MicroRNA 221 expression in theca and granulosa cells: hormonal regulation and function[1]

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ABSTRACT: Small noncoding RNA molecules (miRNA) regulate protein levels in a post-transcriptional manner by partial base pairing to the 3′-UTR of target genes thus mediating degradation or translational repression. Previous studies indicate that numerous miRNA regulate the biosynthesis of intraovarian hormones, and emerging evidence indicates that one of these, miRNA-221 (*MIR221*), may be a modulator of ovarian function. However, the hormonal control of ovarian *MIR221* is not known. The objectives of this study were to investigate the developmental and hormonal regulation of *MIR221* expression in granulosa (GC) and theca cell (TC) and its possible role in regulating follicular function. Bovine ovaries were collected from a local abattoir and GC and TC were obtained from small (<6 mm) and large (≥8 mm) follicles. In Exp. 1, GCs of small follicles had 9.7-fold greater ($P < 0.001$) levels of *MIR221* than those of large follicles, and TCs of large follicles had 3.7-fold greater ($P < 0.001$) levels of *MIR221* than those of small follicles. In large follicles, abundance of *MIR221* was 66.6-fold greater ($P < 0.001$) in TCs than in GCs. In small follicles, *MIR221* abundance did not differ ($P = 0.14$) between GC and TCs. In vitro Exp. 2, 3, and 4 revealed that treatment of bovine TCs with various steroids, phytoestrogens, IGF1, forskolin, and dibutyryl cyclic adenosine monophosphate had no effect ($P > 0.35$) on *MIR221* expression, whereas treatment with fibroblast growth factor 9 (FGF9) and FGF2 increased ($P < 0.001$) TC *MIR221* abundance 1.7- to 2.5-fold. In Exp. 5, FGF9 increased ($P < 0.05$) GC *MIR221* abundance by 1.7- and 2.0-fold in small and large follicles, respectively. The role of *MIR221* in GC steroidogenesis was investigated in Exp. 6 and it was found that transfection with a *MIR221* mimic reduced ($P < 0.01$) GC estradiol and progesterone production induced by FSH and IGF1, whereas transfection with *MIR221* inhibitor had little or no effect. We conclude that thecal *MIR221* expression is increased by FGF9 and increased *MIR221* may act to inhibit GC steroidogenesis in cattle.

Key words: cattle, follicle growth, granulosa cell, microRNA 221 (MIR221), steroidogenesis, theca cell

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INTRODUCTION

Recent research has demonstrated that microRNAs (miRNA) function to silence RNA and post-transcriptionally regulate gene expression in numerous tissues and biological functions in humans and animals, adding another dimension to gene regulation within the animal genome (Hossain et al., 2009; Li et al., 2011). These small noncoding RNA molecules regulate protein levels in a post-transcriptional manner by partial base pairing to the 3′-UTR of target genes thus mediating degradation or translational repression (He and Hannon, 2004; Ha and Kim, 2014). Studies have indicated that numerous miRNA regulate the biosynthesis of intraovarian hormones (Yao et al., 2010; Donadeu et al., 2012; Yin et al., 2012; Hu et al., 2013) and change within follicles. For example, *MIR21*, *MIR26a*, and *MIR143* are elevated in ovaries of anestrous sheep (Di et al., 2014), whereas *MIR21*, *MIR132*, *MIR212*, and *MIR224* are increased during follicular growth in mares (Schauer et al., 2013). Furthermore, *MIR92a* and *MIR92b* are lower in theca cells (TC) of women with polycystic ovarian syndrome (Lin et al., 2015).

Emerging evidence indicates that *miRNA-221* (*MIR221*) may be a modulator of ovarian function. It was first reported that over expression of *MIR221* was associated with human ovarian cancer (Dahiya et al., 2008). More recently, *MIR221* abundance was found to be severalfold greater in granulosa cells (GC) of subordinate than of dominant follicles at d 3 of an estrous cycle in cattle but on d 7 *MIR221* abundance in GC was severalfold less in subordinate than in dominant follicles (Salilew-Wondim et al., 2014). Blood plasma levels of *MIR221* are greater in FSH-treated heifers and also increase between d 3 and 7 postestrus (Noferesti et al., 2015). However, no study has determined the hormonal regulation of ovarian cell *MIR221* in cattle. Thus, the aims of the present work were to investigate the hormonal regulation of *MIR221* expression in TC and GC, and its possible role in follicular steroidogenesis.

MATERIALS AND METHODS

Reagents and Hormones

Reagents and hormones used for cell preparation and culture were: gentamicin (catalog number G1397), glutamine (catalog number G6392), Ham's F-12 (catalog number N4888), Dulbecco modified Eagle medium (DMEM) (catalog number D5921), sodium bicarbonate (catalog number S8761), trypan blue (catalog number T8154), protease (catalog number P5147), collagenase (catalog number C0130), hyaluronidase (catalog number H3506), deoxyribonuclease (DNase) (catalog number DN25), TRI reagent (catalog number T9424), and penicillin-streptomycin (catalog number P0781) from Sigma-Aldrich Chemical Company (St. Louis, MO); ovine FSH (175 x NIH-FSH-S1 U/mg) from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA); recombinant human IGF1 (catalog number 291-G1/CF), recombinant human fibroblast growth factor 9 (FGF9; catalog number 273-F9/CF), and recombinant bovine FGF2 (catalog number 2099-FB/CF) from R&D Systems (Minneapolis, MN); forskolin (catalog number F6886), dibutyryl cyclic adenosine monophosphate (dbcAMP) (catalog number D0260), estradiol (catalog number E8875), α-zearalenol (catalog number Z0166), and β-zearalenol (catalog number Z2000) from Sigma-Aldrich Chemical Company; genistein (catalog number EI-147) from Biomol Research Labs, Inc. (Plymouth Meeting, PA); androstenedione (catalog number A6030) and testosterone (catalog number A6950) from Steraloids (Wilton, NH); and fetal calf serum (FCS) (catalog number SR30-1572) from Equitech-Bio, Inc. (Kerrville, TX).

Cell Culture

Ovaries from beef heifers were collected from a local abattoir and transported to the lab on ice in saline with antibiotics (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 (<6 mm) and large (≥8 mm) follicles (appeared healthy with good vascularity and moderately transparent follicular fluid) and GC and TC were collected as previously described (Stewart et al., 1995; Schreiber et al., 2012; Zhang et al., 2017). Isolated GC and TC were washed twice in serum-free medium (38.5 mM of sodium bicarbonate, 0.12 mM of gentamicin and 2.0 mM of glutamine within 1:1 DMEM and F12) and then re-suspended in serum-free medium containing collagenase (1.25 mg/mL) and DNase (0.5 mg/mL) to prevent cell clumping as previously described (Lagaly et al., 2008; Schreiber et al., 2012).

Viability of TC from large follicles and GC from small and large follicles was determined by trypan blue exclusion method (Adashi et al., 1987), and averaged 90.7 ± 2.2%, 81.8 ± 2.9% and 74.0 ± 6.1%,

respectively. On average, 2.0×10^5 viable cells were plated on 24-well Falcon multiwell plates (No. 3047; Becton Dickinson, Lincoln Park, NJ) in 1 mL of medium and cultured in an environment of 38.5°C with 5% CO_2 and 95% air in 10% FCS for the first 48 h to 144 h until cells reached 80% confluency with medium changes every 24 h. Cells were washed twice with 0.5 mL of serum-free medium, treatments were applied in serum-free medium for an additional 24 h or 48 h, and medium was either aspirated or collected from each well depending on the particular experiment. This culture system was developed to yield hormonally responsive nonluteinized GC and TC (Langhout et al., 1991; Stewart et al., 1995; Spicer and Chamberlain, 1998). First, progesterone production does not increase with time using this culture paradigm (Langhout et al., 1991; Stewart et al., 1995). Secondly, the morphology of the GC and TC retains a fibroblastic appearance (Chamberlain and Spicer, 2001). Third, aromatase activity of GC remains responsive to FSH, insulin and IGF-I and increases between d 3 and 4 of culture (Spicer et al., 1993; Spicer and Chamberlain, 1998), and the TC remain responsive to LH and IGF1 in terms of *CYP17A1* mRNA and androstenedione production (Stewart et al., 1995; Spicer et al., 2008).

Experimental Design

Experiment 1 was designed to determine if the abundance of *MIR221* differed between GC and TC and between small and large follicles. Therefore, GC and TC samples were collected from small and large follicles as described earlier. For large-follicle GC and TC, each of seven samples was collected from one large healthy follicle from one ovary. For small-follicle GC and TC, each sample had pooled cells from three to five small follicles from one ovary. Each cell type had seven samples collected from at least three animals. These fresh cells were lysed in TRI reagent and extracted for RNA as described below.

Because preliminary microarray data from our laboratory indicated FGF9 may induce *MIR221* in TC, Exp. 2 was designed to test the effect of IGF1, FGF9, and FGF2 on *MIR221* expression in large-follicle TC. Cells were cultured for 48 h in 10% FCS medium, washed twice with serum-free medium, and six treatments in a 2 × 3 factorial arrangement were applied for 24 h as follows: control, FGF9 (30 ng/mL), FGF2 (30 ng/mL), IGF1 (30 ng/mL), and IGF1 plus FGF9 or FGF2. Co-treatment with IGF1 was evaluated because many studies show a synergistic effect of IGF1 on hormone-induced cellular responses in ovarian cells (Spicer and Echternkamp, 1995; Spicer and Chamberlain, 1998). After 24 h of treatment, medium was aspirated and cellular RNA was collected as described below. Doses of FGF9 and FGF2 were selected based on previous studies showing that the doses used are effective in eliciting a functional response in cultured TC and GC (Vernon and Spicer, 1994; Schreiber et al., 2012; Totty et al., 2017).

To further explore possible hormonal regulation of *MIR221* expression in TC and because a previous study reported an inhibitory effect of cAMP on *MIR221* abundance (Castagnino et al., 2013), Exp. 3 was designed to test the effect of FGF9, dbcAMP, and forskolin-mediated cAMP on *MIR221* expression in large-follicle TC. Forskolin is an inducer of adenyl cyclase activity increasing intracellular cAMP levels (Hedin and Rosberg, 1983; Adashi et al., 1989), whereas dbcAMP is a cAMP analog that mimics the effect of cAMP (Hedin and Rosberg, 1983; Schreiber et al., 2012). Cells were cultured for 72 h in 10% FCS medium, washed twice with serum-free medium, and six treatments were applied in a 2 × 3 factorial arrangement in serum-free medium as follows: control, dbcAMP (0.1 mg/mL; 0.2 mM), forskolin (4.1 µg/mL; 10 µM), and control plus FGF9 (30 ng/mL), dbcAMP plus FGF9 (30 ng/mL), forskolin plus FGF9 (30 ng/mL). After 24 h of treatment, medium was aspirated and cellular RNA was collected as described below. Doses of FGF9, dbcAMP, and forskolin were selected based on previous studies showing that the doses used are effective in eliciting a functional response in cultured TC and/or GC (Duleba et al., 1985; McArdle et al., 1991; Vernon and Spicer, 1994; Schreiber et al., 2012).

Because a previous study has shown that estrogens directly repress *MIR221* in human breast cancer cell lines (Di Leva et al., 2010) and androgens repress *MIR221* in human prostate cell lines (Ambs et al., 2008), Exp. 4 was designed to test the effect of steroids and phytoestrogens on *MIR221* abundance in large-follicle TC. Cells were cultured for 144 h in 10% FCS medium, washed twice with serum-free medium, and six treatments were applied in serum-free medium as follows: control, genistein (300 ng/mL), estradiol (300 ng/mL), α-zearalenol (300 ng/mL), β-zearalenol (300 ng/mL), and androstenedione (300 ng/mL). After 24 h of treatment, medium was aspirated and cells were lysed in 0.5 mL of TRI reagent for RNA extraction as described below. Doses of the various steroids

were selected based on previous studies showing that the doses used are effective in eliciting a functional response in cultured TC and/or GC (Spicer, 2005; Ranzenigo et al., 2008; Mlynarczuk et al., 2011; Aad et al., 2012; Zhang et al., 2017). Because previous studies have suggested that the phytoestrogens genistein, α-zearalenol, and β-zearalenol have estrogenic activity (Ranzenigo et al., 2008; Mlynarczuk et al., 2011; Pizzo et al., 2015; 2016), we decided to evaluate the possible effects of these phytoestrogens in addition to estradiol.

Experiment 5 was designed to test the effect of FGF9 on GC *MIR221* expression in small and large follicles. Cells were cultured as in Exp. 2, washed twice with serum-free medium, and treated with either 0 or 30 ng/mL of FGF9. After 24 h of treatment, medium was aspirated and cellular RNA was collected as described below.

Because previous studies have indicated microRNAs can impact GC steroid production (Sirotkin et al., 2009; Xu et al., 2011; Dai et al., 2013), Exp. 6 was designed to determine the effects of *MIR221* on steroidogenesis of small-follicle GC using transfection of *MIR221* mimics and inhibitors. Cells were cultured as previously described for Exp. 3, washed twice with serum-free medium, and treated with transfection complexes (control, mimic, inhibitor) (see below) and incubated for 12 h, and then treatments of FGF9 (0 or 30 ng/mL) and IGF1 (0 or 30 ng/mL) were applied in a 3 × 2 × 2 factorial arrangement (see below). After 40 h of treatment, medium was collected for progesterone and estradiol determinations and cells were counted. For transfections, control, *MIR221* mimic, or *MIR221* inhibitor transfection medium was applied to GC for 12 h before treatment. Briefly, 1,300 µL Opti-MEM medium (catalog number 31985070; Invitrogen, Carlsbad, CA) and 39 µL Lipofectamine (catalog number 56531; Invitrogen) were combined, then 52 µL *MIR221* mimic (5 pmol; AGCUACAUUGUCUGCUGGGUUU; catalog number 4464066) or inhibitor (5 pmol; UUUCCCUGCUGUCUUTGTUGCT; catalog number 4464084) purchased from (Life Technologies Corp., Carlsbad, CA) was added. Control transfection complex contained 1,300 µL Opti-MEM medium and 39 µL Lipofectamine. Attached GC were washed twice with 0.5 mL of serum-free medium, then 200 µL of serum-free medium and 50 µL of miRNA-transfection medium was applied to each well. GC were incubated with 250 µL of transfection medium for 12 h, after which an additional 250 µL of medium was added to each well containing control (no added hormones), IGF1, FGF9, or IGF1 plus FGF9 for an additional 40 h. Final concentrations of IGF1 and FGF9 were 30 ng/mL. A similar transfection procedure has been used and shown to be effective in this culture system (Spicer et al., 2006; 2008).

RNA Extraction and Real-Time PCR

For gene expression experiments, medium was aspirated and cells from two replicate wells were lysed in 0.5 mL of TRI reagent as previously described (Aad et al., 2012; Totty et al., 2017; Zhang et al., 2017). Briefly, 0.25 mL TRI reagent was added to all wells, cells were lysed by repeated pipetting and then combined with their respective replicates with each treatment containing four wells that generated two replicate samples of RNA. RNA from cell lysates were isolated using TRI reagent protocol and Phase Lock Gel Heavy tubes (5 Prime Inc., Germantown, MD) and quantitated as previously described (Voge et al., 2004; Aad et al., 2012; Schreiber et al., 2012; Zhang et al., 2017).

Quantification of *MIR221* expression was determined using two-step RT-PCR using TaqMan Small RNA Assays (Life Technologies Corp.; catalog number 4427925) specific for *MIR221*. Complementary DNA (cDNA) was synthesized from total RNA samples using stem-loop primers, and TaqMan MicroRNA Reverse Transcription Kit (Life Technologies Corp.; catalog number 4366596). Reactions were performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA) in a 96-well plate with the following cycling conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min; reaction products were stored at −20 °C. Subsequent real-time quantitative PCR was performed using target-specific TaqMan Assays (Life Technologies Corp.; catalog number 4373318 with FAM dye) and the TaqMan Universal PCR Master Mix II, no UNG (Life Technologies Corp.; catalog number 4440040). Each 20 µL reaction volume contained the appropriate cDNA, specific TaqMan Assay, and TaqMan Universal PCR Master Mix II. All sample assays were performed in triplicate to determine an average threshold cycle (C_T) value. PCR cycling conditions performed on CFX96 Real-Time System consisted of 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min in 96-well plates (Bio-Rad). The PCR intra-assay CV averaged 0.98%, and *U6* snRNA (Life Technologies Corp.; catalog number 4395470) was used as an endogenous control to correct for discrepancies in RNA quantification and loading as previously described (Yang et al., 2012).

Radioimmunoassays

Progesterone concentrations in medium were determined using a double-antibody radioimmunoassay (RIA) as previously described (Langhout et al., 1991; Schreiber et al., 2012) and the intra-assay coefficient of variation averaged 10.6 ± 0.3%. Estradiol concentrations in medium were determined using a double-antibody RIA as previously described (Spicer et al., 1993; Schreiber and Spicer, 2012) and the intra-assay coefficient of variation averaged 9.0 ± 0.5%.

Determination of Cell Numbers

Cell numbers were determined using a Coulter Counter (Model Z2; Beckman Coulter, Hialeah, FL) as previously described (Langhout et al., 1991; Lagaly et al., 2008; Spicer et al., 2011).

Statistical Analysis

For Exp. 1, main effects in ANOVA were cell type, follicle size, pool, and their interactions. For Exp. 2 and 5, main effects in ANOVA were treatment, pool, and their interactions. For Exp. 3 and 4, main effects of FGF9 and treatment were analyzed in a 2 × 3 factorial ANOVA. For Exp. 6, main effects in a 3 × 2 × 2 factorial ANOVA with transfection complex (control, mimic, or inhibitor), FGF9 (+ or −), IGF1 (+ or −), pool, and their interactions. For the in vitro Exp. 2 to 5, each treatment was applied to three or four independent pools of large-follicle TC or GC collected from seven to eight follicles for each pool (biological replicate) and contained two replicates per treatment. For Exp. 5 and 6, each pool (biological replicate) of small-follicle GC was collected from 10 to 30 ovaries and contained two or three replicates per treatment. Treatment effects and interactions on dependent variables (e.g., *MIR221* abundance and steroid production) were assessed using the GLM and ANOVA procedure of SAS (version 9.2, SAS Institute Inc., Cary, NY). To correct for heterogeneity of variance, data were analyzed after transformation to natural log $(x + 1)$ when necessary. Mean differences were assessed using Fisher's protected least significant differences test (Ott, 1977), if significant treatment effects in ANOVA were detected. Steroid production was expressed as ng/10^5 cells per 24 h and cell numbers at the end of the experiment were used for this calculation. Significance was declared at $P < 0.05$ and trends identified at $P < 0.10$. Data are presented as means ± pooled SEM of measurements from replicated experiments.

RESULTS

Changes in Abundance of MIR221 in GC and TC During Follicular Growth (Exp. 1)

A size by cell-type interaction on *MIR221* abundance was observed (P < 0.001). GCs of small follicles had 9.7-fold greater ($P < 0.001$) abundance of *MIR221* than those of large follicles, and TC of large follicles had 3.7-fold greater ($P < 0.001$) abundance of *MIR221* than those of small follicles (Figure 1). In large follicles, abundance of *MIR221* was 66.6-fold greater ($P < 0.001$) in TC than in GC. In small follicles, *MIR221* abundance did not differ ($P = 0.14$) between GC and TC (Figure 1).

Effects of Growth Factors, cAMP Inducers, and Steroids on MIR221 Abundance in Large-Follicle TC

Exp. 2. No significant interaction ($P = 0.67$) between FGF9 and IGF1 treatments on TC *MIR221* abundance was observed. However, FGF9 and FGF2 treatments increased ($P < 0.001$) *MIR221* expression by 1.7- and 2.5-fold (Figure 2), respectively. No significant effect of IGF1 on *MIR221* abundance was observed ($P = .36$).

Exp. 3. Main effect of FGF9 influenced ($P = 0.03$) TC *MIR221* abundance but dbcAMP and forskolin treatments ($P = 0.50$) and their interaction with FGF9 ($P = 0.52$) did not affect *MIR221* abundance (Figure 3). Averaged across treatments,

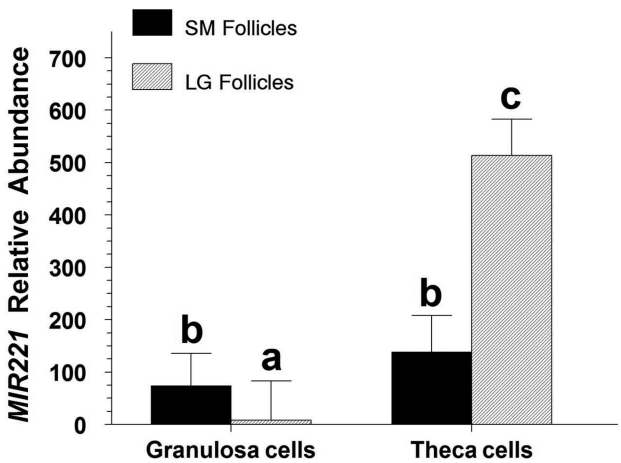

Figure 1. Abundance of *MIR221* in freshly collected granulosa and theca cells from small (SM; <6 mm) and large (LG; ≥8 mm) bovine follicles (Exp. 1). Values are normalized to constitutively expressed *U6* snRNA and expressed as fold of large-follicle granulosa cell values. [a,b,c]Means (±pooled SEM) without a common letter differ ($P < 0.001$).

FGF9 increased ($P = 0.02$) *MIR221* abundance by 1.91-fold.

Exp. 4. Treatment of various steroids and phytoestrogens did not affect ($P = 0.41$) *MIR221* abundance (data not shown). Relative abundance of *MIR221* averaged 1.00, 1.34, 1.30, 0.95, 1.24, and 1.58 ± 0.22 for control, genistein, estradiol, α-zearalenol, β-zearalenol, and androstenedione, respectively.

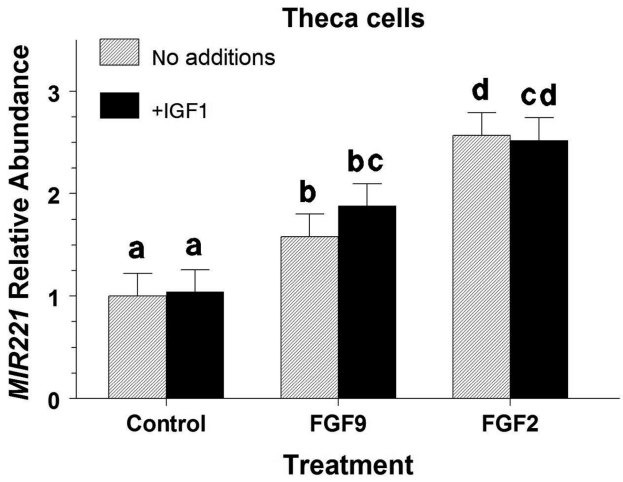

Figure 2. Effect of FGF9 and FGF2 on *MIR221* abundance in bovine large-follicle theca cells treated with or without IGF1 (Exp. 2). Theca cells were isolated and cultured for 48 h in 10% FCS after which cells were treated with 30 ng/mL of FGF9 or FGF2 and/or 30 ng/mL of IGF1 for 24 h. Values are normalized to constitutively expressed *U6* snRNA and expressed as fold of control values with no additions. [a,b,c,d]Means (±pooled SEM) without a common letter differ ($P < 0.05$).

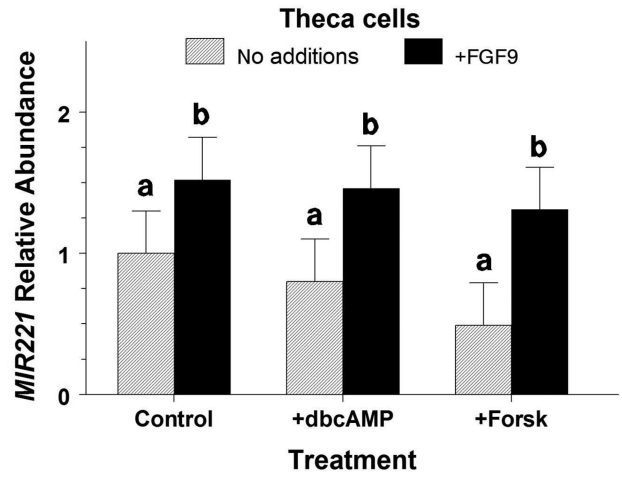

Figure 3. Lack of effect of pharmacologic cAMP agents on *MIR221* abundance in bovine large-follicle theca cells treated with or without FGF9 (Exp. 3). Theca cells were isolated and cultured for 72 h in 10% FCS and then treated with 0.1 mg/mL of dbcAMP, 4.1 µg/mL of forskolin (Forsk), and/or 30 mg/mL of FGF9 for 24 h. Values are normalized to constitutively expressed *U6* snRNA and expressed as fold of control values with no additions. [a,b]Means (±pooled SEM) without a common letter differ ($P < 0.05$).

Effect of FGF9 on MIR221 Abundance in Small- and Large-Follicle GC (Exp. 5)

Data for small and large-follicle GC were analyzed separately. In GC from small and large follicles, FGF9 increased *MIR221* abundance by 1.7-fold ($P = 0.04$) and 2.1-fold ($P = 0.02$), respectively (Figure 4).

Effect of Transfection of GC With MIR221 Mimic and Inhibitor on Steroidogenesis (Exp. 6)

A significant treatment × IGF1 × FGF9 interaction existed ($P < 0.01$) such that FGF9 inhibited estradiol production by 86% to 89% regardless of the presence of IGF1 or *MIR221* mimic or inhibitor (Figure 5A). Transfection of GC with *MIR221* mimic inhibited ($P < 0.001$) estradiol production in control and IGF1-treated GC by 42% and 31%, respectively, and further reduced ($P < 0.001$) the FGF9 inhibition of estradiol production by 26% to 35%. Transfection of GC with *MIR221* inhibitor only inhibited ($P = 0.04$) the IGF1-induced increase in estradiol production by 11% (Figure 5A).

No significant treatment × IGF1 × FGF9 ($P = 0.19$) or any two-way interaction existed ($P = 0.19$). Treatment ($P < 0.001$) and FGF9 affected ($P < 0.001$) progesterone production but IGF1 did not ($P = 0.10$). Specifically, FGF9 inhibited ($P < 0.01$) progesterone production 61% to 73% regardless of the presence of IGF1 or *MIR221* mimic or inhibitor (Figure 5B). Transfection of GC with *MIR221* mimic inhibited ($P < 0.001$) progesterone production in control and IGF1-treated

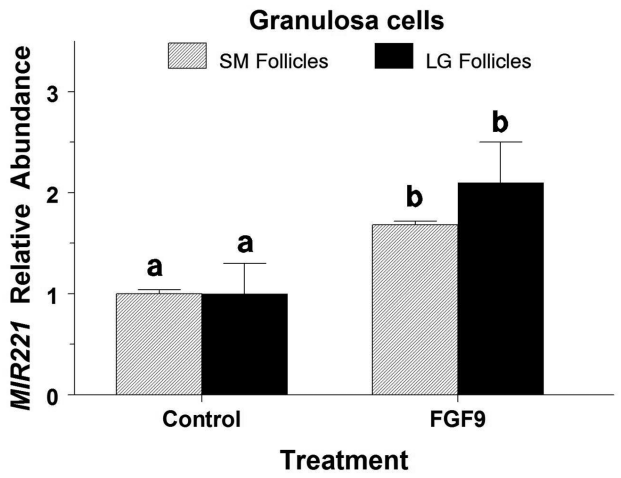

Figure 4. Effect of FGF9 on *MIR221* abundance in bovine granulosa cells from small (SM) and large (LG) follicles (Exp. 5). Granulosa cells were isolated and cultured for 48 h in 10% FCS after which cells were treated with either 0 or 30 ng/mL of FGF9 for 24 h. Values are normalized to constitutively expressed *U6* snRNA and expressed as fold of control values. [a,b]Within follicle size group, means (±pooled SEM) without a common letter differ ($P < 0.05$).

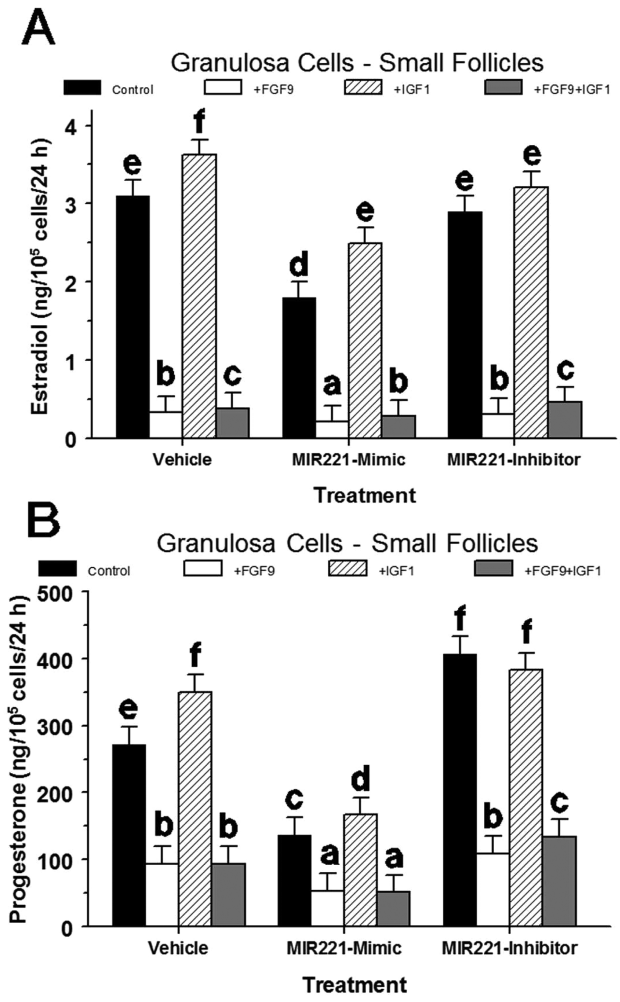

Figure 5. Effect of *MIR221* mimic and *MIR221* inhibitor on estradiol (A) and progesterone (B) production in bovine small-follicle granulosa cells treated with or without IGF1 and FGF9 (Exp. 6). Granulosa cells were isolated and cultured in 10% FCS and then transfected with either *MIR221* mimic or *MIR221* inhibitor for 12 h after which cells were treated with 0 or 30 ng/mL of FGF9, and 0 or 30 ng/mL of FSH + IGF1 for 40 h. [a,b,c,d,e,f]Within a panel, means (±pooled SEM) without a common letter differ ($P < 0.05$).

GC by 50% and 52%, respectively, and further decreased ($P < 0.001$) progesterone production by 44% to 46%, respectively, in the presence of FGF9. Treatment with *MIR221* inhibitor increased progesterone production in control cultures (by 50%; $P < 0.01$) and in those treated with IGF1 plus FGF9 (by 43%; $P = 0.02$) but did not affect progesterone production treated with IGF1 ($P = 0.49$) or FGF9 ($P = 0.20$) alone (Figure 5B).

DISCUSSION

MIR221 is expressed in a wide variety of tissues, including the breast (Di Leva et al., 2010), prostate (Sun et al., 2009), endometrium (Pan et al., 2007; Kuokkanen et al., 2010), and ovary (Dahiya et al., 2008; Salilew-Wondim et al., 2014). The present findings provide novel information on the dynamic regulation of *MIR221* levels in bovine GC and TC. Specifically the present results indicated that: (1) *MIR221* abundance in TC was severalfold greater than in GC of large follicles; (2) *MIR221* increased with follicle size in TC but decreased with follicle size in GC; (3) IGF1, estrogenic compounds, dbcAMP, and forskolin had no effect on TC *MIR221* abundance; (4) FGF9 increased both TC and GC *MIR221* abundance; and (5) transfection of GC with *MIR221* mimic reduced GC estradiol and progesterone production, whereas transfection with *MIR221* inhibitor increased progesterone production. These findings suggest that TC may be the main source of *MIR221* in large follicles and that FGF9 may modulate GC and TC function by influencing *MIR221* expression in cattle.

Previous studies indicate that a single microRNA will target, on average, 200 transcripts (Krek et al., 2005) in part because as little as 6 bp that match with the target mRNA can be sufficient to suppress gene expression (Lewis et al., 2003; Brennecke et al., 2005). Also, it is thought that miRNA expression broadly contributes to tissue specificity of mRNA expression in many human tissues (Sood et al., 2006). A potential 5,760 gene targets for human *MIR221* have been proposed (http://www.microrna.org/microrna/getTargets.do?matureName=hsa-miR-221&startIndex=0&organism=9606) and research with human SNU-398 heptocarcinoma cells reported 125 genes to be regulated by *MIR221* (Lupini et al., 2013). Using TargetScan (release 7.1, Whitehead Institute for Biomedical Research; Agarwal et al., 2015) (see: http://www.targetscan.org/cgi-bin/targetscan/vert_71/targetscan.cgi?species=Cow&gid=&mir_sc=miR-221%2F222&mir_c=&mir_nc=&mir_vnc=&mirg=) 43 target genes of bovine *MIR221* were identified. Therefore, further research will be required to identify and verify specific target genes of bovine *MIR221* in TC and GC.

Determining the expression of *MIR221* in GC and TC and the hormonal regulation of *MIR221* expression is crucial to understanding its role in ovarian follicular function. In a recent study using cattle, *MIR221* expression in GC was up regulated (by 1.8-fold) in subordinate vs. dominant follicles on d 3 postestrus only to be 3.2-fold less in GC of subordinate vs. dominant follicles on d 5 postestrus (Salilew-Wondim et al., 2014). Previously, dominant follicles on d 3 to 4 postovulation had significantly less *FGF9* mRNA than subordinate follicles (Schütz et al., 2016). Thus, based on results of previous and present studies, changes in follicular cell *MIR221* expression may involve

changes in FGF9, which belongs to the 23-member FGF family (Chaves et al., 2012). To date, FGF1, FGF2, FGF7, FGF8, FGF9, FGF10, FGF17, and/or FGF18 have been described in ovaries of many species including rodents (Drummond et al., 2007) and domestic animals (Machado et al., 2009; Portela et al., 2010; Grado-Ahuir et al., 2011). Functions of FGF members in ovarian biological processes include regulation of steroidogenesis (Vernon and Spicer, 1994; Schreiber and Spicer, 2012; Evans et al., 2014), apoptosis and cell survival (Portela et al., 2010; Jiang and Price, 2012), and control of ovarian cell proliferation (Buratini et al., 2005; Schreiber and Spicer, 2012; Schreiber et al., 2012). It has been suggested that FGF9 is acting as an antidifferentiation factor stimulating GC and TC proliferation while inhibiting steroidogenesis in cattle (Schreiber and Spicer, 2012; Schreiber et al., 2012; Schütz et al., 2016). Based on results of the present study, both small and large follicles have greater *MIR221* expression in TC than GC. Interestingly, *MIR221* abundance increased in TC and decreased in GC as follicles enlarged, suggesting that control of *MIR221* expression may differ in GC and TC. However, in the preovulatory dominant and subordinate follicles of heifers, TC and GC *MIR221* abundance did not differ (Gebremedhn et al., 2015). In the present study, FGF9 and FGF2 increased *MIR221* expression by 2- to 3-fold, whereas IGF1 had no effect. Whether changes in FGF2 and FGF9 (or other FGF) are regulating changes in GC or TC *MIR221* abundance during follicular growth will require further elucidation.

In contrast to previous studies in nonovarian tissue, the present study indicates TC *MIR221* expression is not regulated by steroids. Previously, estrogen receptor α stimulation directly repressed *MIR221* expression in human breast cancer cell lines (Di Leva et al., 2010), whereas in human prostate cell lines, *MIR221* expression is repressed by androgens (Ambs et al., 2008). Because our findings indicated that estrogens and androgens had no effect on *MIR221* abundance in TC, it appears that as a follicle grows, changes in steroid hormones do not regulate the changes in *MIR221* expression in bovine GC and TC. This latter suggestion is supported by the findings of Gebremedhn et al. (2015) who reported that abundance of *MIR221* in TC and GC did not differ between dominant and subordinate preovulatory follicles in heifers. Together, the present and previous results suggest that steroid regulation of *MIR221* expression may be tissue specific.

In the current study, enhancers of cAMP production/action had no effect on *MIR221* abundance in TC, which is in contrast to findings of Castagnino et al. (2013) who found that 1 mM dbcAMP and forskolin inhibited *MIR221* expression in cultured murine vascular smooth muscle cells. In cultured murine GC, treatment with 1 mM 8-bromo-cAMP had no effect on another microRNA, *MIR21* (Carletti et al., 2010). Similarly, changes in *MIR125b*, *MIR21*, *MIR145*, and *MIR34a* in cultured bovine TC were independent of 10 µM forskolin treatment (McBride et al., 2012). In line with these findings, data from the present study indicate that the signal transduction of cAMP does not affect *MIR221* abundance in vitro, as dbcAMP and forskolin did not alter *MIR221* expression. We recognize that our study has limitations including the fact that only one dose of dbcAMP, forskolin, growth factors, and steroids were used, and thus, future studies should evaluate the effects of additional doses of these factors.

Small noncoding RNA molecules regulate protein levels in a post-transcriptional manner by partial base pairing to the 3′-UTR of target genes thus mediating degradation or translational repression (He and Hannon, 2004; Ha and Kim, 2014) and research indicates that miRNA can have both inhibiting and stimulating roles (Sirotkin et al., 2009; Xu et al., 2011; Dai et al., 2013) on ovarian steroid hormone production. For example, in human KGN (granulosa) cells, *MIR133a* stimulates estradiol production (Dai et al., 2013), whereas in porcine GC, *MIR378* inhibits estradiol production (Xu et al., 2011). In the present study, *MIR221* mimic reduced estradiol and progesterone production by bovine GC, suggesting that *MIR221* may be inhibiting steroidogenesis in GC via translational repression of *CYP19A1* and *CYP11A1*. Other studies have suggested direct targeting of gene(s) associated with steroid biosynthesis or indirect by affecting its precursors or processing and metabolism (Sirotkin et al., 2009; Yan et al., 2012; Dai et al., 2013). Further research will be required to ascertain whether *MIR221* directly targets *CYP19A1* (for estradiol production) or *CYP11A1* (for progesterone production) in bovine GC. In androgen-independent human prostate cancer cell lines, the *MIR221* cluster is known to interfere with androgen receptor transcriptional activity without affecting the androgen receptor itself (Sun et al., 2009). Transfection of GC with *MIR221* inhibitor had little or no effect on steroid production in the present study, further implying that the main source of follicular fluid *MIR221* may originate from TC.

Many miRNA have been suggested to be involved in regulating ovarian follicular function in cattle (Hossain et al., 2009; Salilew-Wondim et al., 2014; Gebremedhn et al., 2015), sheep (Baillet et al., 2008; Di et al., 2014), mares (Schauer et al., 2013), humans (Sirotkin et al., 2009; Lin et al., 2015), and rodents (Herrera et al., 2005; Yao et al., 2010). Examination of miRNA expression in follicles of sheep and cattle revealed some miRNA are expressed more abundantly in TC while others are expressed more abundantly in GC (McBride et al., 2012; Sohel et al., 2013; Gebremedhn et al., 2015). In addition, previous studies indicate that miRNA, secreted by exosomes (Sohel et al., 2013), are found circulating in serum and can be used as biomarkers for human disease (Friedman et al., 2012; Sochor et al., 2014; Langhe et al., 2015) including ovarian cancer (Hong et al., 2013) and polycystic ovarian syndrome (Ding et al., 2015; Jiang et al., 2016). Perhaps miRNA produced in TC are secreted via exosomes into the follicular fluid and act in a paracrine manner to regulate gene expression in GC (Camussi et al., 2010). In support of this suggestion, recent studies show that *MIR221* can be detected in bovine follicular fluid (Gebremedhn et al., 2015). In addition, changes in blood levels of *MIR221* have been observed during the estrous cycle in cattle (Noferesti et al., 2015) and may also contribute to changes in *MIR221* in follicular fluid. Additional research will be required to verify these suggestions.

In summary, the results from the present study demonstrate that *MIR221* abundance in GC decreases and in TC increases with follicular development in cattle, and that FGF9 and FGF2 increase TC *MIR221* abundance while IGF1, steroids, and cAMP agonists have no effect on TC *MIR221*. We conclude that FGF9 increased GC and TC *MIR221* expression, and that increased *MIR221* may act to inhibit GC steroidogenesis in cattle.

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