HORMONAL REGULATION OF MICRORNA-221 AND ITS EFFECT ON BOVINE OVARIAN THECA CELL FUNCTION

By

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Abstract: Development of ovarian follicles in cattle is controlled by systemic and locally produced hormones acting at the molecular level controlling numerous gene transcripts which spatial-temporal expression leads to one follicle ovulating and other follicles undergoing regression. MicroRNA-221 (miRNA-221) is increased in fibroblast growth factor 9 (FGF9) granulosa and theca cells of subordinate follicles compared with dominant follicles in cattle. The objectives of this study were to investigate the hormonal regulation of miRNA-221 expression in theca cells and its possible role in regulating follicular function. Bovine ovaries were collected from a local abattoir and theca cells were obtained from large (8 to 22 mm) follicles, cultured for 48 h in 10% fetal calf serum (FCS), and treated with various hormones in serum-free medium for an additional 24 or 48 h in five experiments. Medium was collected for analysis of progesterone and androstenedione concentrations via radioimmunoassay, or cellular RNA was collected for gene expression analysis of miRNA-221 via real-time PCR. In Exp. 1, FGF9 increased ($P = 0.08$) abundance of miRNA-221 2.0-fold after 12 h and 2.4-fold after 24 h compared with controls. In Exp. 2, forskolin and dibutyryl cyclic adenosine monophosphate (dbcAMP) had no effect ($P > 0.10$) on miRNA-221 expression in bovine theca cells, but FGF9 treatments increased ($P < 0.05$) miRNA-221 abundance 1.94-fold. In Exp. 3, IGF1 had no effect ($P > 0.10$) on basal or FGF9-induced miRNA-221 expression (3.3-fold increase) in bovine theca cells, however, 10% FCS increased ($P < 0.05$) miRNA-221 abundance by 3-fold greater than control cultures; the combined treatment of FGF9 and 10% FCS did not differ ($P > 0.10$) from either treatment alone. In Exp. 4, estradiol, androstenedione, and phytoestrogens had no effect ($P > 0.10$) on miRNA-221 abundance. In Exp. 5, neither miRNA-221 mimic nor inhibitor affected cell numbers; nonetheless FGF9 stimulated an increase in cell proliferation ($P < 0.01$). Similarly, neither miRNA-221 mimic nor inhibitor affected steroidogenesis whereas FGF9 inhibited ($P < 0.01$) IGF1-induced production of androstenedione and progesterone. In summary, exposure of bovine theca cells to FGF9 *in vitro* increased expression of miRNA-221 however miRNA-221 was not regulated by steroids or cAMP. The role of miRNA-221 in follicular function will require further study.

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CHAPTER I

INTRODUCTION

Cattle are a monovulatory species that develop follicles in wave-like patterns during the diestrous stage of their estrous cycle (De Rensis and Peters, 1999; Evans, 2003). Follicular development in cattle is characterized by recruitment of a group of follicles, selection and growth of one of those follicles, which becomes dominant and destined to ovulate (Ahmad et al., 1997). The dominant follicle will secrete an increased amount of estrogen and inhibin-α as a result of its larger diameter, thus stifling the growth of subordinate follicles (Mihm et al., 2002; Ginther et al., 2003). The recruitment and selection of follicles require a closely regulated sequence of endocrine and biochemical interactions that induce changes within the follicular environment (Palma et al., 2012). These cyclic changes affect numerous processes such as cellular growth, proliferation, differentiation, steroidogenesis and atresia to determine the final fate of follicles (Hossain et al., 2009). Nonetheless, the mechanism of selecting a single dominant follicle remains unknown.

Follicular recruitment, selection, and dominance are contingent on the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Hunter et al., 2004). FSH is the primary hormone that modulates follicular growth by stimulating cell proliferation and preventing atresia (Silva et al., 2006). Additionally, FSH stimulates antral follicles to develop LH receptors in granulosa cells and induces cytochrome P450 side-chain cleavage (CYP11A1; converts cholesterol to pregnenolone) and aromatase (CYP19A1; converts androgens into estrogens) steroidogenic enzymes (Garverick et al., 2002). Initially, LH induces intrafollicular steroid production via stimulation of androgen synthesis of theca cells. Subsequently, LH receptors in granulosa cells are developed on future dominant follicles preceding morphological dominance (Ginther et al., 2003; Fortune et al., 2004). Preovulatory follicles will have a dramatic increase in LH receptors in preparation for ovulation and luteinization (Lei et al., 2001).

Additionally, growth factors including insulin-like growth factor 1 (IGF1), transforming growth factor β (TGFβ) superfamily, and members of the fibroblast growth factor (FGF) family have an important and essential role in follicular development and modulation of follicle function. IGF1 enhances follicular cell proliferation and steroid production in response to gonadotropins (Adashi et al., 1991; Spicer et al., 1993; Gong et al., 1994; Stewart et al., 1996). The TGFβ superfamily contains a large group of proteins that include activins, inhibins, bone morphogenetic proteins (BMPs), and growth differentiation factors (GDFs), and are thought to be involved in follicular activation, hormone synthesis, ovulation, and oocyte maturation (Juengel et al., 2004; Shimasaki et al., 2004; Mazerbourg and Hsueh, 2006; Young and McNeilly, 2010). FGF family of signaling molecules has been implicated in a wide variety of biological processes such as cell proliferation, tissue repair, injury responses and disease via intracrine, paracrine and endocrine actions (Itoh and Ornitz, 2011). The FGF family consists of 23 members and to date, FGF1, FGF2, FGF7, FGF8, FGF9, FGF10, FGF17, and FGF18, have been described in the human,

rodent and other domestic animal ovaries (Drummond et al., 2007; Machado et al., 2009; Portela et al., 2010; Grado-Ahuir et al., 2011; Chaves et al., 2012). Functions of FGF members in the ovary 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), control of cell proliferation (Buratini et al., 2005; Schreiber and Spicer, 2012) and luteal development (Woad et al., 2012). A microarray study indicates that FGF9 may play a role in the development of follicular cysts (Grado-Ahuir et al., 2011). Further research indicated that FGF9 stimulates granulosa and theca cell proliferation while inhibiting steroidogenesis (Schreiber and Spicer, 2012; Schreiber et al., 2012). Irregular expression of these developmentally related genes and products of those genes in the follicle could lead to dysfunction in cellular communication and disruption of normal follicle recruitment and development (Toloubeydokhti et al., 2008). The regulatory mechanism of post-transcription of such genes associated with follicular recruitment, selection, and dominance during the estrous cycle is poorly understood (Salilew-Wondim et al., 2014).

Ovarian follicular development is mediated by hormonal coordination between the pituitary, the ovary (Hirshfield, 1991; Hunter et al., 2004) and numerous growth factors (Webb et al., 1999; Fortune et al., 2004). The biosynthesis of these hormones and growth factors are modulated by expression of numerous mRNA transcripts in the ovary (Imbar and Eisenberg, 2014). Emerging evidence indicates that non-coding microRNAs (miRNA) regulate cellular processes and major signaling pathways by complementary binding to messenger RNA (mRNA) to mediate degradation or transcriptional repression of mRNA (Hwang and Mendell, 2007). MicroRNA molecules are the most prevalent group of small RNAs in the ovary (Ro et al., 2007), and may influence fundamental ovarian physiological processes like steroidogenesis and apoptosis in granulosa cells. Of particular interest is miRNA-221 because it is overexpressed in

ovarian carcinoma patients (Dahiya et al., 2008) and its differential expression in subordinate verses dominant follicles in cattle (Salilew-Wondim et al., 2014).

Because FGF9 signaling is involved in ovarian folliculogenesis and steroidogenesis, our lab investigated the relationship between miRNA-221 and FGF9 in theca cells of cattle. This review will summarize the roles that gonadotropins, growth factors, and microRNAs play during folliculogenesis.

CHAPTER II

REVIEW OF LITAUTURE

1. Ovarian Follicular Growth and Development in Cattle

The coordinated process of follicular development is governed by endocrine signals and paracrine communication between the oocyte, granulosa and theca cells (Young and McNeilly, 2010; Palma et al., 2012). Follicles are the fundamental units of the ovary encompassing an oocyte with several layers of granulosa cells and a layer of theca cells. The number of both theca and granulosa cell layers, and size of the oocyte is dictated by the stage of follicular growth (Buccione et al., 1990). Follicles start to develop from resting primordial follicular pools. As primordial follicles morphologically transform into primary follicles, oocyte maturation continues and granulosa cells proliferate as secondary follicles develop. The preantral (secondary) follicles then develop into antral follicles through continual differentiation and development (Aerts and Bols, 2010). During growth of primary follicles, theca cells start to emerge and form a layer around the granulosa-oocyte structure. A locally produced growth factor, GDF-9, is critical for the development of the theca layer, without which follicles do not develop beyond the primary stage (Dong et al., 1996; Nilsson and Skinner, 2002; Silva et al., 2005).

In cattle, a monovulatory species, follicular development is characterized by recruitment of a group of follicles, selection and growth of one of those follicles, which becomes dominant (Ahmad et al., 1997). The estrous cycle of cattle consists of two physiological states, the follicular phase and luteal phase (Hansel and Convey, 1983; Mihm et al., 2002). Additionally, the estrous cycle is further categorized into four stages: metestrus, diestrus, proestrus and estrus. Ovarian follicles grow and develop in a wave-like pattern throughout the estrous cycle, which is established by a combination of endocrine signals and intraovarian factors (Adams, 1999; Ginther, 2000). Characteristically two to three follicular waves occur during each estrous cycle in cattle (Mihm et al., 2002; Evans, 2003). During metestrus and after ovulation, LH is secreted in low quantities whereas FSH levels are transiently increased stimulating the initial follicular wave (Lucy et al., 1992; Ginther et al., 1996; McGee and Hsueh, 2000; Mihm and Bleach, 2003). The transient rise in FSH after ovulation (metestrus) activates different intracellular pathways for the recruited cohort of follicles to avoid an apoptotic demise (Kaipia and Hsueh, 1997; Ginther et al., 2003). In cattle a cohort of antral follicles normally contain 5 to 10 follicles that are 4 to 6 mm in diameter (Savio et al., 1988; Sirois and Fortune, 1988; Sunderland et al., 1994). Additional follicular waves are initiated during maximal progesterone in diestrus; and these waves occur around d 11, or d 9 and d 16 for cattle with two or three waves, respectively (Sirois and Fortune, 1988; Mapletoft et al., 2002). Neither complete follicular development nor ovulation can occur under progesterone dominance, but rather transient rises in FSH and increases in estradiol (Ginther et al., 1996; Mihm and Bleach 2003). As progesterone levels decrease during proestrus, a dramatic increase in gonadotropin-releasing hormone (GnRH) stimulates anterior pituitary and systemic levels of FSH and LH to increase and the concomitant LH and FSH surges occur (Hansel and Convey, 1983; Ginther et al., 1996).

After recruitment, the follicles of a wave are gonadotropin-dependent and undergo a common growth phase (Gastal et al., 1997; Ginther et al., 1997; McNatty et al., 2007). In monovulatory species, the selection of follicles is a dynamic process where one follicle develops beyond the common growth phase to become the only follicle with ovulatory capacity while the remaining subordinate follicles begin to regress (Ginther et al., 2000; Ireland et al., 2000). Diameter deviation is preceded by intrafollicular biochemical events that ensure future dominance of the selected follicle (Fortune et al., 2004). The dominant follicle secretes an increased amount of estradiol and inhibin-α compared with subordinate follicles of the same wave (Ginther et al., 2003; Fortune et al., 2004). Estradiol synthesis is dependent on gonadotropin and IGF1 modulation. Researchers at the University of Missouri discovered that newly selected dominant follicles had greater LH receptor mRNA abundance in both theca and granulosa cells compared with recruited follicles (Bao et al., 1997). Both inhibin-α and estradiol suppress circulating FSH concentrations, which stifle growth of subordinate follicles leading to atresia (Bergfelt and Ginther, 1993). Once a growing follicle reaches 5 mm in diameter, it has the capacity to inhibit FSH concentrations (Bergfelt et al., 2000; Ginther, 2000). Thus, the dominant follicle must develop survival mechanisms that do not involve FSH to become ovulatory.

Evidence implies that changes in the intrafollicular insulin-like growth factor (IGF) system within the future dominant follicle are a crucial aspect of selection, subsequent growth, differentiation and ovulation (Fortune et al., 2004; Spicer, 2004). The IGF system consists of IGF1 and IGF2, IGF type I and type II receptors, binding proteins (IGFBP 1-6) and IGFBP proteases (Webb et al., 1999; Spicer, 2004). In cattle, IGF1 has paracrine-autocrine actions stimulating granulosa and theca cell growth and amplifying gonadotropin-induced differentiation and steroid production (Monget and Monniaux, 1995; Spicer and Echternkamp, 1995; Glister et al., 2001). IGFBPs are potent modulators of IGF1 bioavailability within the follicle (Armstrong and Webb, 1997; Spicer, 2004). The IGFBPs have the potential to inhibit gonadotropin-

dependent follicular development and differentiation via inhibiting the action of IGF1 and IGF2 (Adashi et al., 1991; Stewart et al., 1996; Armstrong and Webb, 1997; Clemmons et al., 1998). In cattle, four main IGFBPs (BP-2, -3, -4 and -5) have been identified in follicular fluid (Echternkamp et al., 1994; Mihm et al., 2000; Austin et al., 2001). Studies have demonstrated that IGFBP-2, -4, and -5 decrease in selected estrogen-active dominant follicles (Stewart et al., 1996; Rivera and Fortune, 2001; Spicer et al., 2001; Echternkamp et al., 2004; Spicer, 2004), which in turn increase the bioavailability of IGF1, suggesting that differentiation of the dominant follicle is enhanced by changes in intrafollicular amounts of IGF1 and IGFBPs. The reduction in IGFBPs is in part due to alterations in ovarian synthesis of these binding proteins (Spicer, 2004; Voge et al., 2004) in addition to action of IGFBP proteases (Fortune et al., 2004; Spicer, 2004).

Pregnancy-associated plasma protein-A (PAPPA) is an IGFBP protease that is found in follicular fluid of cattle (Mazerbourg et al., 2000; Spicer, 2004) and this protease cleaves and degrades IGFBP complexes to liberate IGF1 (Mazerbourg et al., 2000; Qin et al., 2000; Ginther et al., 2001). *In vitro* studies indicate that regulation of PAPPA mRNA abundance in bovine granulosa and theca cells differ (Aad et al., 2006). In theca cells, estradiol inhibits both IGFBP-2 and -4 mRNA (Voge et al., 2004) and PAPPA mRNA expression (Aad et al., 2006), suggesting that estradiol may have a role in an intraovarian feedback system regulating bioactivity of IGFs in the theca layer of recruited follicles. In granulosa cells, IGFBP-2 and -4 mRNA (Voge et al., 2004) and PAPPA mRNA (Aad et al., 2006) abundance are not affected by FSH or estradiol suggesting that changes in PAPPA activity may be driven by changes in protease inhibitors since FSH treatment *in vivo* increases intrafollicular PAPPA activity (Rivera and Fortune, 2003a). Liberated IGF1 then synergizes with FSH leading to greater follicular estradiol production (Rivera and Fortune, 2003a). This biochemical selection triggers morphological selection as the follicle continues to grow and FSH returns to basal levels (Fortune et al., 2004).

As reviewed in this section, the process of recruitment, selection and growth of follicles are under control of synchronized paracrine and endocrine factors (Mihm et al., 2002; Hunter et al., 2004), and involve a balance between many cellular processes and signaling pathways that are controlled via strictly modulated expression of a multitude of genes located within the ovary (Bonnet et al., 2008; Mihm et al., 2008). Nonetheless, the estrous cyclicity of cattle may not always follow the expected hormone expression and successive events. Decreased expression of estrus and silent ovulation are related to environmental factors such as heat stress (Madan and Johnson, 1973; Her et al., 1988). Heat stress affects follicular function directly at the ovarian level as well as indirectly via secretion of pituitary hormones (Hansen, 2009). In lactating dairy cows, heat stress increases numbers of small and medium follicles, and recruitment of these follicles into a growth wave appears to be due to a reduction in circulating concentrations of inhibin and increased FSH secretion and thus extend the length of the estrous cycle (Roth et al., 2000). Similarly, *in vitro* bovine follicular cells have reduced steroidogenesis with elevated (i.e., 41° C) temperature and perhaps alter oocyte competence (Wolfenson et al., 1997; Bridges et al., 2005). Follicular cysts are another dysfunction related to cyclicity, and as a result negatively impact reproduction (Bartlett et al., 1986; Peter, 2004; Vanholder et al., 2005). Cystic follicles develop due to a dysfunction of the hypothalamic-pituitary-ovarian axis, and therefore many factors (cellular and molecular) are likely involved in their pathogenesis (Kesler and Graverick, 1982; Vanholder et al., 2005). Understanding the expression of genes modulating follicle development in cattle has been highly revealing. Grado-Ahuir and colleagues (2011) utilized microarrays to identify 163 differentially expressed genes in ovarian granulosa cells between normal dominant and cystic follicles of cattle. Further, microarrays were used to elucidate the global pattern of gene expression in aberrant persistent dominant follicles and found changes in expression of 475 genes involved in nutrient metabolism, amino acid transport and apoptosis in granulosa cells (Lingenfelter et al., 2008). A number of microarray studies evaluating altered gene expression during ovarian follicle development revealed as many as 261 genes differentially

expressed between dominant and subordinate follicles (Evans et al., 2008; Hayashi et al., 2010). Evans et al. (2008) further identified 83 genes involved in signal transduction, which are differentially expressed between subordinate and dominant follicles. The microarray approach has lead to the discovery of new genes involved in ovarian follicle development, and may help to identify new solutions for increased success of synchronization programs that regulate antral follicle growth and ultimately ovulation (Moore and Thatcher, 2006).

2. Endocrine and Paracrine Factors Controlling Ovarian Follicular Growth

2.1 Gonadotropins

In cattle, follicles that develop beyond 4 mm in diameter are regarded as gonadotropin-dependent, which includes those follicles involved in recruitment, selection and dominance (Savio et al., 1988; Zeleznik and Benyo, 1994; Campbell et al., 1995; Gong et al., 1996). FSH is gonadotropic-glycoprotein hormone that is necessary for folliculogenesis, especially for emergence and early follicular growth (Bao and Garverick, 1998). The impact of FSH is determined by circulating concentrations of FSH, and its receptor expression on granulosa cells (Bodenstiener et al., 1996; O'Shaughnessy et al., 1996). A FSH knockout mouse model indicates that FSH is necessary for continued folliculogenesis (Kumar et al., 1997; Danilovich et al., 2000; Burns et al., 2001). Granulosa cell proliferation, prevention of atresia, synthesis of LH receptors (LHCGR), expression of two steroidogenic enzymes, CYP19A1 and CYP11A1, are all stimulated by FSH in cattle (Silva and Price, 2000; Silva et al., 2006). Binding of FSH to its receptor activates numerous intracellular pathways via cyclic adenosine 3'-5' monophosphate (cAMP) (Izadyar et al., 1998; Conti 2002). Subsequently, protein kinase A (PKA), C (PKC), and B (PKB; AKT), extracellular signal-regulated kinases (ERK) and phosphatidylinositol 3-kinase (PI3K) are stimulated by FSH's signal transduction (Izadyar et al., 1998; Conti 2002; Silva et al., 2006; Castanon et al., 2012). Alterations in concentration of FSH and FSH receptor modulate key

aspects of follicular divergence and survival of future dominant follicles (Adams et al., 1992; Bao et al., 1997; Mihm et al., 1997).

Luteinizing hormone (LH) becomes more important as follicles continue to develop and mature (Lucy et al., 1992). A transient increase in systemic LH concentration is required to induce androgen biosynthesis and cause ovulation (Hansel and Convey, 1983). Recruited follicles express mRNA for LHCGR in theca cells, and future dominant follicles attain greater numbers of LHCGR before diameter deviation and contain a greater amount of LH receptor protein when compared with subordinate follicles (Stewart et al., 1996; Bao et al., 1997). LH-suppressed heifers develop smaller dominant follicles that have a diameter of 7 to 9 mm and lower intrafollicular concentrations of estradiol, androstenedione and IGF1 but increased IGFBP-2 concentrations (Ginther et al., 2003). Similarly, when exogenous LH treatments are applied during the midluteal phase, follicular fluid concentrations of estradiol and androstenedione are increased (Manikkam et al., 2001). These studies indicate that LH is an important component of the dominant follicle, which stimulates steroidogenesis and growth factor systems for continued development (Sartori et al., 2001; Ginther et al., 2003).

2.2 Steroids

Ovarian factors such as steroids modulate release of gonadotropins from the anterior pituitary gland and have paracrine effects on granulosa and theca cells. Estradiol secreted from the dominant follicle stimulates LH pulses while having an inhibitory affect on FSH secretion in cattle (Rahe et al., 1980; Austin et al., 2002). To further suppress circulating FSH concentrations, the dominant follicle secretes the protein hormone inhibin (Austin et al., 2002). Collectively, inhibin and estradiol reduces FSH release and as a result starve FSH-dependent subordinate follicles causing their growth to be discontinued. This negative feedback is thought to be the main developmental mechanism for selection of a single dominant follicle in monovular species

(Ginther et al., 2003). However, recent evidence indicates that changes in the IGF type II receptor may alter the number of follicles recruited for ovulation (Aad et al., 2012). Estradiol also is involved in intraovarian communication acting in both a paracrine and autocrine manner. Paracrine signaling of estradiol suppresses progesterone production and enhances androstenedione production induced by LH, insulin and IGF1 in bovine theca cells (Roberts and Skinner, 1990; Spicer, 2005). Additionally, paracrine actions of estradiol affect the IGF system via inhibiting PAPPA mRNA abundance (Aad et al., 2006) and reducing IGFBP-2 and -4 mRNA abundance in cultured theca cells (Voge et al., 2004). Within granulosa cells, estradiol has an autocrine effect on differentiation as it stimulates IGFBP-4 synthesis in bovine granulosa cells (Spicer and Chamberlin, 2002). Thus, granulosa-cell derived estradiol may have a role in follicular development, via a paracrine effect on theca cells and an autocrine influence on granulosa cells.

Progesterone is another steroid hormone involved in intraovarian communication via paracrine and autocrine modes of action, as well as its endocrine role that regulates the estrous cycle. During emergence of the first follicular growth wave, treatment of progesterone paired with estradiol subdues pulsatile secretion of LH while minimally affecting FSH release, which arrests dominant follicle development (Austin et al., 2002). *In vivo*, treatment of progesterone decreases LH pulse frequency and reduces estradiol concentrations in follicular fluid (Manikkam and Rajamahendran, 1997; Austin et al., 2002). Progesterone treatments also amplified the intrafollicular concentration of IGFBPs and apoptosis in granulosa cells (Manikkam and Rajamahendran, 1997; Austin et al., 2002). These studies indicate that progesterone has a direct inhibitory endocrine effect on gonadotropin release, which indirectly inhibits dominant follicle development. Because progesterone receptors (PR) increase during follicular growth, autocrine and paracrine actions of progesterone throughout follicular development are indicated (Berisha et al., 2002; Sriraman et al., 2010)

Androstenedione, produced by theca cells, is a steroid precursor for estradiol production, which is critical for follicular development and ovulation and thus reproductive success (Senger, 2005; King et al., 2007; Walters et al., 2008). When co-cultured with granulosa cells, theca cells exhibit increased amounts of androstenedione produced, suggesting a synergistic relationship between the two cell types (Tajima et al., 2006). The majority of healthy follicles and even some atretic follicles are able to secrete androstenedione in response to LH however, only 1 to 3 follicles have granulosa cells capable of converting it to estradiol (McNatty et al., 1984). Moreover, in the ovarian microenvironment, theca-derived androgens act as important paracrine factors by increasing FSH receptors in granulosa cells and increasing aromatase activity (Luo and Wiltbank, 2006). Co-cultures of bovine granulosa and theca cells show that theca cells can significantly alter inhibin production by granulosa cells (Tajima et al., 2006). Thus, paracrine actions of androgens can impact both endocrine and intraovarian function of follicles.

2.3 Insulin-like Growth Factors

Insulin-like growth factor 1 (IGF1) is a protein hormone containing 70 amino acids. This growth factor has a similar structure and function to insulin. Produced primarily by the liver, IGF1 is an endocrine hormone that mediates many biological functions including absorption of glucose, stimulation of myogenesis, inhibition of apoptosis and stimulation of lipid synthesis (Etherton, 2004; Reyna et al., 2010). Biosynthesis of IGF1 in the liver is stimulated by growth hormone (GH), and factors of malnutrition and lack of protein in a diet suppress IGF1 production (Miura et al., 1992; Sorensen and Knight, 2002). Although IGF1 or IGF2 mRNA exists in granulosa cells of many species (Giudice, 2001; Spicer, 2004), in cattle, the vast majority of IGF1 found in follicular fluid is derived from the systemic circulation (Spicer et al., 1992).

The IGF type I and II receptors are localized in bovine oocytes, cumulus cells and both granulosa and theca cell (Yoshida et al., 1998; Perks et al., 1999; Armstrong et al., 2001; Schams

et al., 2002). *In vitro* studies indicate a synergistic relationship between IGF1 and gonadotropins in the stimulation of steroidogenesis in both granulosa (Baranao and Hammond, 1984; Spicer et al., 1993; Ranzenigo et al., 2008) and theca cells (Stewart et al., 1995; Spicer and Francisco, 1997; Spicer and Chamberlain, 1998; Spicer et al., 2008). Specifically, IGF1 and FSH synergize to stimulate aromatase activity in granulosa cells (Spicer et al., 2002). As reviewed earlier, the bioactivity of IGF1 is mediated by IGFBPs and IGFBP proteases (e.g., PAPPA). IGFBPs have a greater affinity for IGF1 and IGF2 than IGF receptors and as such serve as a modulator of cellular processes that are dependent on these growth factors such as steroid production or cell proliferation.

2.4 Other Growth Factors

In addition to IGF1, multiple peptide growth factor systems appear to be regulating ovarian follicular development.

TGFβ. Oocytes, granulosa and theca cells synthesize transforming growth factor-β (TGFβ) during follicle development. TGFβ and its family of proteins are involved in controlling follicular growth by modulating recruitment, steroidogenesis, follicular cell proliferation, luteinization, ovulation and oocyte maturation (Knight and Glister, 2006; Zheng et al., 2008). The TGFβ family consists of extracellular signaling molecules that include three isoforms of TGFβ: anti-mullerian hormone (AMH), inhibins (α and β) and activins (α, β and αβ) (Knight and Glister, 2006; Mazerbourg et al., 2004). Influencing the activity of activins is a single-chain polypeptide called follistatin (Michel et al., 1993). Additionally, 20 bone morphogenetic proteins (BMP) and nine growth differentiation factors (GDF) are a part of the TGFβ system (Knight and Glister, 2006). With the exception of inhibin, TGFβ family members cause biological effects by forming heteromeric complexes with a type I and type II receptor on the cell surface (Lebrun et al., 1997; Miyazono, 1997; Mazerbourg et al., 2006).

TGFβ family members, specifically GDF9, inhibins, activins, TGFβ, BMP-2,-4,-6,-7 and AMH, are expressed and affect ovarian follicular development (Knight and Glister, 2006; Spicer et al., 2008; Otsuka et al., 2011; Knight et al., 2012). Observations indicate that BMPs act directly on theca cells to reduce androgen production, in addition to modulating the expression of factors secreted from granulosa cells that act in a paracrine manner on theca steroidogenesis (Glister et al., 2005; Campbell et al., 2006). GDF9 alone, and in combination with IGF1, stimulates bovine thecal cell proliferation and decreases theca cell progesterone and androstenedione synthesis (Spicer et al., 2008). Inhibin enhances LH-induced androgen production by theca cells of cattle, whereas activin reduces this effect (Hsueh et al., 1987; Wrathall and Knight, 1995). The activin-follistatin complex, which consists of one activin dimer and two follistatin molecules, inhibits the activin-stimulated increase of FSH receptor mRNA (Xiao et al., 1992; Tano et al., 1995). Testosterone inhibits AMH mRNA expression in bovine granulosa cells (Crisosto et al., 2009) and AMH reduces the sensitivity of small antral follicles to FSH (Visser and Themmen, 2005). TGFβ is produced by theca cells in cattle and suppresses theca cell androgen production (Demeter-Arlotto et al., 1993; Knight and Glister, 2006). In addition, TGFβ inhibits FSH-induced estradiol production by bovine granulosa cells (Ouellette et al., 2005). Collectively, these studies indicate that the predominant effect of members of the TGFβ family is to inhibit differentiated function (e.g., increased steroidogenesis) of granulosa and theca cells.

FGFs. The fibroblast growth factor (FGF) family consists of 23 members in vertebrates, and thus is one of the largest families of growth factors (Drummond et al., 2007; Chaves et al., 2012). The FGF family members are heparin-binding peptides that share 13 to 70% homology and have a molecular mass ranging from 17 to 34 kDa (Itoh and Ornitz, 2004). FGFs and their receptors are distributed in various tissues and are involved in a wide variety of biological processes including cell proliferation, tissue repair, injury response, differentiation, and cell migration through intracrine, autocrine, paracrine and endocrine mechanisms of action (Itoh and

Ornitz, 2011). FGFs mediate biological responses by binding and activating both types high affinity and low affinity FGF receptors (FGFR) (Cotton et al., 2008; Beenken and Monhammadi, 2009). FGFR1 to FGFR4 tyrosine-kinase receptors consist of an intracellular domain, a single transmembrane domain and an extracellular portion containing three immunoglobulin-like domains, which serve for the interaction and specificity with the FGFs (Wing et al., 2003; Itoh, 2010; Chaves et al., 2012). Subsequent to binding to their receptors, FGFs induce functional dimerization, phosphorylation of cytoplasmic tyrosine residues and activation of key downstream signaling pathways as well as signal transducers and activators of transcription (Turner and Grose, 2010).

The FGF family is emerging as a group of factors involved in the regulation of ovarian function. Early observations by Gospodarowicz et al. (1989) revealed an ovarian source of basic FGF (bFGF or FGF2). More recently, research shows FGF family members have important roles in reproductive function of mammals including human, caprine, ovine, bovine, and murine species (Chaves et al., 2012). To date FGF1, FGF2, FGF7, FGF8, FGF9, FGF10, FGF17, and FGF18 have been described in the ovary of humans, rodents or domestic animals (Drummond et al., 2007; Machado et al., 2009; Portela et al., 2010; Grado-Ahuir et al., 2011; Chaves et al., 2012). Functions of FGF members in the ovary include regulation of steroidogenesis (Savion et al., 1981; Vernon and Spicer, 1994; Schreiber and Spicer, 2012; Evan et al., 2014), apoptosis and cell survival (Tilly et al., 1992; Portela et al., 2010; Jiang and Price, 2012), control of cell proliferation (Gospodarowicz et al., 1977; Buratini et al., 2005; Schreiber and Spicer, 2012) and luteal development (Woad et al., 2012). These processes are critical during follicular development, suggesting that FGFs play an important role in folliculogenesis.

In a microarray study, FGF9 mRNA abundance was down regulated in granulosa cells of cystic follicles compared with dominant follicles of cows (Grado-Ahuir et al., 2011), suggesting FGF9 may play a role in ovarian folliculogenesis. Indeed, FGF9 mRNA abundance is greater in

small versus large follicle granulosa and theca cells in cattle (Schreiber et a., 2012). Previous research revealed FGF9 having a wide array of functions including male sex determination (Cotinot et al., 2002), lung development (Colvin et al., 2001), and glial cell growth (Naruo et al., 1993). Additionally, FGF9 is widely expressed in embryos and fetuses (Colvin et al., 1999; Manon-Pepin et al., 2003). With regard to follicular development, studies in mice indicate that FGF9 protein is localized to theca, stroma, and basement membranes surrounding granulosa and theca cells (Drummond et al., 2007). FGF9 interacts with FGFR1 to FGFR4 (Ornitz et al., 1996). In cattle, FGFR1 through FGFR3 have been localized to granulosa and theca cells (Parrott and Skinner, 1998; Berisha et al., 2004), whereas theca cells have been found to express only FGFR4 (Buratini et al., 2005). Exogenous FGF9 stimulates *in vitro* cell proliferation while inhibiting steroid production of bovine granulosa and theca cells (Schreiber et al., 2012; Schreiber and Spicer 2012). Thus, FGF9 may directly impact ovarian follicular function by acting as an anti-differentiation factor in cattle.

3. MicroRNAs in Follicular Development

MicroRNAs (miRNAs) play a diverse role in gene regulation in both humans and animals (Erdmann et al., 2014; Fatima et al., 2014). Emerging evidence indicates non-coding miRNAs regulate cellular processes and major signal pathways by complementary binding to mRNA to mediate degradation or silencing of target genes (Ambros and Chen, 2007; Bushati and Cohen, 2007; Bartel, 2009). The sequential and profound changes in granulosa and theca cell function within follicles are finely synchronized at the molecular level (Mihm and Bleach, 2003; Rivera and Fortune, 2003b; Knight and Glister 2006), nonetheless little is known about the mechanisms regulating ovarian miRNA expression and their interaction with mRNA.

3.1 MicroRNA Biogenesis

Studies have implicated miRNAs influence on gene expression in a variety of tissues and biological processes in both humans and animals (Ambros, 2004; Fatima et al., 2014; Sun et al., 2014). A primary miRNA (pri-miRNA) is transcribed from intergenic regions of genes (Berezikov et al., 2007; Saini et al., 2007) by RNA polymerase II or III, resulting in the formation of an imperfect stem-loop hairpin structure up to several hundred nucleotides in length (Kim, 2005; Krol et al., 2010). The pri-miRNA is cleaved by a nuclear protein complex consisting of Drosha enzyme and numerous other proteins, which yields a precursor miRNA (pre-miRNA) transcript that is 60-110 base pairs in length (Yi et al., 2003; Lund et al., 2004). Exportin-5 mediates the transport of pre-miRNA out of the nucleus into the cytoplasm where Dicer, an enzyme that cleaves the hairpin loop structure, gives rise to a double-stranded miRNA complex (Khvorova et al., 2003). Only a single strand functions as a mature miRNA as it associates with the miRNA-induced silencing complex (RISC) and targets the 3' untranslated region of mRNA (Khvorova et al., 2003). The non-coding miRNAs modulate protein levels post-transcriptionally by degrading or repressing translation of targeted transcript. It is predicted that 1 to 5% of genes encode for miRNA, and they regulate the activity of ~50% of all known protein-coding genes in mammals (Berezikov et al., 2005; Lewis et al., 2003). Some miRNAs are thought to act in an epigenetic manner via changing de novo methyltransferase gene expression and thus its activity (Fabbri et al., 2007). More recently, several miRNAs have been identified as biomarkers of various diseases in humans including melanoma (Friedman et al., 2012), breast cancer (Sochor et al., 2014), polycystic ovary syndrome (PCOS) (Sang et al., 2013), and ovarian caner (Langhe et al., 2015).

3.2 MicroRNAs in Ovarian Function

Intraovarian factors are responsible for regulating events ranging from cell proliferation, differentiation, apoptosis, and hormone secretion. Studies indicate that miRNAs, through post-transcriptional regulation of their specific target genes, play an essential role in the outcome of all

cellular and tissue activities under normal and pathologic ovarian function (Toloubeydokhti et al., 2008). Initial studies elucidated the role of miRNA in reproductive tissues through globally knocking down miRNA via manipulations of the Dicer enzyme (Luense et al., 2009). Knocking out Dicer in mice results in post-implantation embryonic lethality (Bernstein et al., 2003). Additionally, a conditional knockdown (cKO) of Dicer in mice revealed multiple defects in ovarian functions, including abnormal estrous cycles, short estrus and longer metestrus, and abnormal response to gonadotropins (Nagaraja et al., 2008). In a study using granulosa cells, Dicer cKO mice increased primordial follicle pool capability, accelerated early follicle recruitment, and increase follicle degeneration (Lei et al., 2010). Further, *in vivo* and in cultured cells, KO of Dicer1 was associated with altered expression of fundamental genes involved in granulosa cell proliferation and steroidogenesis as well as genes involved in oocyte development (Lei et al., 2010). Genome-wide miRNA expression has been examined in whole ovaries from cattle (Hossain et al., 2009; Tripurani et al., 2010) and pigs (Li et al., 2011). These studies identified novel and ovarian-specific miRNA species. A large number of ovarian miRNAs are encoded from chromosomes X and 2 in several species (Ahn et al., 2010; Huang et al., 2011; Li et al., 2011). Furthermore, differential miRNA profiles in follicular tissue are associated with normal development and atresia of dominant follicles in cattle (Sontakke et al., 2014).

Expression of miRNA in the ovary may be under hormonal regulation. Following LH/hCG treatment of mouse granulosa cells, miRNA-132 and miRNA-212, which share the same seed sequence, were differentially up regulated, implying their role in ovarian response to LH (Fiedler et al., 2008). A study observing the effect of 12 h FSH exposure in rat granulosa cells indicated 17 miRNAs were up regulated and 14 miRNAs down regulated (Yao et al., 2010). An additional bi-phasic regulation of miRNAs by FSH was revealed in this same study as miRNA-29a and miRNA-30d expression decreased 12 h post-FSH treatment but at 48 h post-FSH stimulation miRNA-29a and miRNA-30d abundance was increased 2- and 3-fold, respectively

(Yao et al., 2010). These findings suggest miRNAs are involved in the fine-tuning of gonadotropin actions in ovarian follicular cells. In line with this research, 51 miRNAs suppress estradiol secretion in cultured human granulosa cells (Sirotkin et al., 2009). Likewise, 19 miRNAs reduced the release of all three intraovarian steroid hormones *in vitro* (Sirotkin et al., 2009). In addition, Sirotkin et al. (2009) observed an overexpression in miRNA-24, miRNA-25, miRNA-122, miRNA-182, miRNA-18, miRNA-125 and miRNA-32 resulted in an increase of progesterone secretion. Together these studies implicate miRNAs as regulators of steroidogenesis in ovarian follicles.

To identify molecular mechanisms that drive follicular development, a gene expression comparison of known growth factors may aid in elucidating whether miRNA are involved in follicular growth. The use of microarray technology provides a snapshot of the global view of cellular activity while simultaneously recording the expression levels of thousands of genes. A recent microarray study (Spicer et al., unpublished) investigated differently expressed genes between control and FGF9-treated bovine ovarian theca cells. It was found that a total of 736 genes were differentially expressed, with 359 genes up regulated and 377 genes down regulated in FGF9-stimulated theca cells compared to control theca cells; miRNA-221 was up regulated by 2.42-fold in FGF9-stimulated bovine theca cells. Further study will be needed to identifying the function of this miRNA in ovarian cells and determine its physiological role in the FGF9 effect observed by Schreiber and associates (2012).

Both miRNA-221 and its paralog, miRNA-222, are clustered in an intergenic region on the X chromosome of humans, mice and cattle (http://uswest.ensembl.org/Bos_taurus/Location/View?db=core;r=X:103538835-103538944;redirect=no). The mature miRNA-221 is 23 nucleotides in length and is highly conserved with greater than 93% homology among chimpanzee, cattle, dogs and humans, suggesting a necessary and sufficient role in biological functions (Tabasi and Erson, 2009). This

miRNA is expressed is a wide variety of tissues, including cord blood cells (Felli et al., 2005), brain tissue (Ciafre et al., 2005), kidney and bladder tissue (Gottardo et al., 2007), lung cells (Garofalo et al., 2008), and pancreatic cells (Lee et al., 2007). In addition, miRNA-221 temporal expression regulates male germ cell differentiation (Smorag et al., 2012), and over expression of miRNA-221 is associated with human ovarian cancer (Dahiya et al., 2008). There is a growing body of evidence that miRNA-221 may be a potent modulator of ovarian biological functions. Salilew-Wondim et al. (2014) reported that miRNA-221 is increased in granulosa and theca cells of subordinate follicles at d 3 of the estrous cycle, whereas at d 7 of the estrous cycle miRNA-221 was lower in granulosa and theca cells of subordinate follicles compared to dominant follicles (Salilew-Wondim et al., 2014). Changing expression of miRNA-221 in both dominant and subordinate follicles throughout the estrous cycle implies it is involved in ovarian follicular development.

To date, no studies have been conducted in cattle to investigate the possible hormonal regulation of miRNA-221 or evaluate the effects of miRNA-221 and its association with FGF9-induced repression of steroids in theca cells.

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CHAPTER III

HORMONAL REGULATION OF MICRORNA-221 AND ITS EFFECTS ON BOVINE OVARIAN THECA CELL FUNCTION

1. Abstract

Development of ovarian follicles in cattle is controlled by systemic and locally produced hormones acting at the molecular level controlling numerous gene transcripts which spatial-temporal expression leads to one follicle ovulating and other follicles undergoing regression. MicroRNA-221 (miRNA-221) is increased in granulosa and theca cells of subordinate follicles compared with dominant follicles on day 3 of the estrous cycle in cattle. The objectives of this study were to investigate the hormonal regulation of miRNA-221 expression in theca cells and its possible role in regulating follicular function. Bovine ovaries were collected from a local abattoir and theca cells were obtained from large (8 to 22 mm) follicles, cultured for 2 to 7 days in 10% fetal calf serum (FCS), and treated with various hormones in serum-free medium for an additional 24 or 48 h in five experiments. Medium was collected for analysis of progesterone and androstenedione concentrations via radioimmunoassay, or cellular RNA was collected for gene expression analysis of miRNA-221 via real-time PCR. In Exp. 1, FGF9 increased ($P = 0.08$) abundance of miRNA-221 2.0-fold after 12 h and 2.4-fold after 24 h compared with controls. In Exp. 2, forskolin and dibutyryl cyclic adenosine monophosphate (dbcAMP) had no effect ($P > 0.10$) on miRNA-221 expression in bovine theca cells, but FGF9 treatments increased ($P < 0.05$) miRNA-221 abundance 1.94-fold. In Exp. 3, IGF1 had no effect ($P > 0.10$) on basal or FGF9-

induced miRNA-221 expression (3.3-fold increase) in bovine theca cells, however, 10% FCS increased ($P < 0.05$) miRNA-221 abundance by 3-fold greater than control cultures; the combined treatment of FGF9 and 10% FCS did not differ ($P > 0.10$) from either treatment alone. In Exp. 4, estradiol, androstenedione, and phytoestrogens had no effect ($P > 0.10$) on miRNA-221 abundance. In Exp. 5, neither miRNA-221 mimic nor inhibitor affected cell numbers; nonetheless FGF9 stimulated an increase in cell proliferation ($P < 0.01$). Similarly, neither miRNA-221 mimic nor inhibitor affected steroidogenesis whereas FGF9 inhibited ($P < 0.01$) IGF1-induced production of androstenedione and progesterone. In summary, exposure of bovine theca cells to FGF9 *in vitro* increased expression of miRNA-221, however miRNA-221 was not regulated by steroids or cAMP. The role of miRNA-221 in follicular function will require further study.
Keywords: microRNA 221 (*MIR221*); theca cell; cattle; steroidogenesis.

2. Introduction

In cattle, follicular growth beyond the early antral stage is characterized by two or three waves of follicular growth in each estrous cycle in response to endocrine and intraovarian factors (De Rensis and Peters, 1999; Evans 2003). An initial phase of growth occurs in recruited follicles within a wave, which is followed by the selection of a single follicle to continue growing as a dominant follicle and become ovulatory. The remaining subordinate follicles will cease to develop and undergo atresia. In some cases, follicular development goes awry and follicles progress into ovarian cysts (Silvia et al., 2002; Vanholder et al., 2006). Each of these developmental steps involves sequential and profound changes in the expression of thousands of genes (Lingenfelter et al., 2008; Li et al., 2009; Grado-Ahuir et al., 2011). Any variations in the activity of these genes may be vital in determining the survival of dominant follicles and the demise of subordinate follicles. One gene discovered by Grado-Ahuir et al. (2011) to be involved with cyst formation was fibroblast growth factor 9 (FGF9). This FGF is one of 23 members and to date, FGF1, FGF2, FGF7, FGF8, FGF9, FGF10, FGF17, and FGF18, have been described in

the human, rodent and other domestic animal ovaries (Drummond et al., 2007; Machado et al., 2009; Portela et al., 2010; Grado-Ahuir et al., 2011; Chaves et al., 2012). 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), control of cell proliferation (Buratini et al., 2005; Schreiber and Spicer, 2012) and luteal development (Woad et al., 2012). Further research suggests that FGF9 stimulates granulosa and theca cell proliferation while inhibiting steroidogenesis (Schreiber and Spicer, 2012; Schreiber et al., 2012).

Recent research has demonstrated microRNAs (miRNAs) have a significant impact on gene expression in numerous tissues and biological functions in humans and animals. These small non-coding RNA molecules that 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 miRNAs are involved in key physiological processes as they modulate cell proliferation and differentiation. It is anticipated that miRNAs are important regulators in the biosynthesis of intraovarian hormones and growth factors. Several findings already indicate effects of specific miRNAs on granulosa cell function (Donadeu et al., 2012), including steroid production and proliferation (Yao et al., 2010; Xu et al., 2011; Yan et al., 2012; Yin et al., 2012; Dai et al., 2013). In addition, miRNA-21, miRNA-26a and miRNA-143 are elevated in ovaries of anestrous sheep (Di et al., 2014) whereas miRNA-21, miRNA-132, miRNA-212 and miRNA-224 are increased during follicular growth in mares (Schauer et al., 2013). Furthermore, miRNA-92a and miRNA-92b are lower in theca cells of women with PCOS (Lin et al., 2015).

In a microarray study, our lab recently discovered that FGF9 induces microRNA-221 (miRNA-221) in bovine theca cells (Spicer et al., unpublished). This miRNA is expressed in a wide variety of tissues, including cord blood cells (Felli et al., 2005), brain tissue (Ciafre et al.,

2005), kidney and bladder tissue (Gottardo et al., 2007), lung cells (Garofalo et al., 2008), and pancreatic cells (Lee et al., 2007). Emerging evidence indicates that miRNA-221 may be a modulator of ovarian functions. Over expression of miRNA-221 is associated with human ovarian cancer (Dahiya et al., 2008). Furthermore, miRNA-221 abundance in theca and granulosa cells in subordinate follicles of cattle was greater than in dominant follicles at d 3 of an estrous cycle but on d 7 miRNA-221 abundance in both theca and granulosa cells was less in subordinate than dominant follicles (Salilew-Wondim et al., 2014). Difference in expression of miRNA-221 between dominant and subordinate follicles on d 3 and d 7 of the estrous cycle implies miRNA-221 may be involved in ovarian follicular development in cattle. In addition, the homolog to miRNA-221, miRNA-222 is increased in serum of women with PCOS (Long et al., 2014).

To date, no studies have been done in cattle to investigate the hormonal control of miRNA-221 in theca cells or to determine its effect on ovarian theca cells. Thus, the aims of the present work were to investigate the hormonal regulation of miRNA-221 expression in theca cells and its possible role in regulating follicular function.

3. Materials and Methods

Reagents and Hormones

Reagents and hormones used for cell preparation and culture were: gentamicin, glutamine, Ham's F-12, Dulbecco modified Eagle medium (DMEM), sodium bicarbonate, trypan blue, protease, collagenase, hyaluronidase, deoxyribonuclease (DNase), and penicillin-streptomycin from Sigma-Aldrich Chemical Company (St. Louis, MO); LH (ovine NIDDK-oLH 26, activity 1.0 x NIH-LH-S1 U/mg) from National Hormone and Pituitary Program (Torrance, CA); recombinant human insulin-like growth factor (IGF)-1 and recombinant human fibroblast growth factor (FGF)-9 from R&D Systems (Minneapolis, MN); estradiol, forskolin, dibutyryl

cyclic adenosine monophosphate (dbcAMP), α-zearalenol, β-zearleanol, and androstenedione from Sigma-Aldrich Chemical Company (St. Louis, MO); genistine from Biomol Research Labs, Inc. (Plymouth Meeting, PA) and fetal calf serum (FCS) from Equitech-Bio, Inc. (Kerrville, TX).

Reagents used in sample preparation, cell transfection and RNA extraction were: TriZol® Solution, Lipofectamine, Opti-MEM ® medium and DEPC-treated water from Life Technologies, Inc. (Gaithersburg, MD), isopropyl alcohol and ethanol from Pharmco Products Inc. (Brookfield, CT), and chloroform from Sigma-Aldrich Chemical Co.

The reagents used for radioimmunoassays (RIA) were: Androstenedione Double Antibody-^{125}I RIA Kit (ICN Biomedicals, Costa Mesa, CA), [^{125}I]iodo-progesterone (ICN Biomedicals, Irvine, CA), anti-progesterone rabbit antiserum (X-16) provided by Dr. P. Natashima Rao (Southwestern Foundation for Research Education, San Antonia, TX), goat anti-rabbit antibody, and normal rabbit serum (NRS) (Linco Research, Inc., St. Charles, MO).

Cell Culture

Ovaries from non-pregnant beef heifers were collected from a local abattoir and transported to the lab in 0.9% saline solution with 1% streptomycin/penicillin at 4°C. Theca cells were collected from large follicles (8 to 22 mm) that appeared healthy with good vascularity and moderately transparent follicular fluid. Large follicles were bisected longitudinally with a scalpel after aspiration of follicular fluid and granulosa cells were scraped free from theca interna via blunt dissection. The theca interna tissue was detached via microdissection and torn using rat-tooth forceps, and then enzymatically digested for 1 h at 37°C on a rocking platform. Non-digested thecal tissue was removed via filtration through a sterile syringe filter holder with metal screen of 149 μm mesh (Gelman Sciences, Ann Arbor, MI). Theca cells were centrifuged at 500 rpm for 7 min, the pellet was washed with medium (1:1 DMEM and Ham's F-12 containing 0.12 mM and 2.0 mM of gentamicin and glutamine, respectively, along with 38.5 mM sodium

bicarbonate) and suspended in serum-free medium containing DNase and collagenase at 1.25 mg/mL and 0.5 mg/mL, respectively, to prevent clumping.

Viability of theca cells from large follicles was determined by trypan blue exclusion method on a 0.1 mm deep hemacytometer (American Optical Corporation, Buffalo, NY), and averaged 90.5 ± 1.5% for the 5 experiments.

Approximately 2.0 x 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 a environment of 38.5°C with 5% CO_2 and 95% air in 10% FCS for the first 48 h with medium change at 24 h. Cells were then washed twice with 0.5 mL of serum-free medium and the different hormonal treatments were applied in serum-free medium for 24 h or 48 h depending on the experiment. At the conclusion of the treatment period, medium was either aspirated or collected from each well depending on experiment.

For transfections, miRNA-221 mimic or inhibitor and transfection complex was combined and applied to theca cells for 4 h before treatment. Briefly, 1300 µL Opti-MEM medium and 39 µL Lipofectamine were combined, then 52 µL miRNA-221 mimic (5 pmol; AGCUACAUUGUCUGCUGGGUUU) or inhibitor (5 pmol; UUUCCCUGCUGUCUUTGTUGCT) was added. Control transfection complex contained 1300 µL Opti-MEM medium and 39 µL Lipofectamine. Theca cells were washed twice with 0.5 mL of serum-free medium, then 200 µL of serum-free medium and 50 µL of miRNA-transfection complex medium was applied to each well. Theca cells were incubated 4 h after which treatments were applied for 48 h.

RNA Extraction

For gene expression experiments, medium was aspirated and cells from two replicate wells were lysed in 0.5 mL of TriZol reagent solution. Briefly, 0.25 mL TriZol reagent was

added to all wells and cells lysed by repeated pipetting and then combined with their respective replicates. Combined wells were then transferred to 1.5 mL eppendorf tubes. Each treatment containing 4 wells that generated 2 replicate samples of RNA. Cell lysates were incubated in TriZol reagent for approximately 5 min at room temperature, and then 0.10 mL chloroform was added to each sample followed by a 15 s vortex. After being pipetted into Phase Lock tubes (5 Prime, Gaithersburg, MD) and incubated for 2 min at room temperature, samples were centrifuged at 3500 x g for 30 min at 4°C using eppendorf centrifuge 5417C (Brinkmann Instruments, Westbury, NY). The upper aqueous phase of each sample was transferred to a new eppendorf tube and RNA precipitated with 0.250 mL of isopropanol (99%) coupled with gently mixing by inversion. Samples were incubated at room temperature for 10 min and then centrifuged at 3500 x g for 10 min at 4°C. The RNA pellet was washed after discarding the supernatant with 0.5 mL of 70% ethanol and allowed to dry at room temperature. When the RNA pellets were completely dry, RNA was re-suspended in 16.5 µL of DEPC-treated water and stored at -80°C.

RNA was quantitated by spectrophotometry at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). To determine the purity and concentration of RNA in ng/µL, aliquots of 1.5 µL of each sample were used. Purity was based on a ratio of 260/280 nm wherein values between 1.8 and 2.2 were considered acceptable. RNA samples were then diluted to 10 ng/µL in DEPC-treated water.

Real-time PCR Analysis

Quantification of miRNA-221 expression was determined using two-step RT-PCR using TaqMan Small RNA Assays (Applied Biosystems, Foster City, CA). Complementary DNA (cDNA) was synthesized from total RNA samples using stem-loop primers, and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Each 15 µL reaction volume

contained 10 ng total RNA, 5X RT primers, 50 U MultiScribe reverse transcriptase, 100 mM dNTPs, 20 U RNase inhibitor, and 10X reverse transcription buffer (Applied Biosystems). 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.

Real-time quantitative PCR was performed using target-specific TaqMan Assays and the TaqMan Universal PCR Master Mix II, No UNG (Applied Biosystems, Foster City, CA). Each 20 μL reaction volume contained 1.33 μL of the appropriate cDNA, 20X specific TaqMan Assay, and 2X 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 a 96-well plate (Bio-Rad, Hercules, CA). U6 was used as endogenous control for theca cell samples to correct for discrepancies in RNA quantification and loading as previously described (Young et al., 2012).

Radioimmunoassays (RIA)

Progesterone RIAs were conducted using rabbit antiserum (X-16), which served as the first antibody (diluted 1:3,000 with assay buffer (PBS, EDTA, NaN_3 and gelatin)), raised against BSA-11glutamate derivative as previously described by Baraño and Hammond (1985). The second antibody used was goat anti-rabbit antibody (diluted 1:10 with assay buffer). [^{125}I]Iodo-progesterone tracer was diluted 1:200 in assay buffer. The standard curve for progesterone was prepared from 80.0 ng/mL of stock and serially diluted with assay buffer to concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.625, 0.31, and 0.16 ng/mL. In duplicate, 20 μL to 100 μL of medium samples were combined with the appropriate volume of assay buffer to make a total volume of 100 μL. One hundred microliters of tracer and first antibody were added to all samples,

then samples were vortexed and incubated at 37° C for 1 h. Subsequently, 200 μL of second antibody was added to all samples, vortexed and incubated overnight at 4° C. The following day, 50 μL of normal rabbit serum (NRS) (diluted 1:5 with assay buffer from a 15% NRS stock) were added to all samples and then incubated for 1 h at 4° C. Samples were centrifuged at 4° C in a Sorvall Model RC-3 (Thermo Fisher Scientific, Inc., Miami, OK) at 1800 x g for 25 min. Supernatant was aspirated and precipitates were counted for 2 min using a Cobra AII Auto-Gamma counter (Packard Instrument Co., Downers Grove, IL). Intra-assay coefficient of variation averaged 10.6 ± 0.3%.

Androstenedione assays were conducted using an Androstenedione Double Antibody-^{125}I RIA Kit (ICN Biomedicals, Costa Mesa, CA) according to the manufacture's protocol with modification as previously described (Stewart et al., 1995). Assay tubes were centrifuged for 20 min at 1000 x g as described for the progesterone RIA. Precipitates were counted as described for the progesterone RIA. Intra-assay coefficient of variation averaged 6.8 ± 0.4%.

Cell Counting

To determine cell numbers, culture medium was aspirated and all wells were washed twice with 0.5 mL saline (0.9%). Then 0.5 mL of trypsin (0.25% solution) was added to each well and allowed to incubate for 20 min at 37° C. Wells were then scraped, aspirated, washed an additional time, and diluted 1:10 in saline. Cell numbers were determined using a Z2 Coulter ® Particle Count and Size Analyzer (Beckman Coulter, Hialeah, FL) as previously described (Langhout et al., 1991; Lagaly et al., 2008; Spicer et al., 2011).

Experimental Design

Exp. 1 was designed to determine the time-course of the FGF9 effect on miRNA-221 abundance in large-follicle theca cells. Cells were cultured for 144 h in 10% FCS medium then washed twice with serum-free medium and serum starved for 24 h before two treatments were

applied as follows: control and FGF9 (30 ng/mL). After 0 h, 4 h, 12 h and 24 h of treatment, medium was aspirated and cells were lysed in 0.5 mL of TRI reagents for RNA extraction as described earlier.

Exp. 2 was designed to test the effect of FGF9, dbcAMP and forskolin-mediated cAMP on miRNA-221 expression in large-follicles theca cells. Cells were cultured for 72 h in 10% FCS medium, washed twice with serum-free medium, and six treatments were applied in a 2 x 3 factorial arrangement as follows: control, dbcAMP (0.1 mg/mL), forskolin (4.1 μg/mL), 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 cells were lysed in 0.5 mL TRI reagent for RNA extraction as described earlier. Doses of dbcAMP, and forskolin were selected based on previous studies (Schreiber and Spicer 2012; Schreiber et al., 2012).

Exp. 3 was designed to test the effect of IGF1 and 10% FCS on FGF9-induced miRNA-221 expression in large-follicle theca cells. Cells were cultured for 72 h in 10% FCS medium, washed twice with serum-free medium, and six treatments in a 2 x 3 factorial arrangement were applied for 24 h as follows: control, FGF9 (30 ng/mL), IGF1 (30 ng/mL), IGF1 plus FGF9 (30 ng/mL), 10% FCS, and 10% FCS plus FGF9 (30 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 earlier.

Exp. 4 was designed to test the effect of steroids and phytoestrogens on miRNA-221 abundance in large-follicle theca cells. Cells were cultured for 168 h in 10% FCS medium then washed twice with serum-free medium, and six treatments were applied as follows: control, genistein (300 ng/mL), estradiol (300 ng/mL), α-zearalenol (300 ng/mL), β-zearleanol (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 earlier. Doses of the

various steroids were selected based on previous studies (Ranzenigo et al., 2008; Aad et al., 2012).

Exp. 5 was designed to determine the effect of miRNA-221 mimics and inhibitors on FGF9 and LH plus IGF1-induced cell numbers and steroidogenesis of large-follicle theca cells. Cells were cultured as previously described for Exp. 2. Transfection complexes (control, mimic, inhibitor) were applied and incubated for 4 h, and then treatments in a 2 x 2 x 3 factorial arrangement were applied for 48 h as follows: control, FGF9 (0 or 27.3 ng/mL), IGF1 (0 or 27.3 ng/mL), FGF9 plus IGF1. After 48 h of treatment, medium was collected for progesterone and androstenedione determinations and cells were counted.

Statistical Analysis

Data are presented as means ± SEM of measurements from replicated experiments. Each experiment of theca cells was performed with three or four different pools of theca cells collected from seven to eight follicles for each pool. Each experiment contained two or three replicates per treatment. Treatment effects and interactions on dependent variables (e.g., miRNA-221 abundance, steroid production and cell numbers) were assessed using the general liner models (GLM) and ANOVA procedure of SAS (version 9.2, SAS Institute Inc., Cary, NY). For Exp. 1, main effects in ANOVA were duration of treatment, FGF9 (+ or -), pool (biological replicate), and their interactions. For Exp. 2 and Exp. 3, main effects in ANOVA were treatment, pool, and their interactions. For Exp. 4, main effects in ANOVA were treatment, pool, and their interaction. For Exp. 5, main effects in a 2 x 2 x 3 factorial ANOVA were transfection complex (control, mimic, or inhibitor), FGF9 (+ or -), IGF1 (+ or -), pool, and their interactions. Mean differences were determined by Fisher's protected least significant differences test (Ott, 1977), if significant treatment effects in ANOVA were detected. Steroid production was expressed as ng or pg/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 $P < 0.10$.

4. Results

Exp. 1: Time-course of FGF9 effect on miRNA-221 abundance in large-follicle theca cells.

Main effect of time ($P < 0.05$) and FGF9 treatment ($P < 0.10$) but not their interaction ($P > 0.10$) influenced miRNA-221 gene expression. In FGF9-treated theca cells, miRNA-221 abundance increased 2.0-fold after 12 h and 2.4-fold after 24 h, but after 4 h of treatment, miRNA-221 abundance did not differ ($P > 0.10$) between FGF9 and control. In control-theca cells, miRNA-221 did not significantly change between 0 and 24 h (Fig. 1).

Exp. 2: Effect of dbcAMP and forskolin on miRNA-221 abundance in large-follicle theca cells.

Main effect of FGF9 treatment influenced ($P < 0.05$) theca cell miRNA-221 abundance but dbcAMP and forskolin treatments and their interaction with FGF9 did not significantly affect *MIR221* mRNA abundance (Fig. 2). Specifically, FGF9 increased ($P < 0.05$) *MIR221* mRNA abundance by 1.94-fold.

Exp. 3: Effect of IGF1 and 10% FCS on FGF9-induced miRNA-221 abundance in large-follicle theca cells.

A significant interaction between FGF9, IGF1 and 10% FCS on miRNA-221 abundance, was observed. Specifically, IGF1 had no effect ($P > 0.10$) on control or FGF9-induced miRNA-221 expression in bovine theca cells, however, 10% FCS increased ($P < 0.05$) miRNA-221 abundance over 3-fold in control cultures; the combined treatment of FGF9 and 10% FCS did not differ ($P > 0.10$) from either treatment alone (Fig. 3).

Exp. 4: Effect of steroids and phytoestrogens on miRNA-221 abundance in large-follicle theca cells.

Treatment of various steroids and phytoestrogens did not affect ($P > 0.10$) miRNA-221 abundance. miRNA-221 abundance averaged 2.79, 3.73, 3.64, 2.66, 3.46, and 4.41 $\pm$ 0.62 for control, genistine, estradiol, α-zearalenol, β-zearalenol, and androstenedione, respectively.

Experiment 5: Effect of miRNA-221 mimics and inhibitors on FGF9 and IGF1 induced cell numbers and steroidogenesis of large-follicle theca cells.

Neither treatment (miRNA-221 mimic or inhibitor transfection) nor LH plus IGF1 affected cell numbers significantly (Fig. 4). However, FGF9 increased cell numbers by 37% as compared with controls ($P < 0.01$). No significant interactions between main effects on cell numbers were observed.

Neither treatment (miRNA-221 mimic or inhibitor transfection) nor its interaction with FGF9 or LH plus IGF1 affected ($P > 0.10$) androstenedione production. However, FGF9, LH plus IGF1 and their interaction significantly affected androstenedione production. Specifically, LH plus IGF1 increased androstenedione production by 5.8-fold ($P < 0.01$), whereas FGF9 decreased LH plus IGF1 androstenedione production ($P < 0.01$) by 58% and had no effect ($P > 0.10$) on basal androstenedione production.

Neither treatments (miRNA-221 mimic or inhibitor transfection) nor the interaction with FGF9 or LH plus IGF1 affected ($P > 0.10$) progesterone production (Fig. 6). However, FGF9, LH plus IGF1 and their interaction affected ($P < 0.05$) progesterone production. Specifically, LH plus IGF1 increased progesterone production by 3-fold ($P < 0.05$) whereas, FGF9 decreased ($P < 0.05$) LH plus IGF1 induced progesterone production by 56% and basal progesterone production by 46%.

5. Discussion

The results of the present study indicated that in cultured bovine theca cells: (1) FGF9 increases miRNA-221 abundance from 12 h to 24 h; (2) dbcAMP and forskolin had no effect on miRNA-221 abundance; (3) 10% FCS like FGF9 increased miRNA-221 abundance; (4) IGF1 and estrogenic compounds had no affect on miRNA-221 abundance; (5) miRNA-221 mimics and inhibitors did not affect cell numbers and steroid production. These findings suggest neither steroids nor cAMP affect miRNA-221 mRNA abundance in theca cells. Further, FGF9 may modulate ovarian function in cattle by influencing miRNA-221 and other factors that mediate cell cycle progression in theca and/or granulosa cells.

The present findings are important as they provide novel information on the dynamic regulation of miRNA-221 levels in bovine theca cells. MicroRNAs add another dimension to gene regulation within the animal genome (Hossain et al., 2009). Alterations in the expression of a single miRNA could have a significant impact on cellular activity. Studies have proposed several miRNAs to be involved in regulating ovarian follicle functions; nonetheless the majority of this evidence is derived from studies using granulosa cell cultures (Donadue et al., 2012). Examination of miRNA expression in follicles of sheep and cattle revealed miRNAs to be primarily expressed in theca cells, while only a small proportion are expressed in granulosa cells (McBride et al., 2012). This differential expression may be due to the diverse nature of theca cells, which includes steroidogenic cells and vascular endothelial cells, the later of which have been described as targets of numerous miRNAs (Suárez and Sessa, 2009). Furthermore, miRNAs produced in theca cells may act in a paracrine manner to regulate gene expression in granulosa cells (Camussi et al., 2010). Whether miRNA-221 affects granulosa cell function will require further studies.

Expression analysis of miRNAs in different ovarian cells and tissue types enables the discovery of their functional regulation as well as their mechanisms of action (Hossain et al., 2009). Throughout different stages of ovarian development from fetal, newborn and adult animals there is altered expression of small RNAs and various ovarian related genes (Herrera et al., 2005; Baillet et al., 2008). Hossain et al. (2009) discovered ovarian miRNA-29a, miRNA-140, miRNA-199, miRNA-378, miRNA-F0132, and miRNA-F2422 to be differentially expressed between fetal and adult cows, suggesting miRNAs are involved in regulating ovarian follicle development. Determining the temporal expression and hormonal regulation of miRNA-221 in bovine theca cells is a crucial step to understanding its role in ovarian follicle function. In a recent study using in cattle, miRNA-221 expression was up regulated in subordinate versus dominant follicles on d 3 of an estrous cycle (Salilew-Wondim et al., 2014). Also, FGF9 has been shown to stimulate cell proliferation while decreasing steroidogenesis in both bovine granulosa and theca cells (Schreiber and Spicer, 2012; Schreiber et al., 2012). Our results confirm these results and show that miRNA-221 expression increases in response to stimulation of FGF9, and that miRNA-221 may not be involved in FGF9-induced cell proliferation and/or reduction of steroidogenesis of the theca cell.

It has been implicated that FSH and LH/hCG regulate the expression of miRNAs in ovarian granulosa cells. Using mouse mural granulosa cells treated with human chorionic gonadotropin (hCG), several miRNAs were differentially expressed in response to hCG treatment (Fiedler et al., 2008). Once LH/hCG binds the LH receptor the cAMP signal transduction pathway is activated. In the study of Fiedler et al. (2008), miRNA-21, miRNA-132 and miRNA-212 were up regulated by hCG treatments. In a subsequent study, miRNA-21 levels spontaneously increased in cultured granulosa cells within 12 h after plating, a response that was not affected by the presence of serum in culture medium or by treatment with 8-bromo-cAMP (Carletti et al., 2010). Similarly, changes in miRNA-125b, miRNA-21, miRNA-145 and

miRNA-34a in cultured bovine theca cells were independent of forskolin treatment (McBride et al., 2012). In line with these findings, data from the present study indicates that the signal transduction of cAMP does not affect miRNA-221 abundance *in vitro*, as dbcAMP and forskolin did not alter miRNA-221 expression. Thus, it appears that several ovarian miRNAs are not regulated by cAMP signal transduction.

Expression of a number of miRNAs in steroidogenic cells of the testis, ovary and adrenal glands are subject to hormonal regulation (Hu et al., 2013). In addition, estrogen receptor α has been demonstrated to directly repress miRNA-221 in human breast cancer cell lines (Di Leva et al., 2010), and in human prostate cell lines miRNA-221 is repressed by androgens (Ambs et al., 2008). Contrary to these observations, it appears as a follicle grows, changes in steroid hormones do not regulate miRNA-221 expression in theca cells, because our findings show that steroids and phytoestrogens have no effect on miRNA-221 abundance in bovine theca cells. Specifically, estradiol, genistein, α-zearalenol, β-zearalenol and androstenedione did not alter miRNA-221 expression in theca cells. Together, the present and previous results suggest that steroid regulation of miRNA-221 may be tissue specific.

Many growth factors have an essential and important role in the modulation of theca functions, including IGFs, TGFβ, FGFs and EGFs (Young and McNeily, 2010). To elucidate if growth factors alter miRNA-221 expression, the present study investigated miRNA-221 abundance induced by IGF1 and 10% FCS in bovine theca cells, and results indicated that IGF1 had no affect on the expression of miRNA-221. This is the first research conducted investigating the effect of IGF1 on miRNA-221. Previously, it has been shown that IGF1 and miRNA-1 are involved in a feedback loop where miRNA-1 and its target IGF1 are mutually regulated (Elia et al., 2009). FCS is a protein supplement that has various growth factors, such as EGFs and FGFs, for supporting viability and maturation in cell culture (Leibfried-Rutledge et al., 1986). Theca cells contain EGF receptors and EGF/TGFβ affect steroidogenesis in theca cells (Erickson and

Case, 1983; Skinner and Coffey, 1988). The expression of miRNA-221 *in vitro* is increased by FCS and as a result initiates a cell proliferation of airway smooth muscle cells (Perry et al., 2014). Consistent with this finding, the present research indicated that 10% FCS increased miRNA-221 abundance in cultured theca cells and that the effect of FGF9 is similar to but not additive to the stimulus of 10% FCS. This latter finding suggests that FGF9 is present in 10% FCS. However, it is possible that other growth factors present in 10% FCS influence miRNA-221 abundance. Further work will be required to verify this suggestion.

Research on the role of miRNAs on ovarian steroid hormone secretion indicates that miRNAs can have both inhibiting and stimulating roles (Sirotkin et al., 2009). Mechanisms of miRNA effects on steroidogenesis could be direct via targeting gene(s) associated with steroid biosynthesis or release, or indirect by affecting its precursors or processing and metabolism (Sirotkin et al., 2009). The conversion of cholesterol to progesterone in theca cells involves numerous possible steps for modulation by miRNAs. Progesterone is then converted to androgens, which are then aromatized to estradiol by granulosa cells (Hillier, 2001; Macklon and Fauser, 2001; Jamnongjit and Hammes, 2006; Stouffer et al., 2007). The transformation of follicles into corpora lutea is characterized by a dramatic increase in progesterone secretion. In androgen-independent human prostate cancer cell lines, the miRNA-221 cluster is known to interfere with androgen receptor transcriptional activity without affecting the androgen receptor itself (Sun et al., 2009). With regard to miRNAs physiological role in modulating progesterone expression in the endometrium, miRNA-221 is differentially expressed in the late proliferative versus mid-secretory phases in women (Pan et al., 2007; Kuokkanen et al., 2010). In the present study, miRNA-221 mimic and inhibitor had no effect on progesterone or androstenedione production, suggesting that miRNA-221 may not be involved in regulating steroidogenesis in theca cells. In comparison, transfection with miRNA-222 (miRNA-221 paralog) in the steroidogenic human granulosa-like tumor cell line, KGN, increased estradiol secretion (Sang et

al., 2013). Transfection of miRNA-221 and miRNA-222 in human breast cancer cell lines suppressed ERα protein, but did not affect ERα mRNA in ERα positive MCF-7 and T47D cells (Zhao et al., 2008). Perhaps in the present study, miRNA-222 compensated for the knockdown of miRNA-221 thus explaining why both progesterone and androstenedione secretion was not affected by the miRNA-221 mimic or inhibitor. Alternatively, miRNA-221 may be affecting steroid production in granulosa cells rather than theca cells. Future research will be required to clarify these possibilites.

Follicle growth includes morphological and functional changes in different types of follicular cells, specifically proliferation of the surrounding granulosa and theca cells. Modifications in follicular cell morphology and functions are closely regulated by coordinate endocrine and paracrine factors, which are modulated by the expression of a number of genes (Bonnet et al., 2008). In the present study miRNA-221 mimic and inhibitor had no effect on theca cell proliferation. To date, no other studies have investigated the effect of miRNA-221 on theca cell proliferation. In non-ovarian cells, miRNA-221 and miRNA-222 promote growth in human non-small cell lung carcinoma, H460, whereas in five other lung cancer cell lines miRNA-221 suppressed growth in four of five other cell lines (Yamashita et al., 2015). The lack of effect of miRNA-221 was observed in the H838 cell line and is consistent with the present research. In another study, prostate carcinoma cell lines treated with miRNA-221 and miRNA-222 mimics have an increase growth and progression of cancer, in part by blocking cell cycle cyclin-dependent kinase (CDK) inhibitors (Galardi et al., 2007). CDK inhibitors, CDKN1B and CDKN1C, and miRNA-221 are described to have an inverse correlation. CDKN1C is an important factor regulating cell cycle; cells lacking CDKN1C show increased cell growth concomitant with decreased differentiation (Yan et al., 1997; Zhang et al., 1997). Further studies will be required to elucidate if miRNA-221 affects theca or granulosa cell proliferation by directly down-regulating CDKN1C.

In summary, the results from the present study demonstrate that neither cAMP nor ovarian steroids alter miRNA-221 abundance. Additionally, IGF1 had no effect on miRNA-221 expression. Taken together, these findings suggest that ovarian hormones, except for FGF9, do not affect miRNA-221 abundance. FGF9 increased abundance of miRNA-221 in the absence and presence of IGF1. The miRNA-221 mimic and inhibitor studies also indicate that miRNA-221 is not involved in regulating the biosynthesis of thecal steroids, or theca cell proliferation. More research is required to elucidate whether miRNA-221 elicits a response in theca cells or granulosa cells.

Figure 1: Time course of FGF9-induced miRNA-221 abundance in bovine large-follicle theca cells. Theca cells were isolated and cultured for 144 h in 10% FCS and then serum-starved for 24 h after which cells were treated with 30 ng/mL FGF9 for 0 to 24 h. ANOVA revealed a significant time effect and a tendency for a FGF9 effect ($P < 0.10$).

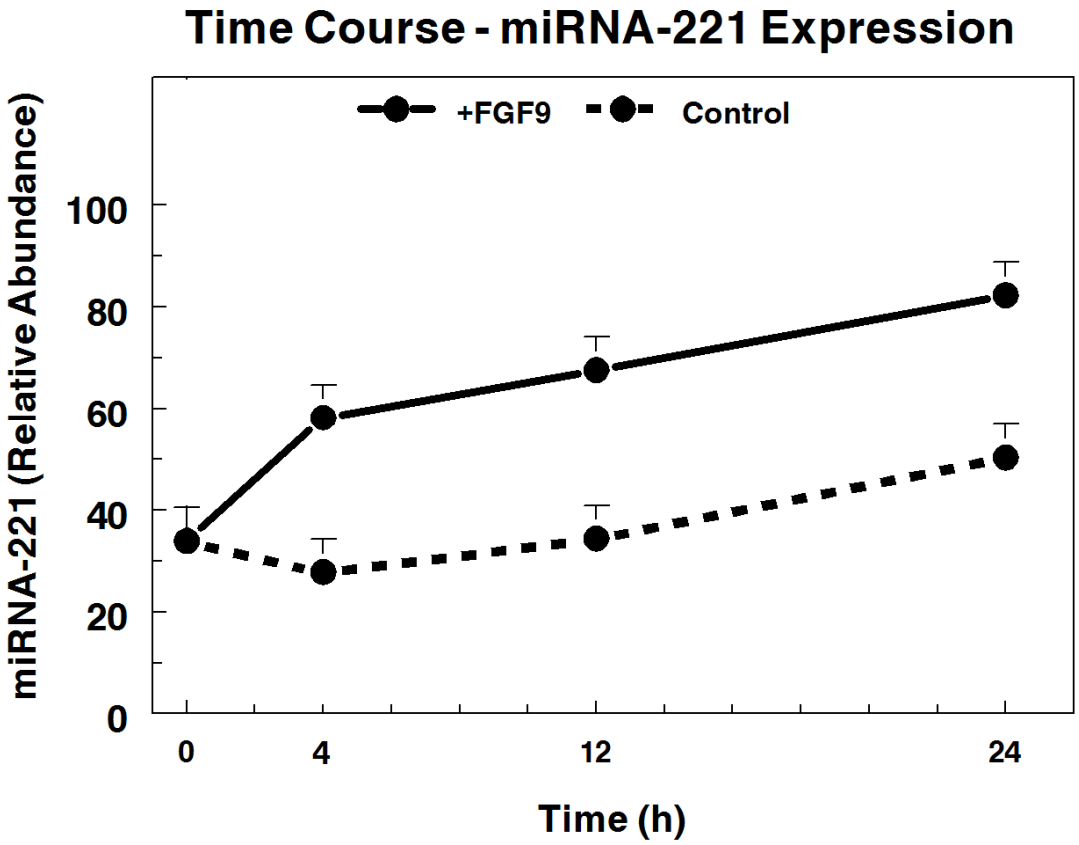
Time Course - miRNA-221 Expression
+FGF9
Control
miRNA-221 (Relative Abundance)
100
80
60
40
20
0
0
4
12
24
Time (h)

FIGURE 1

Figure 2: Lack of effect of pharmacologic cAMP agents on miRNA-221 abundance in bovine large-follicle theca cells treated with or without FGF9. Theca cells were isolated and cultured for 72 h in 10% FCS and then treated with 0.1 mg/mL dbcAMP, 4.1 μg/mL forskolin and 30 mg/mL FGF9 for 24 h. ANOVA revealed a significant FGF9 effect.

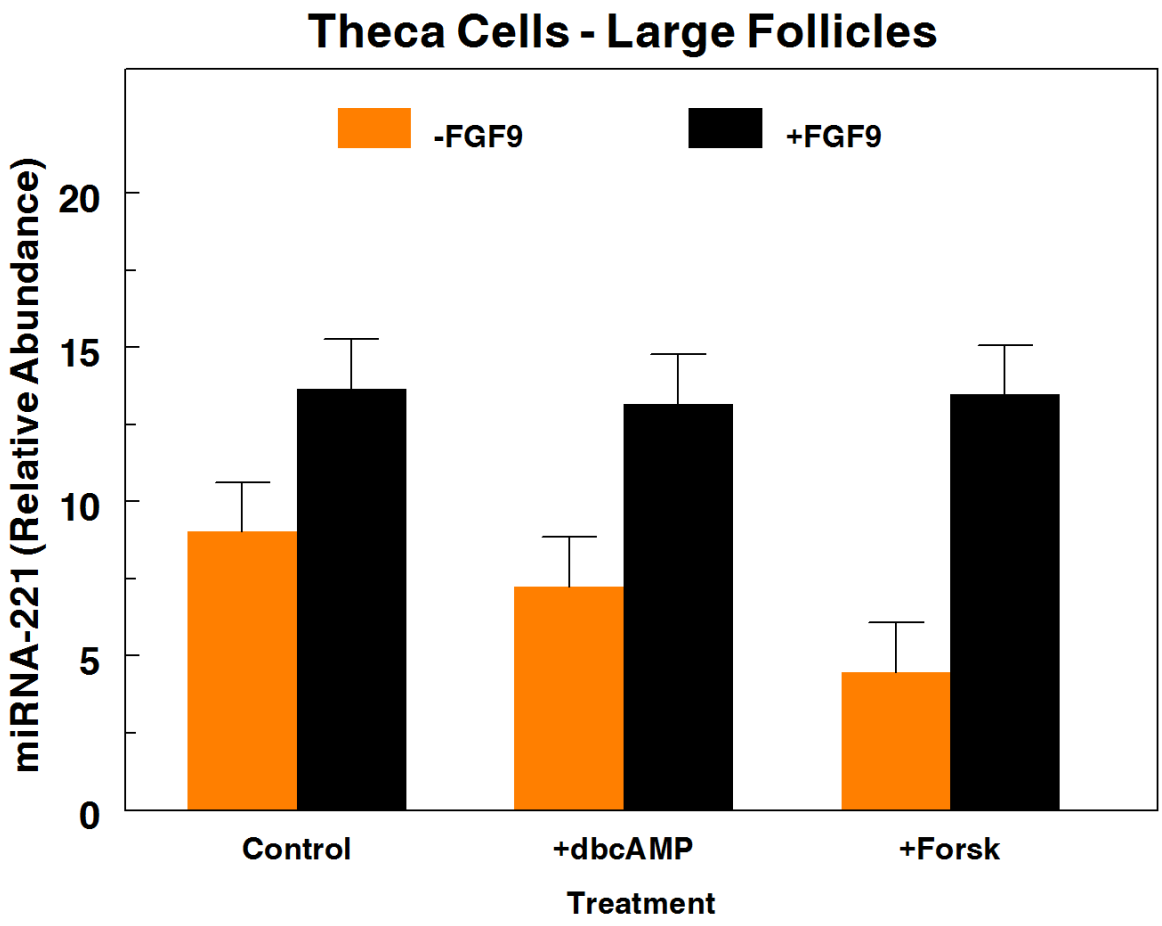
Theca Cells - Large Follicles
-FGF9
+FGF9
miRNA-221 (Relative Abundance)
0
5
10
15
20
Control
+dbcAMP
+Forsk
Treatment

FIGURE 2

Figure 3: Effect of IGF1 and 10% FCS on miRNA-221 abundance in bovine large-follicle theca cells treated with or without FGF9. Theca cells were isolated and cultured for 72 h in 10% FCS after which cells were treated with 30 ng/mL FGF9, 30 ng/mL IGF1 and 10% FCS for 24 h. ANOVA revealed a significant interaction between treatments. Asterisk (*) indicates mean differs ($P < 0.05$) from its respective control mean.

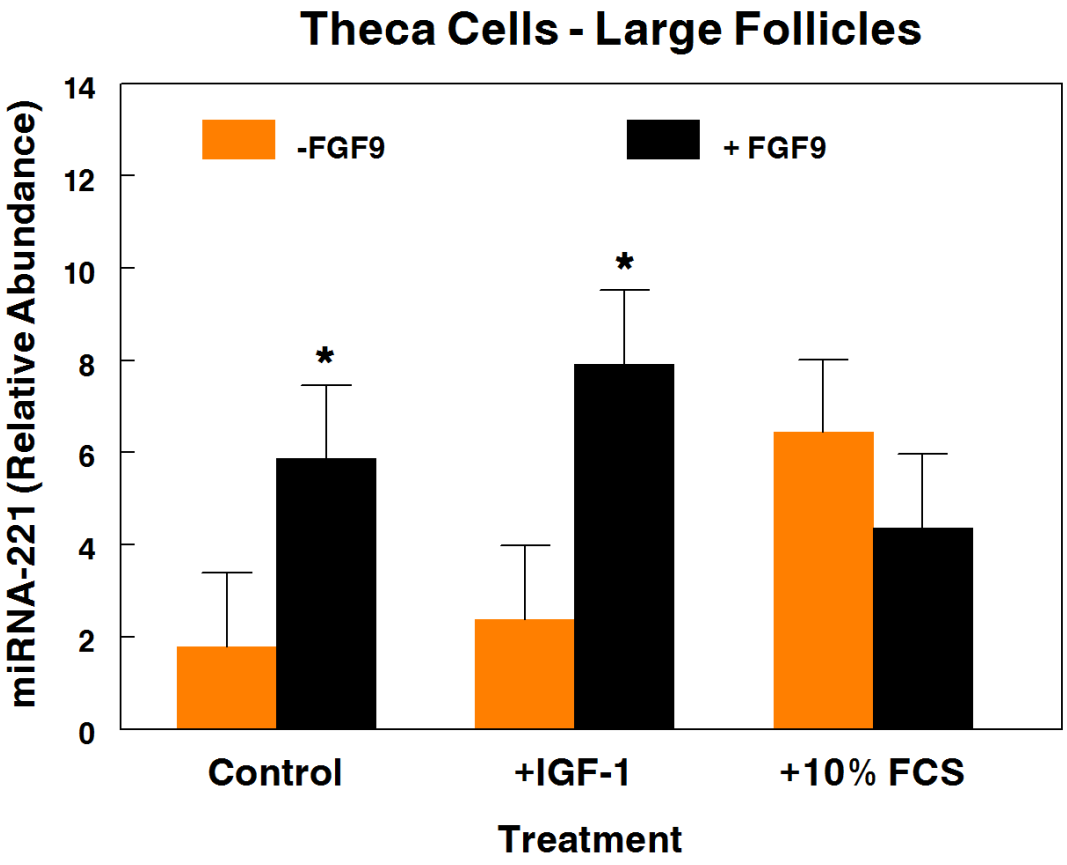
Theca Cells - Large Follicles
miRNA-221 (Relative Abundance)
14
12
10
8
6
4
2
0
-FGF9
+ FGF9
*
*
Control
+IGF-1
+10% FCS
Treatment

FIGURE 3

Figure 4: Effect of miRNA-221 mimic and inhibitor on cell proliferation in bovine large-follicle theca cells treated with or without IGF1 and FGF9. Theca cells were isolated and cultured for 72 h in 10% FCS and then transfection complex of miRNA-221 mimic or inhibitor (62.3 nM) was applied for 4 h after which cells were treated with 0 or 27.3 ng/mL FGF9, and 0 or 27.3 ng/mL LH+IGF1 for 48 h. ANOVA revealed that neither miRNA-221 mimic nor inhibitor effected cell number, nonetheless FGF9 increased cell numbers by 37%. Single asterisk (*) indicates that mean differs ($P < 0.05$) from its respective untreated controls. Double asterisk (**) indicates that mean differs ($P < 0.05$) from its respective untreated controls as well as from the LH+IGF1 treatment.

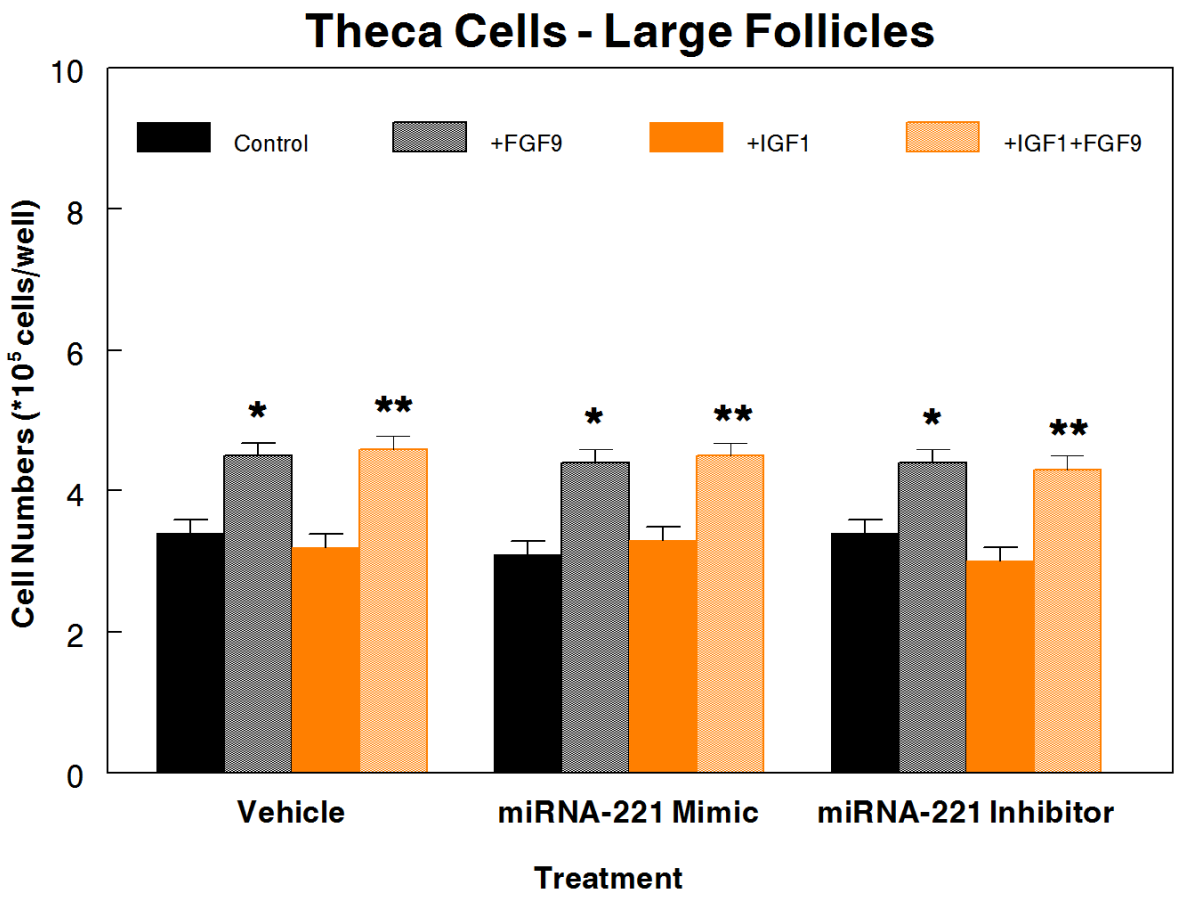
Theca Cells - Large Follicles
Control
+FGF9
+IGF1
+IGF1+FGF9
Cell Numbers (*10^5 cells/well)
10
8
6
4
2
0
*
**
*
**
*
**
Vehicle
miRNA-221 Mimic
miRNA-221 Inhibitor
Treatment

FIGURE 4

Figure 5: Effect of miRNA-221 mimic and inhibitor on androstenedione production in bovine large-follicle theca cells treated with or without IGF1 and FGF9. Theca cells were isolated and cultured for 72 h in 10% FCS and then transfection complex of miRNA-221 mimic or inhibitor (62.4 nM) was applied for 4 h after which cells were treated with 0 or 27.3 ng/mL FGF9, and 0 or 27.3 ng/mL LH+IGF1 for 48 h. ANOVA revealed significant FGF9 and LH+IGF1 treatment effects and a significant interaction between FGF9 and LH+IGF1 treatments. Single asterisk (*) indicates that mean differs ($P < 0.05$) from its respective untreated controls. Double asterisk (**) indicates that mean differs ($P < 0.05$) from its respective untreated controls as well as from the LH+IGF1 treatment.

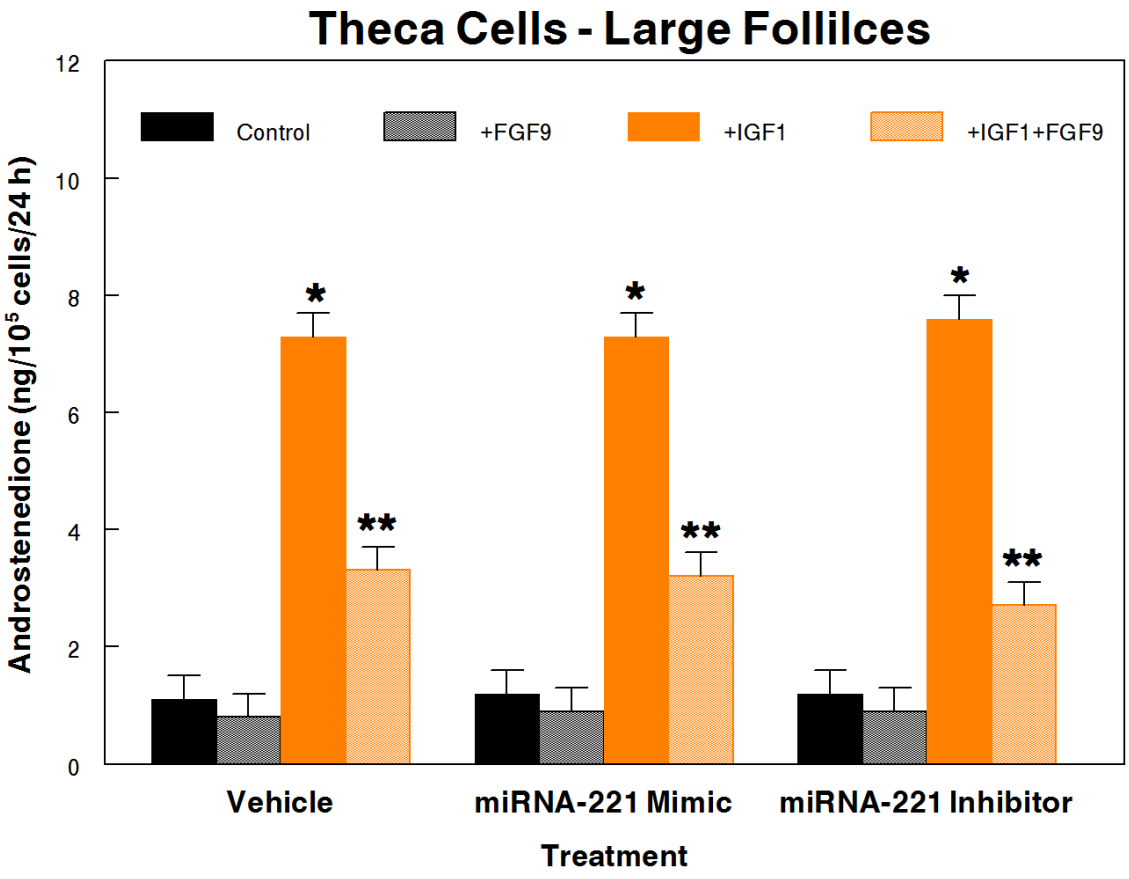
Theca Cells - Large Follilces
Control
+FGF9
+IGF1
+IGF1+FGF9
Androstenedione (ng/10^5 cells/24 h)
0
2
4
6
8
10
12
*
**
Vehicle
miRNA-221 Mimic
miRNA-221 Inhibitor
Treatment

FIGURE 5

Figure 6: Effect of mimic and inhibitor miRNA-221 on progesterone production in bovine large-follicle theca cells treated with or without IGF1 and FGF9. Theca cells were isolated and cultured for 72 h in 10% FCS and then transfection complex of miRNA-221 mimic or inhibitor (62.4 nM) was applied for 4 h after which cells were treated with 0 or 27.3 ng/mL FGF9, and 0 or 27.3 ng/mL LH+IGF1 for 48 h. ANOVA revealed significant FGF9 and LH+IGF1 treatment effects and a significant interaction FGF9 and LH+IGF1 between treatments. Single asterisk (*) indicates that mean differs ($P < 0.05$) from its respective untreated controls. Double asterisk (**) indicates that mean differs ($P < 0.05$) from its respective untreated controls as well as from the LH+IGF1 treatment.

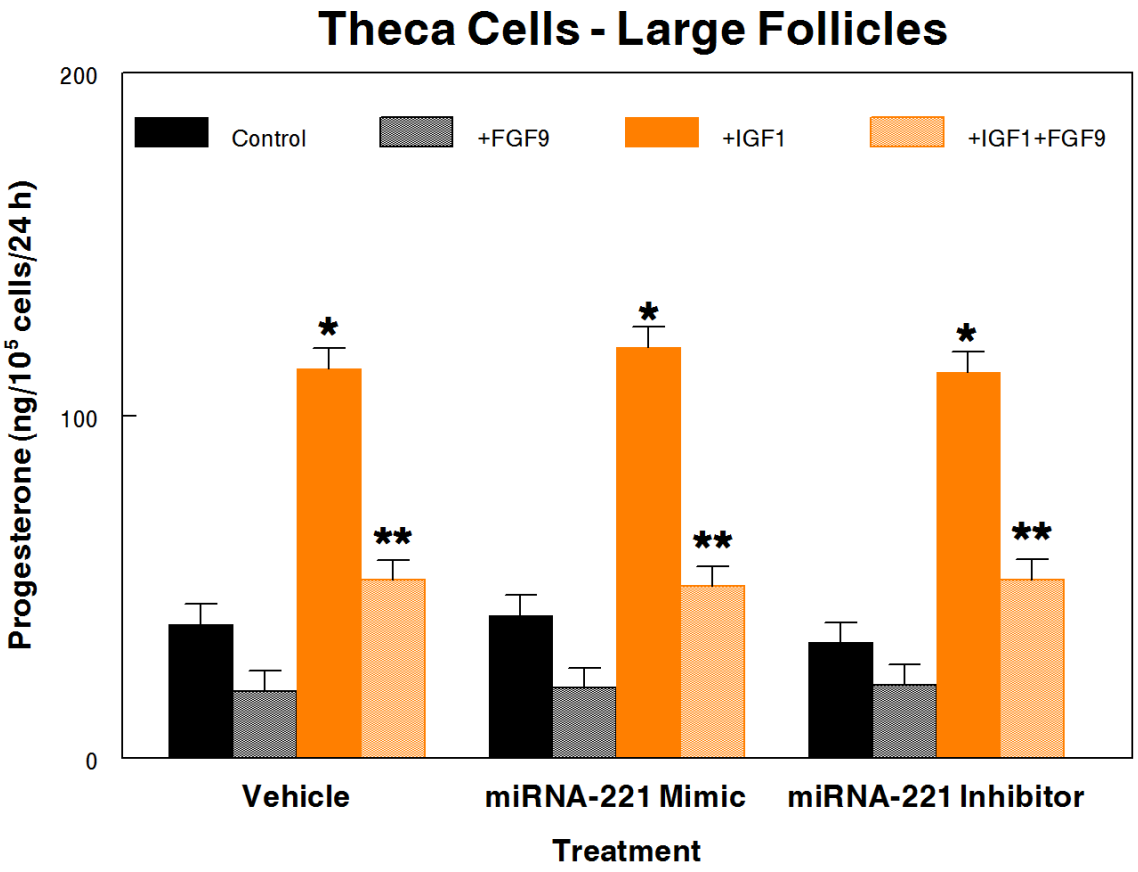
Theca Cells - Large Follicles
Progesterone (ng/10^5 cells/24 h)
200
100
0
Control
+FGF9
+IGF1
+IGF1+FGF9
*
**
Vehicle
miRNA-221 Mimic
miRNA-221 Inhibitor
Treatment

FIGURE 6

6. References

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CHAPTER IV

SUMMARY AND CONCLUSION

In cattle, follicular growth is dynamically modulated by intraovarian events that occur on a cyclical basis. The process of follicular recruitment, selection and growth is under control of synchronized endocrine and intraovarian factors. During waves of follicular growth, changes in the balance between many cellular processes and signaling pathways are controlled by precisely regulated expression and interaction of numerous genes within the ovary. One gene discovered to be involved with cyst formation is FGF9. FGF9 is one of 23 members of the FGF signaling family, which plays key roles in regulating ovarian follicular development. To date FGF1, FGF2, FGF7, FGF8, FGF9, FGF10, FGF17, and FGF18 have been identified in the ovary of humans, rodents or domestic animals. Members of the FGF family regulate steroidogenesis, cell survival and apoptosis, cell proliferation, and luteal development within the ovary. With regard to follicular development, research indicates FGF9 protein localizes to theca, stroma, and the basement membrane surrounding granulosa and theca cells. *In vitro*, FGF9 stimulates cell proliferation while inhibiting steroidogenesis in bovine granulosa and theca cells. Recent research indicates miRNAs have significant impact on gene expression in numerous tissues in humans and animals modulating cell proliferation and differentiation. In a microarray study it was discovered that FGF9 induces miRNA-221 in bovine theca cells. Also, miRNA-221 is overexpressed in

human ovarian cancer and is differentially expressed in dominant and subordinate follicles during the estrous cycle of cattle; indicating that miRNA-221 likely modulates ovarian function.

In the present study, hormonal regulation of miRNA-221 was evaluated in bovine theca cells *in vitro* and it was found that miRNA-221 was not altered by the cAMP signaling cascade or ovarian steroid hormones. However, FGF9 increased miRNA-221 in the presence and absence of IGF1. The miRNA-221 mimic and inhibitor study indicated that miRNA-221 is not involved in regulating the biosynthesis of thecal steroids or cell numbers, suggesting that miRNA-221 may not be a part of the FGF9-mediated reduction of steroidogenesis or stimulation of cell proliferation in bovine theca cells. Understanding the role of miRNA-221 in regulating ovarian follicular growth and development will require further study.

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