Hormonal regulation of *vascular endothelial growth factor A (VEGFA)* gene expression in granulosa and theca cells of cattle¹

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ABSTRACT: Vascular endothelial growth factor A (VEGFA) stimulates angiogenesis and is associated with increased vascularity in ovarian follicles of cattle. The objectives of this study were to investigate the developmental and hormonal regulation of *VEGFA* expression in ovarian granulosa and theca cells (TC) of cattle. Bovine ovaries were collected from a local slaughterhouse and granulosa cells (GC) and TC were collected from small (SM; 1 to 5 mm) and large (LG; 8 to 20 mm) follicles. Cells were collected fresh or cultured in serum-free medium and treated with various factors that regulate angiogenesis and follicular development. RNA was collected for analysis of *VEGFA* mRNA abundance via quantitative PCR. In SM-follicle GC (SMGC), prostaglandin E2 (PGE2) and FSH decreased ($P < 0.05$) *VEGFA* mRNA abundance by 30 to 46%, whereas in LG-follicle GC (LGGC), PGE2 and FSH were without effect ($P > 0.10$). In SMGC, dihydrotestosterone (DHT), sonic hedgehog (SHH), and growth differentiation factor-9 (GDF9) decreased ($P < 0.05$) *VEGFA* expression by 30 to 40%. Fibroblast growth factor-9 (FGF9) and estradiol (E2) were without effect ($P > 0.10$)

on *VEGFA* mRNA in both SMGC and LGGC, whereas progesterone increased ($P < 0.05$) *VEGFA* mRNA in LGGC but had no effect in LGTC. Bone morphogenetic protein-4 (BMP4), LH, and FGF9 increased ($P < 0.05$) abundance of *VEGFA* mRNA by 1.5- to 1.9-fold in LGTC. Insulin-like growth factor-1 (IGF1) was without effect ($P > 0.10$) on *VEGFA* mRNA in both TC and GC. An E2F transcription factor inhibitor, HLM0064741 (E2Fi), dramatically (i.e., 8- to 13-fold) stimulated ($P < 0.01$) the expression of *VEGFA* mRNA expression in both SMGC and LGTC. Abundance of *VEGFA* mRNA was greater ($P < 0.05$) in LGGC and SMGC than in LGTC. Also, SMTTC had greater ($P < 0.05$) abundance of *VEGFA* mRNA than LGTC. In conclusion, *VEGFA* mRNA abundance was greater in GC than TC, and *VEGFA* expression decreased in TC during follicle development. Some treatments either suppressed, stimulated, or had no effect on *VEGFA* expression depending on the cell type. The inhibition of E2F transcription factors had the greatest stimulatory effect of all treatments evaluated, and thus, E2Fs may play an important role in regulating angiogenesis during follicle growth in cattle.

Key words: cattle, follicle, granulosa cell, theca cell, vascular endothelial growth factor

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J. Anim. Sci. 2019.97:3034–3045

doi: 10.1093/jas/skz164

¹The authors thank Dr. A. F. Parlow, National Hormone & Pituitary Program (Torrance, CA) for purified FSH and LH and Creekstone Farms (Arkansas City, KS) for their generous donation of bovine ovaries. This study was supported in part by the Oklahoma State University Wentz Project scholarship program (to J.N. and A.H.), and the Oklahoma State University Agricultural Experiment Station Project OKL02970. This study was approved for publi-

cation by the Director, Oklahoma Agricultural Experiment Station.

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Received March 26, 2019.

Accepted May 10, 2019.

INTRODUCTION

The vascular endothelial growth factor (VEGF) family is made up of polypeptide proteins that act as angiogenic factors (Berisha et al., 2000; Ferrara, 2004; Niu and Chen, 2010; Araújo et al., 2013). The founding member of this family is VEGFA (Ferrara, 2004; Arcondeguy et al., 2013) and has been confirmed as an angiogenic factor through embryonic research; during embryogenesis, the disruption of VEGFA or its receptors results in early embryonic death due to deficient vascularization (Ng et al., 2006). The reproductive system requires expansive vascularization (Shweiki et al., 1993; Yamada et al., 1995; Van Blerkom, 2000; Grazul-Bilska et al., 2007; Tal et al., 2015; Berisha et al., 2016), and VEGFA plays an important role as a mitogen for endothelial cells (Ng et al., 2006) and blood vessel formation (Holmes and Zachary, 2005). Also, VEGFA shows regulatory properties on folliculogenesis and luteogenesis in mammals (Roberts et al., 2007; Yang et al., 2008; Araújo et al., 2013). In fact, VEGFA properties were first reported in the formation of the bovine and ovine corpus luteum which includes the development of a new vascular system from pre-existing vessels (Reynolds and Redmer, 1998). In cattle, as follicles grow, VEGFA protein concentrations in follicular fluid and *VEGFA* mRNA abundance in GC increases in large (>12 mm) follicles compared with medium (6 to 10 mm) follicles (Berisha et al., 2000), but what regulates this increase in VEGFA is unknown. Berisha et al. (2000) hypothesized that VEGFA supports the selection and growth of the dominant follicle in cattle by aiding the proliferation and formation of new capillaries fundamental for the delivery of oxygen, nutrients, steroid precursors, and growth factors to the growing follicle. Indeed, increased follicle vascularization is correlated with increased VEGFA expression (Grazul-Bilska et al., 2007).

Research done by Shweiki et al. (1993) on angiogenic processes found that *VEGFA* mRNA was expressed in cells surrounding the expanding vasculature during neovascularization of ovarian follicles, and neovascularization of the corpus luteum. Using in situ hybridization, *VEGFA* was shown to be expressed in steroidogenic and steroid-responsive cell types including theca cells (TC) and granulosa cells (GC) (Shweiki et al., 1993). The increase in *VEGFA* expression in some cells corresponds with steroidogenic activity in the cell, which suggests *VEGFA* is hormonally regulated (Shweiki et al., 1993). Subsequently, VEGFA has

been localized in preantral follicles of different species including humans (Harata et al., 2006), cows (Greenaway et al., 2005), pigs (Barboni et al., 2000), and rats (Celik-Ozenci et al., 2003; Araújo et al., 2013). Studies also show the presence of VEGFA in follicular fluid of cattle (Berisha et al., 2000, 2008; Grazul-Bilska et al., 2007), pigs (Mattioli et al., 2001; Galeati et al., 2003), and humans (Friedman et al., 1997; Coppola et al., 2005) as well as the ability of bovine (Schams et al., 2001), primate (Duffy and Stouffer, 2003), and porcine (Galeati et al., 2003; Grasselli et al., 2010) GC from antral follicles to produce VEGFA in vitro. More recently, a study in mice showed that loss of *VEGFA* gene expression in GC arrests follicular development leading to decreased fertility (Sargent et al., 2015). However, the hormones and growth factors that regulate follicular VEGFA production in cattle is unknown, and therefore, additional studies are required to determine the hormonal control of VEGFA production in bovine GC and TC.

The E2F transcription factor family is made up of 8 members and is crucial in regulating cellular proliferation, differentiation, DNA repair, cell cycle, and cell apoptosis (Lv et al., 2017), and some E2Fs have been implicated in ovarian cancer (Reimer et al., 2006), but little is known about the role of E2Fs in normal ovarian function. The E2F family is divided into 3 groups based on their roles in transcriptional regulation (Ertosun et al., 2016). Activator E2Fs include E2F1, E2F2, and E2F3a and they possess functional transactivation domains (Ertosun et al., 2016). Repressor E2Fs include E2F3b, E2F4, and E2F5; these are constitutively expressed and E2F4 and E2F5 repress the transcription of cell proliferation genes in quiescent cells (Ertosun et al., 2016). The final group is the inhibitor E2Fs, which includes E2F6, E2F7a, E2F7b, and E2F8 (Ertosun et al., 2016). These inhibitor proteins compete with other E2Fs to bind to target sequences, and E2F7 and E2F8 prevent expression of E2F1 (Ertosun et al., 2016). In nonovarian tissues, VEGFA and E2Fs interact with each other (Zhu et al., 2003; Weijts et al., 2012; Wu et al., 2014), and therefore we evaluated the effect of inhibition of E2Fs, using an E2F inhibitor, on *VEGFA* gene expression in bovine TC. We hypothesized that increased intrafollicular estradiol (E2) production and growth factors (e.g., insulin-like growth factor 1; IGF1) directly stimulate *VEGFA* gene expression in bovine GC and TC. Thus, the objective of this study was to investigate the hormonal regulation of *VEGFA* expression in bovine ovarian GC and TC.

MATERIALS AND METHODS

Tissue, Reagents, and Hormones

Ovaries from beef heifers were collected at a slaughterhouse where humane slaughter practices were followed, according to USDA guidelines.

Reagents and hormones used in cell culture were: Ham's F-12, Dulbecco modified Eagle medium (DMEM), gentamicin, glutamine, sodium bicarbonate, trypan blue, protease, collagenase, hyaluronidase, deoxyribonuclease (DNase), penicillin-streptomycin, TRI reagent, prostaglandin E2 (PGE2), E2, progesterone (P4), dihydrotestosterone (DHT), and androstenedione (A4) from Sigma-Aldrich Chemical Company (St. Louis, MO); fetal calf serum (FCS) from Equitech-Bio, Inc. (Kerrville, TX); purified ovine FSH (FSH activity: $15 \times$ NIH-FSH-S1 U/mg) and LH (NIDDK-oLH 26, activity $1.0 \times$ NIH-LH-S1 U/mg) from National Hormone and Pituitary Program (Torrance, CA); E2F inhibitor (E2Fi; HLM0064741; Millipore Sigma, Burlington, MA) shown to inhibit E2F1, E2F2, E2F3 and E2F4 (Ma et al., 2008; Kurtyka et al., 2014); recombinant (carrier-free) mouse growth differentiation factor 9 (GDF9), recombinant human IGF1, bone morphogenetic protein 4 (BMP4), epidermal growth factor (EGF), sonic hedgehog (SHH), and fibroblast growth factor 9 (FGF9) from R&D Systems (Minneapolis, MN); GDF9, IGF1, EGF, BMP4, SHH and FGF9 have been shown to be bioactive in bovine GC and TC cultures (Spicer and Stewart, 1996; Stewart et al., 1995; Spicer et al., 2008, 2009; Schreiber and Spicer, 2012) and share a 67, 100, 68, 97, 88, and 99% amino acid homology, respectively, with their respective bovine proteins.

Cell Culture

Ovaries from nonpregnant beef heifers were collected from an abattoir and transported to the lab on ice in antibiotic saline (0.9% saline solution with 1% penicillin-streptomycin) as previously described (Langhout et al., 1991; Schreiber et al., 2012). Follicular fluid was aspirated from small (SM; 1 to 5 mm) and large (LG; 8 to 20 mm) follicles, which appeared healthy with good vascularity and moderately transparent follicular fluid as previously described (Stewart et al., 1995; Spicer and Chamberlain 1998; Schreiber and Spicer, 2012). To isolate granulosa cells (GC), follicular fluid was centrifuged at $200 \times g$ for 8 min and the supernatant was collected for RIA or removed. Freshly

isolated GC were washed twice in serum-free medium (1:1 DMEM and Ham's F12 with 38.5 mM sodium bicarbonate, 0.12 mM gentamicin, and 2.0 mM glutamine) and then resuspended in serum-free medium with enzymes (collagenase and DNase at 1.25 and 0.5 mg/mL) to prevent clumping as previously described (Schreiber et al., 2012; Robinson et al., 2018).

To isolate TC, follicles were bisected with a scalpel after aspiration of follicular fluid, attached GC were separated from theca interna via blunt dissection, and theca interna tissue removed from the follicle wall, and enzymatically digested in 10 mL of medium on a rocking platform for 1 h at 37 °C as previously described (Stewart et al., 1995; Schreiber et al., 2012; Schutz et al., 2016). Briefly, nondigested thecal tissue was removed via a sterile syringe filter holder with metal screen of 149 μ m mesh (Gelman Sciences, Ann Arbor, MI) and then filtered TC were centrifuged at $50 \times g$ for 8 min, the pellet was washed and then TC resuspended in the same way as were the GC.

Viability of GC and TC used for cell culture was determined by trypan blue exclusion method on a 0.1 mm deep hemocytometer (American Optical Corp, Buffalo, NY), and averaged 52.7, 58.8, and 96.6% for small-follicle GC, large-follicle GC, and large-follicle TC, respectively. On average, 2×10^5 viable cells/well were plated on 24-well Falcon multiwell plates (No. 3047; Becton Dickinson, Lincoln Park, NJ) with 1 mL of medium/well and cultured (at 38.5 °C with 5% CO₂ and 95% air) in 10% FCS for the first 48 to 96 h (until cells reached 80% confluency) with medium changed every 24 h. Cells were then washed twice with 0.5 mL of serum-free medium and the different treatments were applied in serum-free medium for an additional 24 h and cellular RNA collected.

RNA Extraction

For gene expression experiments of fresh cells, GC isolated as described earlier were centrifuged at $200 \times g$ for 8 min at 4 °C, supernatant was aspirated, and 0.5 mL TRI reagent was added to each sample and stored at -80 °C. Fresh thecal tissue was centrifuged at $200 \times g$ for 8 min, with supernatant aspirated out and theca samples were placed in 0.5 mL RNAlater solution at 4 °C overnight before they were homogenized with PCR Tissue Homogenizing Kits (Omni International, Inc., Warrenton, VA) and stored in -80 °C in 0.5 mL TRI reagent for subsequent RNA extraction as previously described (Aad et al., 2012; Schutz et al.,

2016). For gene expression experiments of cultured cells, medium was aspirated from each well, and 0.25 mL/well TRI reagent was added. Each treatment was applied to 4 wells and 2 of these wells were combined to form one sample, generating 2 replicate samples of RNA for each treatment and each experimental replicate. Samples were stored in -80°C for subsequent RNA extraction.

Total RNA was extracted using TRI reagent protocol (Life Technologies, Carlsbad, CA) as previously described (Grado-Ahuir et al., 2011; Aad et al., 2012; Zhang et al., 2017). The RNA pellets were then resuspended in 16.5 μL of diethylpyrocarbonate (DEPC)-treated water (Life Technologies), and stored at -80°C . Quantity of RNA was determined by spectrophotometry at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) as previously described (Aad et al., 2012; Schreiber and Spicer, 2012; Zhang et al., 2017), and RNA samples were then diluted to a final concentration of 10 ng/ μL with DEPC-treated water for subsequent PCR.

Design of Primers and Probes

Bovine *VEGFA* primers and probe for quantitative PCR were designed using Primer Express Software (Foster City, CA), assuring that the primers spanned exon–exon junctions. The *VEGFA* (2736 bp; Accession NM_174216.2) forward primer was constructed between bp 1487 and 1507 with a T_m of 54.3 and a sequence of 5'-AGC GGAGAAAGCATTGTTG-3'. The reverse *VEGFA* primer was constructed between bp 1562 and 1543 with a T_m of 57.7 $^{\circ}\text{C}$ and a sequence of 5'-TTGCAACGCGAGTCTGTGTT-3'. The *VEGFA* probe was constructed between bp 1509 and 1538 with a T_m of 61.9 $^{\circ}\text{C}$ and a sequence of 5'-ACAAGATCCGCAGACGTGTAAATGTTC CTG-3' possessing FAM and TAMRA as the 5' reporter and the 3' quencher dyes; primers and probe were purchased from Integrated DNA Technologies Inc. (San Diego, CA). Efficiency of the PCR was determined to be 96.6% for a range of 10 to 1,000 ng of RNA. A “highly similar sequences” BLAST query search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was conducted for the primers and probe to ensure specificity.

Real-Time PCR Analysis

Quantification of *VEGFA* mRNA abundance was determined using 1-step real-time quantitative

PCR with iTaq Universal Probe One-Step Kit (Bio-Rad, Hercules, CA), performed on a CFX96 Real-Time System in a 96-well plate (Bio-Rad). Each 20 μL reaction contained 50 ng total RNA, 0.16 pmol primers, 0.08 pmol probe, 10 μL 1-step reaction mix, and 0.25 μL iScribe reverse transcriptase, and were run at 95 $^{\circ}\text{C}$ for 10 min, 40 cycles of 95 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 1 min. Each sample was assayed in duplicate to determine an average threshold cycle (C_t) value. Gene expression was normalized to constitutively expressed *18S* rRNA and relative quantity of *VEGFA* mRNA was expressed as $2^{-\Delta\Delta C_t}$ using the relative comparative threshold cycle method as previously described (Voge et al., 2004; Grado-Ahuir et al., 2011; Schreiber and Spicer, 2012). In each PCR run, a nontemplate control and a nonreverse transcriptase control were conducted to ensure no genomic DNA contaminated the master mix. The housekeeping gene, *18S* rRNA was selected because previous studies reported it to be a stable gene over a variety of treatments (Schmittgen and Zakrajsek, 2000; Sekar et al., 2000; Tsuji et al., 2002; Voge et al., 2004; Zhang et al., 2017).

Experimental Design

Experiment 1 was designed to determine if the abundance of *VEGFA* mRNA in GC and TC changes during follicle growth. For LG-follicle GC and TC, each of the 7 samples was collected from 1 LG healthy follicle from 1 ovary. For SM-follicle GC and TC, each sample had pooled cells from 3 to 5 SM follicles from 1 ovary. Each cell type had 7 samples collected from at least 3 animals. These fresh cells were lysed in TRI reagent and extracted for RNA as described earlier.

Experiment 2 was designed to test the effects of known ovarian trophic factors (i.e., DHT, FSH, PGE2, SHH, and GDF9) on *VEGFA* mRNA abundance in SM-follicle GC. Granulosa cells were cultured as described earlier and then 6 treatments were applied in serum-free medium as follows: control, DHT (300 ng/mL), FSH (30 ng/mL), PGE2 (300 ng/mL), SHH (500 ng/mL), and GDF9 (500 ng/mL). Doses of DHT, PGE2, FSH, GDF9, and SHH were based on previous studies (Spicer and Hammond, 1988; Spicer et al., 2006, 2008, 2009; Dentis et al., 2016; Zhang et al., 2017) showing that the doses used effectively alter bovine GC and TC function. After 24 h of treatment, cellular RNA was isolated as described earlier.

Experiment 3 was designed to test the effects of different steroids and intraovarian factors known to regulate cell proliferation (i.e., E2, A4, IGF1, FGF9, and E2Fi) on *VEGFA* mRNA abundance in

SM-follicle GC. Granulosa cells were cultured as in Exp. 2 and then 6 treatments were applied in serum-free medium as follows: control, E2 (300 ng/mL), A4 (300 ng/mL), IGF1 (30 ng/mL), FGF9 (30 ng/mL), and E2Fi (50 μ M). Doses of steroids and IGF1 were based on previous studies (Spicer et al., 1993, 1996; Spicer, 2005; Zhang et al., 2017). Dose of E2Fi was based on previous studies (Ma et al., 2008; Kurtyka et al., 2014; Huang et al., 2017). After 24 h of treatment, cellular RNA was isolated as described earlier.

Experiment 4 was designed to test the effects of known ovarian trophic factors (i.e., IGF1, E2, FSH, PGE2, and LH) on *VEGFA* mRNA abundance in LG-follicle GC. Granulosa cells were cultured as in Exp. 2 and then 6 treatments were applied in serum-free medium as follows: control, IGF1 (30 ng/mL), E2 (300 ng/mL), FSH (30 ng/mL), PGE2 (300 ng/mL), and LH (30 ng/mL). Doses of IGF1, E2, PGE2, FSH, and LH were based on Exp. 2 and previous studies (Spicer et al., 2002; Spicer, 2005; Spicer and Stewart, 1996; Dentis et al., 2016). After 24 h of treatment, cellular RNA was isolated as described earlier.

Experiment 5 was designed to test the effects of known ovarian trophic factors (i.e., E2, DHT, P4, and FGF9) on *VEGFA* mRNA abundance in LG-follicle GC. Granulosa cells were cultured as in Exp. 2 and then 5 treatments were applied in serum-free medium as follows: control, E2 (300 ng/mL), DHT (300 ng/mL), P4 (300 ng/mL), and FGF9 (30 ng/mL). Doses of E2, DHT, P4, and FGF9 were based on Exp. 2 and 3 and previous studies (Spicer and Hammond, 1988; Spicer, 2005; Schreiber and Spicer, 2012; Zhang et al., 2017). After 24 h of treatment, cellular RNA was isolated as described earlier.

Experiment 6 was designed to test the effects of various intraovarian factors known to regulate TC function including IGF1, IGF1 plus LH, E2Fi (50 μ M), BMP4, and FGF9 on *VEGFA* mRNA abundance in LG-follicle TC. Theca cells were cultured as in Exp. 2 and then 6 treatments were applied in serum-free medium as follows: control, IGF1 (30 ng/mL), IGF1 plus LH (30 ng/mL), E2Fi (50 μ M), BMP4 (100 ng/mL), or FGF9 (30 ng/mL). Doses of IGF1, LH, BMP4, and FGF9 were based on Exp. 3 and previous studies (Spicer and Stewart, 1996; Spicer, 2005; Spicer et al., 2006; Ma et al., 2008; Schreiber et al., 2012). After 24 h of treatment, cellular RNA was isolated as described earlier.

Experiment 7 was designed to test the effects of known ovarian trophic factors (i.e., E2, DHT, P4, LH, and EGF) on *VEGFA* mRNA abundance in LG-follicle TC. Theca cells were cultured as in Exp. 2 and then 6 treatments were applied in serum-free medium as follows: control, P4 (300 ng/mL),

E2 (300 ng/mL), LH (30 ng/mL), DHT (300 ng/mL), and EGF (10 ng/mL). Doses of DHT, E2, P4, LH, and EGF were based on Exp. 3 to 6 and previous studies (Spicer and Hammond, 1988; Spicer and Stewart, 1996; Spicer, 2005; Schutz et al., 2016; Zhang et al., 2017). After 24 h of treatment, cellular RNA was isolated as described earlier.

Statistical Analyses

Experiment 1 was analyzed as 2×2 factorial ANOVA with follicle size, cell type, and their interaction as main effects. Data from Exp. 2 to 7 were analyzed as a 1-way ANOVA. For in vitro Exp. 2 and 3, each pool (biological replicate) of small-follicle GC was collected from 10 to 30 ovaries and contained 2 or 3 experimental replicates per treatment. For the in vitro Exp. 4 to 7, each treatment was applied to 3 or 4 independent pools of large-follicle TC or GC collected from 7 to 8 follicles from at least 5 animals for each pool (biological replicate) and contained 2 replicates per treatment. Treatment effects of dependent variables (i.e., *VEGFA* mRNA abundance) were determined using ANOVA and the GLM procedure of SAS for Windows (version 9.3, SAS Institute Inc., Cary, NY). If significant (i.e., $P < 0.05$) main effects in the ANOVA were detected, mean differences were then determined using Fisher's protected least significant differences test (Ott, 1977). Data were tested for homogeneity of variance using Hartley's F max test (Ott, 1977). To correct for heterogeneity of variance (when necessary), data were analyzed after transformation to natural $\ln(x + 1)$ and presented relative fold of controls for each experiment.

RESULTS

Experiment 1: *VEGFA* Abundance in GC and TC During Follicle Growth

Abundance of *VEGFA* mRNA was greater ($P < 0.05$) in GC than TC of both SM and LG follicles (Fig. 1). Abundance of *VEGFA* mRNA in TC was less ($P < 0.05$) in LG than SM follicles, whereas abundance of *VEGFA* mRNA in GC did not differ ($P > 0.10$) between SM and LG follicles (Fig. 1).

Experiment 2: Effect of DHT, FSH, PGE2, SHH, and GDF9 on *VEGFA* mRNA in SM-Follicle GC

In SM-follicle GC, all treatments decreased ($P < 0.05$) *VEGFA* mRNA abundance by 30 to 46% (Fig. 2A).

Figure 1. Vascular endothelial growth factor A (*VEGFA*) expression in freshly collected granulosa cells (GC) and theca cells (TC) from small (SM; 1 to 5 mm) and large (LG; ≥ 8 mm) follicles (Exp. 1). Values are normalized to constitutively expressed *18S* ribosomal RNA and expressed as fold of LG-follicle TC mean. ^{a,b,c}Means ($\pm$ SEM; $n = 7$) without a common letter differ ($P < 0.05$).

Experiment 3: Effect of E2, A4, IGF1, FGF9, and E2Fi on *VEGFA* mRNA in SM-Follicle GC

In SM-follicle GC, E2Fi treatment increased ($P < 0.05$) *VEGFA* mRNA abundance by 13-fold (Fig. 2B), whereas E2, A4, IGF1, and FGF9 had no effect ($P > 0.10$) on *VEGFA* mRNA abundance in SM-follicle GC.

Experiment 4: Effect of IGF1, E2, FSH, PGE2, and LH on *VEGFA* mRNA in LG-Follicle GC

In LG-follicle GC, none of the treatments affected ($P > 0.10$) *VEGFA* mRNA abundance (Fig. 3A).

Experiment 5: Effect of E2, DHT, P4, and FGF9 on *VEGFA* mRNA in LG-Follicle GC

In LG-follicle GC, P4 treatment increased ($P < 0.05$) *VEGFA* mRNA abundance by 32%, whereas E2, DHT, and FGF9 were without effect ($P > 0.10$) (Fig. 3B).

Experiment 6: Effect of IGF1, LH, FGF9, BMP4, and E2Fi on *VEGFA* mRNA in LG-Follicle TC

In LG-follicle TC, E2Fi increased ($P < 0.05$) *VEGFA* mRNA abundance by 8-fold and BMP4 and FGF9 increased ($P < 0.05$) *VEGFA* mRNA abundance by 1.5-fold (Fig. 4A). However, neither IGF1 nor IGF1 plus LH treatment altered *VEGFA* mRNA abundance in TC ($P > 0.10$).

Figure 2. In vitro effects of various hormones on abundance of vascular endothelial growth factor A (*VEGFA*) mRNA in granulosa cells from small follicles (Exp. 2 and 3). Granulosa cells were isolated from small follicles and cultured in 10% fetal calf serum for 2 d and then treated in serum-free medium with: dihydrotestosterone (DHT; 300 ng/mL), FSH (30 ng/mL), prostaglandin E2 (PGE2; 300 ng/mL), sonic hedgehog (SHH; 500 ng/mL), or growth differentiation factor-9 (GDF9; 500 ng/mL) for 24 h (Panel A); or estradiol (E2; 300 ng/mL), androstenedione (A4; 300 ng/mL), insulin-like growth factor 1 (IGF1; 30 ng/mL), an E2F inhibitor (E2Fi; 50 μ M), or fibroblast growth factor 9 (FGF9; 30 ng/mL) for 24 h (Panel B). Values are normalized to constitutively expressed *18S* ribosomal RNA and expressed as fold of control values with no additions. *Within a panel, asterisk indicates that mean ($\pm$ SEM; $n = 6$) differs from control ($P < 0.05$).

Experiment 7: Effect of E2, DHT, P4, LH, and EGF on *VEGFA* mRNA in LG-Follicle TC

In LG-follicle TC, LH alone and EGF alone increased ($P < 0.05$) *VEGFA* mRNA abundance by 1.6- and 1.9-fold, respectively (Fig. 4B), whereas E2, DHT, and P4 were without effect ($P > 0.10$).

DISCUSSION

The results of the current study indicate that in cattle: 1) *VEGFA* gene expression is greater in

Figure 3. In vitro effects of various hormones on abundance of *vascular endothelial growth factor A* (*VEGFA*) mRNA in granulosa cells from large follicles (Exp. 4 and 5). Granulosa cells were isolated from large follicles and cultured in 10% fetal calf serum for 2 d and then treated in serum-free medium with: IGF1 (30 ng/mL), estradiol (E2; 300 ng/mL), FSH (30 ng/mL), prostaglandin E2 (PGE2; 300 ng/mL), or LH (30 ng/mL) for 24 h (Panel A); or control, E2 (300 ng/mL), dihydrotestosterone (DHT; 300 ng/mL), progesterone (P4; 300 ng/mL) or fibroblast growth factor 9 (FGF9; 30 ng/mL) for 24 h (Panel B). Values ($\pm$ SEM; $n = 6$) are normalized to constitutively expressed *18S* ribosomal RNA and expressed as fold of control values with no additions. *Asterisk indicates mean differs from control ($P < 0.05$).

GC than TC and changes with follicle size in TC; 2) autocrine, paracrine, and endocrine regulators of follicular development, such as LH, FSH, BMP4, EGF, and steroids, modulate *VEGFA* mRNA abundance in GC and TC in vitro; and 3) inhibition of E2F transcription factors induce *VEGFA* gene expression in both GC and TC.

To our knowledge, the present study is the first to evaluate hormonal regulation of gene expression of *VEGFA* mRNA in bovine TC, and identify E2F transcription factors as a major inducer

Figure 4. In vitro effects of various hormones on abundance of *vascular endothelial growth factor A* (*VEGFA*) mRNA in bovine theca cells of Exp. 6 and 7. Theca cells were isolated from large follicles and cultured in 10% fetal calf serum for 2 d and then treated in serum-free medium with: control, an E2F inhibitor (E2Fi; 50 μ M), IGF1 (30 ng/mL), IGF1 plus LH (30 ng/mL) or bone morphogenetic protein-4 (BMP4; 100 ng/mL) for 24 h (Panel A); or control, estradiol (E2; 300 ng/mL), dihydrotestosterone (DHT; 300 ng/mL), progesterone (P4; 300 ng/mL), LH (30 ng/mL), or epidermal growth factor (EGF; 10 ng/mL) for 24 h (Panel B). Values ($\pm$ SEM; $n = 6$) are normalized to constitutively expressed *18S* ribosomal RNA and expressed as fold of control values with no additions. Within a panel, asterisks indicates mean differs from control (* $P < 0.05$; ** $P < 0.01$).

of *VEGFA* gene expression in GC and TC. We also observed that several intraovarian factors had inhibitory effects on *VEGFA* gene expression in GC from SM but not LG follicles in vitro, suggesting that *VEGFA* production by less differentiated GC (SM follicles) is more responsive to inhibitory inputs than are more differentiated GC (LG follicles). Perhaps suppression of *VEGFA* production and thus angiogenesis in SM follicles is important to prevent too rapid of growth and/or premature differentiation. Previously, it was reported that

FSH and LH stimulate *VEGFA* mRNA abundance in mice GC (Rico et al., 2014) and bovine LG-follicle GC (Klipper et al., 2010; Yang et al., 2017), whereas in the present study, FSH had an inhibitory effect on *VEGFA* mRNA abundance in SM-follicle GC and no effect in LG-follicle GC. Also in the present study, LH had no effect on *VEGFA* mRNA in LG-follicle GC but stimulated *VEGFA* mRNA in LG-follicle TC. Previous studies have reported that hCG increases *VEGFA* mRNA in bovine LG-follicle GC cultured in the presence of FCS (Garrido et al., 1993; Klipper et al., 2010). Similarly, LH stimulates VEGFs secretion by water buffalo GC cultured in 10% FCS from preovulatory follicles but not from small, medium, or large follicle GC (Babitha et al., 2014). Reasons for the lack of effect of LH on GC in the present study may be due to differences in culture conditions (e.g., serum-free vs. FCS-containing medium) or other factors. Nonetheless, our data support the hypothesis that VEGFA production is hormonally regulated and that this regulation changes with the differentiation state of the GC. Interestingly, BMP4, FGF9, LH, and EGF increased *VEGFA* mRNA abundance in LG-follicle TC and suggest that these hormones may be up-regulating VEGFA production in TC and therefore angiogenesis.

In the present study, we observed less *VEGFA* mRNA abundance in LG- versus SM-follicle TC but similar amounts of *VEGFA* mRNA in GC of LG and SM follicles. Previously, changes in *VEGFA* gene and protein expression during follicular development has been reported for beef cattle (Berisha et al., 2000; Greenaway et al., 2005), ewes (Chowdhury et al., 2010), and water buffalo (Feranil et al., 2005; Babitha et al., 2013). In beef cattle, VEGFA immunolocalization in both GC and TC is greatest in large follicles, intermediate in medium follicles, and lowest in small follicles (Greenaway et al., 2005). Similarly, in dual purpose beef cattle, *VEGFA* mRNA abundance in GC and TC significantly increased as follicles enlarged (from 6 to 10 mm to >14 mm) with increased estradiol concentrations (Berisha et al., 2000). In ewes, *VEGFA* mRNA abundance was greater in theca of large versus small or medium follicles (Chowdhury et al., 2010). In water buffalo, *VEGFA* mRNA increased during follicular development in GC but did not change in TC (Feranil et al., 2005; Babitha et al., 2013). In the present study, *VEGFA* mRNA was greater in GC than TC and greater in TC of SM versus LG follicles, and did not differ in GC between SM and LG follicles. Similarly, VEGFA immunolocalization was greater in GC than TC of

water buffalo and Holstein-Friesian cattle (Feranil et al., 2005). Why the present study and some previous studies differ in their findings will require further elucidation but may be due to breed/species differences and/or due to other methodological differences among studies including how follicles were classified and when follicles were collected during the estrous cycle.

Steroid regulation of *VEGFA* mRNA in the ovary has not been thoroughly evaluated. Previously, E2 stimulated *VEGFA* mRNA expression in rat uterine luminal epithelial cells (Kazi et al., 2009) and in bovine LG-follicle GC cultured in 10% FCS (Garrido et al., 1993). In the present study using a serum-free medium, we observed that DHT inhibited *VEGFA* mRNA abundance in SM-follicle GC but E2 had no effect on *VEGFA* mRNA abundance in SM-follicle GC, LG-follicle GC, or LG-follicle TC. Similarly, in GC from bovine medium-sized follicles cultured in serum-free medium, E2 had no effect on *VEGFA* mRNA expression (Shimizu and Miyamoto, 2007). Androgens (A4 and DHT) also had no effect on *VEGFA* mRNA abundance in LG-follicle GC or TC in the present study. However, P4 significantly increased *VEGFA* mRNA abundance in LG-follicle GC but had no effect in LG-follicle TC. Similarly, P4 induction of *VEGFA* mRNA in GC from medium-sized bovine follicles (Shimizu and Miyamoto, 2007) and human breast cancer cells (Wu et al., 2004) have been reported. Thus, results from the present study support the idea that differences in *VEGFA* expression between GC and TC is not due to changes in estradiol levels, but rather due to changes in P4 levels and other factors. Further research will be required to verify this suggestion.

Growth factor regulation of *VEGFA* gene expression in the ovary has not been thoroughly evaluated. Because fasting increases follicular fluid levels of VEGFs in pigs (Galeati et al., 2003) and decreases systemic IGF1 (Spicer et al., 1992), we evaluated the effect of IGF1 on *VEGFA* mRNA and found that IGF1 had no significant effect on *VEGFA* gene expression in bovine GC or TC. It was concluded from a previous study with water buffalo that IGF1 increases VEGFA secretion in vitro by GC from preovulatory follicles but not by GC from large, medium, or small antral follicles (Babitha et al., 2014). Because FGF9 is an important intraovarian regulator in cattle (Schutz et al., 2016) and half-dose gene of FGF9 in mice caused marked decreases in *VEGFA* mRNA (Behr et al., 2010), we evaluated the effect of FGF9 on *VEGFA* mRNA and found that FGF9 had no

significant effect on *VEGFA* gene expression in GC but FGF9 increased *VEGFA* mRNA abundance in TC. We also discovered that EGF stimulated *VEGFA* mRNA expression in LG-follicle TC and this is consistent with studies in water buffalo GC (Babitha et al., 2014), colorectal cancer cells (Cascio et al., 2009), and human gastric cancer cells (Akagi et al., 2003), but not human breast cancer cells (Lee et al., 2004). We also found that BMP4 increased abundance of *VEGFA* mRNA in LG-follicle TC. Similar to our findings, studies reported that BMP7 increases *VEGFA* mRNA abundance in human GC (Akiyama et al., 2014) and BMP4 enhances *VEGFA* mRNA expression in neuroblastoma cells (HaDuong et al., 2015). Further research will be needed to clarify how intraovarian EGF, FGF9 (and other FGFs), and BMP4 (and other BMPs) regulate *VEGFA* production and angiogenesis by bovine follicles.

In the present study, inhibition of E2F transcription factors had the most dramatic stimulatory effect on *VEGFA* mRNA expression in both GC and TC a novel finding, and suggests an important role for E2F transcription factors in regulating *VEGFA* expression in bovine follicles. As mentioned earlier, 8 E2F transcription factors have been identified as regulators of cell proliferation, differentiation, DNA repair, cell cycle, and cell apoptosis in nonovarian tissue (Lv et al., 2017), and some E2Fs have been implicated in ovarian cancer (Reimer et al., 2006). A previous study by Putowski et al. (2001) reported possible roles of some E2F members in ovarian function. Specifically, using human and rat GC, overexpression of E2F1 (an activator E2F) decreased FSH receptor (*FSHR*) transcription, whereas overexpression of E2F5 (a suppressor E2F) increased *FSHR* transcription (Putowski et al., 2001), supporting the idea that E2F proteins may be involved in follicular growth in species other than cattle. The E2F inhibitor used in the present study has been shown to inhibit at least E2F1, E2F2, E2F3, and E2F4 expression in nonovarian cell lines (Ma et al., 2008; Kurtyka et al., 2014), but other E2Fs were not evaluated. Thus, which of the numerous E2Fs are impacting *VEGFA* mRNA is uncertain and will require further research. Additional studies are also needed to further understand the mechanism through which E2Fs are impacting *VEGFA* mRNA as well as the overall relationship between E2Fs and *VEGFA* and their role in regulating angiogenesis. Because increased blood flow to dominant follicles is an important step in selection (Ginther et al., 2017) and prior to ovulation (Acosta, 2007), the present

findings may be applicable to the study of improving superovulation and synchronization procedures.

In summary, the present study reported novel information on hormonal regulation and possible role of *VEGFA* in ovarian folliculogenesis indicating that *VEGFA* gene expression and its regulation differs between GC and TC and changes with follicular development in cattle. Autocrine, paracrine, and endocrine regulators, such as BMP4, EGF, GDF9, P4, DHT, PGE2, SHH, FSH, and LH appear to modulate *VEGFA* mRNA abundance in GC and/or TC in vitro. Some of these factors (e.g., P4) stimulate *VEGFA* mRNA in LG-follicle GC and have no effect in LG-follicle TC, whereas others (e.g., DHT and PGE2) inhibit *VEGFA* mRNA in SM-follicle GC and have no effect in more differentiated LG-follicle GC, suggesting that these specific factors may be suppressing production of angiogenic factors in early developing follicles to prevent too rapid of growth and/or differentiation. An imbalance of these factors could lead to ovarian abnormalities such as cystic ovaries.

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