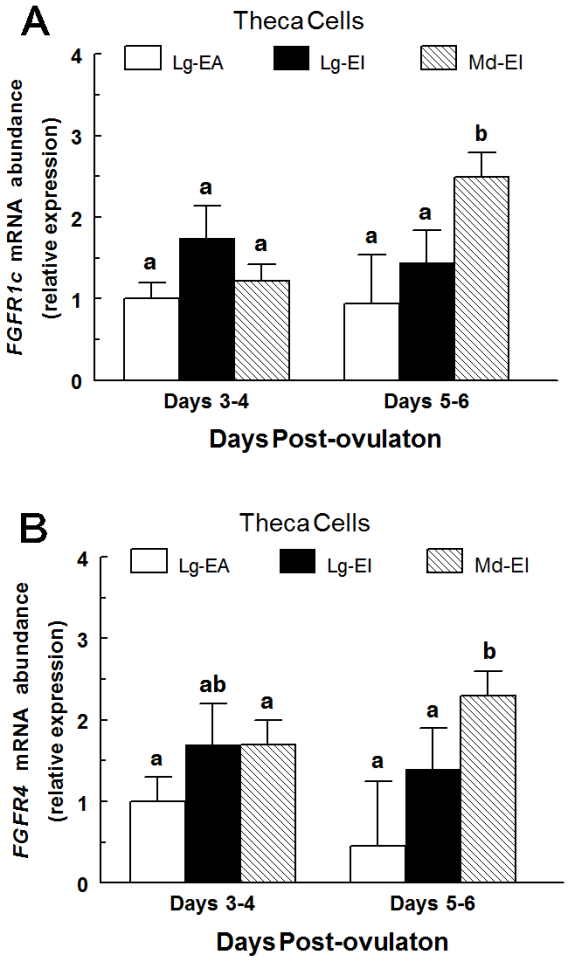

A
Granulosa Cells
FGFR1c
FGFR2c
FGFR mRNA abundance (relative expression)
0
2
4
6
8
10
a
a
b
b
c
b
b
b
Lg-EA
Lg-EI
Md-EI
Sm-EI
Follicle Size - E2 Status
B
Granulosa Cells
FGFR3c
FGFR4
FGFR mRNA abundance (relative expression)
0
1
2
3
4
Lg-EA
Lg-EI
Md-EI
Sm-EI
Follicle Size - E2 Status

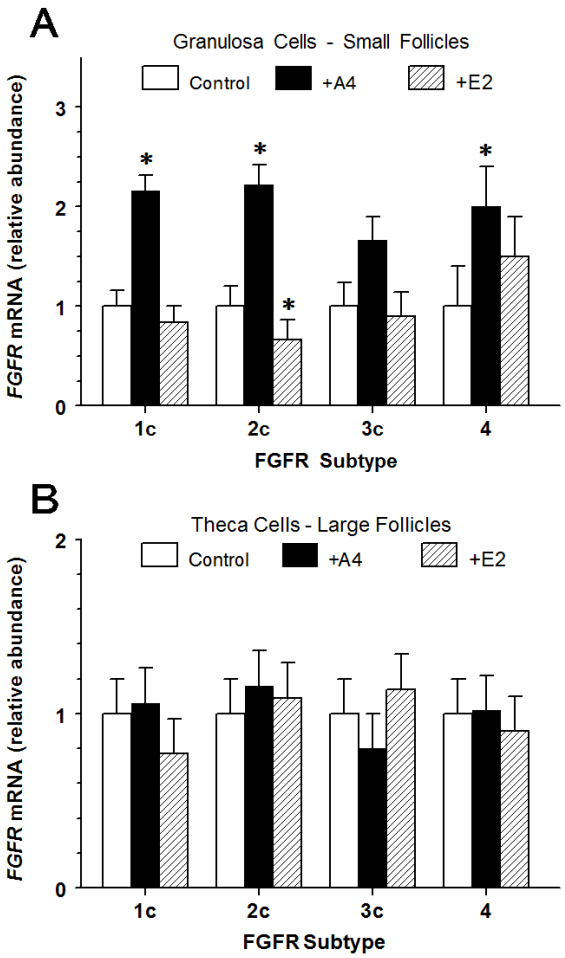
Figure 2



665

670

Figure 3


A
Granulosa Cells - Small Follicles
Control
+A4
+E2
FGFR mRNA (relative abundance)
0
1
2
3
*
*
*
1c
2c
3c
4
FGFR Subtype
B
Theca Cells - Large Follicles
Control
+A4
+E2
FGFR mRNA (relative abundance)
0
1
2
1c
2c
3c
4
FGFR Subtype