GENE EXPRESSION IN OVARIAN GRANULOSA AND THECA CELLS IN CATTLE SELECTED FOR DOUBLE OVULATIONS AND TWIN BIRTHS

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2005

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Submitted to the Faculty of the Graduate College
of the Oklahoma State University in partial
fulfillment of the requirements for the
DOCTOR OF PHILOSOPHY
May, 2008

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"One of the most essential things you need to do for yourself is to choose a goal that is important to you. Perfection does not exist... you can always do better and you can always grow"

Les Brown
Motivational Speaker

ACKNOWLEDGEMENTS

I would first like to express my appreciation to Dr. Leon Spicer, whose mentorship, insight and advice made my studies, research and growth at Oklahoma State University an enjoyable adventure. Gratitude and recognition barely compare to the support that Dr. Spicer provided me during my long stay in the animal science department, first as a Fulbright visiting research scholar, a Masters of Science student and then as a Ph.D. student. The role model I found in Dr. Spicer allowed me to shape my science beliefs; his leadership molded my interested and encouraged my involvement on campus. My deepest thanks to Dr. Sherill Echternkamp whose collaboration, research interests and contributions allowed the accomplishment of this research. On numerous occasions, Dr. Echternkamp's insights into reproductive physiology and Twinning in cattle helped shape the objectives of this research. My deepest gratitude for Dr. David Buchanan, Dr. Guolong Zang, and Dr. Mark Payton for serving on my doctoral committee and providing me with insight on research and teaching, along with valuable suggestions and comments. Dr. Wettemann's suggestions advanced my current research endeavors in thriving for perfection. The constant encouragement and support of the Animal Science faculty and their teaching efforts counterbalanced the long hours and hard work in the accomplishment of this degree.

Thank you to Dustin Allen and Consuelo Santiago for technical assistance in the early stages of this research, and to Alberto Grado, Laura Hulsey, Dana Lagally, Nicole Fry and Amanda Burress for camaraderie and workmanship, and to my fellow graduate students at Oklahoma State University who welcomed me and made my stay enjoyable.

DEDICATIONS

To my parents, Youssef and Afaf and my siblings for believing in my potential to achieve my goals their constant support and prayers, and for their indulgence and support through my USA adventure,

To the my second family, the Beers, for tolerating my presence in their life,

To the Doughan, the Reilly, the Zubaidi, the Naddour, the Balaa and the Abou Jaoudeh families for their support and friendship,

And to all my friends and all those who helped me and supported me during my education and experience in the USA, I dedicate this manuscript.

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CHAPTER I

INTRODUCTION

Reproductive efficiency is a key constituent in successful and economically feasible beef and dairy production. Ovarian follicular growth is controlled by various hormones, including follicle stimulating hormone (FSH), luteinizing hormone (LH), insulin, insulin-like growth factors (IGFs), and their binding proteins and proteases. FSH controls follicular recruitment and growth, while LH is responsible for inducing ovulation. Receptors for both LH and FSH have been detected in the ovarian granulosa cells, and IGFs were shown to stimulate LH and FSH receptors in the follicle to increase its responsiveness to LH and FSH.

Studies from Dr. Leon Spicer's lab showed that insulin-like growth factors (IGF1 and IGF2), the IGF binding proteins (IGFBP-2 through -5) and their proteolysis (via pregnancy-associated plasma protein-A; PAPP-A) play a major role in follicular growth and dominance (for review Spicer and Echternkamp, 1986, 1995; Spicer et al., 2000; Spicer et al., 2001; Spicer, 2004). Briefly, IGF1 stimulates both steroidogenesis and mitogenesis within the bovine follicle (Spicer et al., 1993; Spicer and Chamberlain, 1998) and intrafollicular IGF2 levels are greater than those of IGF1 (Spicer and Echternkamp, 1995; Stewart et al., 1995; Spicer et al., 2004), thus IGF2, like IGF1, modulates ovarian folliculogenesis (Gong et al., 1993; Spicer et al., 1993) and acts in an autocrine and/or

paracrine manner to affect cellular proliferation and differentiation. Since both IGF1 and IGF2 biological actions are via the IGF type 1 receptor (IGF1R; Adashi et al., 1990), and since IGFBPs modulate the IGF effect by sequestering IGF1 or 2 and preventing degradation and signaling of these growth factors, any increase in intrafollicular levels of IGF1 (mainly from plasma) or IGF2 (locally produced) can have a great impact on follicular growth. The mannose-6 phosphate / IGF type 2 receptor (IGF2R), described as a membrane-bound IGF2 binding receptor (LeRoith, 1991), works in the same manner as IGFBPs and modulates free intrafollicular IGF2 levels, but is not well studied in the ovary. Furthermore, the involvement of IGF2R in the control of long term selection for twin ovulations has not been ascertained.

Twinning in cattle has been studied for a number of years through the genetic selection of a herd of cattle with their ability for twin ovulation and births. The Meat Animal Research Center (MARC) at Clay Center, Nebraska, developed a herd of cattle with increased twinning rate to improve the efficiency of the beef cattle industry. Echternkamp et al. (1990 remove Cushman) (2005) demonstrated that twin births is in fact due to the simultaneous ovulation of two follicles preferentially from 2 ovaries, resulting in the survival of multiple offspring. Studies on the underlying reproductive mechanisms of follicular growth, hormonal control and efficiency of multiple versus single bearing cattle were conducted to understand the underlying mechanisms controlling multiple births. The MARC twinning population showed increased plasma and follicular fluid IGF1 levels (Echternkamp et al., 1990), but have not found differences in secretion of gonadotropins between Twinners and single cattle. Whether

increased IGF1 is the sole factor resulting in the selection of two rather than one dominant / ovulatory follicle at each follicular cycle remains to be determined.

In growing follicles, communication between oocyte, granulosa and theca cell compartments modulates proliferation and differentiation of these cell layers (Nilsson and Skinner, 2001; Wijgerde et al., 2005). The hedgehog gene family members, including Sonic (Shh), Indian (Ihh) and Desert (Dhh) hedgehog have been identified in mammalian cells (Pangas, 2007), and were localized to the mouse ovarian cell layers including granulosa, theca and the oocyte (Wijgerde et al., 2005; Russell et al., 2007). In addition, the hedgehog system was identified to stimulate proliferation of ovarian somatic stem cells (Zhang and Kalderon, 2000) and germ cells (King et al., 2001) in Drosophila. Recently, the hedgehog system was identified to stimulate mitogenesis in murine ovarian cells (Russell et al., 2007) and involved in regulating cell proliferation and survival, determining cell fate, differentiation and polarity of embryonic cells (Ingham and Placzek, 2006). Whether the hedgehog system is physiologically regulated during ovarian follicular development in mammals is unknown.

Therefore, we hypothesized that components of the IGF system other than IGF1 as well as gonadotropins receptors might be involved in the control of the development of multiple dominant follicles leading to twinning. The objectives of this study were to characterize the gonadotropin receptors (LHR and FSHR) expression patterns in Twinner versus Control cattle at different stages of ovarian follicular growth to determine the expression and regulation of IGF2R in growing and atretic antral follicles from these two

cattle populations, and to determine the gene expression levels of two of the hedgehog system components, Ihh and Ptch1, in ovarian granulosa and theca cells, respectively.

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CHAPTER II

REVIEW OF LITERATURE

1. Role of steroids and gonadotropins in the control of follicle recruitment, deviation.

In cattle, follicular development during the estrous cycle follows a well-established pattern of two or three follicular waves with 60 to 70 % showing a 2-wave, and 30 to 40 % showing a 3-wave estrous cycle, marked by the development of two versus three consecutive dominant follicles within a cycle (Adams, 1999; Echternkamp, 2000; Wiltbank et al., 2000; Adams et al., 2008). The endocrine control of folliculogenesis involves pituitary, uterine and ovarian factors, such as the gonadotropins (i.e., FSH and LH), insulin-like growth factors, estradiol and prostaglandins, to name a few (Ginther et al., 2003; Hsueh et al., 1983; Hsueh et al., 2000; Mihm et al., 2000; Spicer and Echternkamp, 1986, 1995).

Follicular development starts with the recruitment of a cohort of 3-4 mm antral follicles in response to a transient increase in FSH secretion (Hsueh et al., 1989; Wiltbank et al., 2000). As the estrous cycle progresses, estradiol production by the growing follicles increases (Ireland and Roche, 1983; Kulick et al., 1999; Wiltbank et al., 2000), which causes a negative feedback on FSH secretion (Wiltbank et al., 2000). The decrease in FSH secretion drives the selection of the largest follicles to continue their growth by virtue of increased sensitivity to FSH, via an increase in granulosa cell FSH

and LH receptors (Donadeu and Ginther, 2001; Ginther et al., 2003; Ginther et al., 2002; Wiltbank et al., 2000). Simultaneously, basal levels of pituitary LH start to increase, further driving the selected growing follicles to acquire theca cell LH receptors (Donadeu and Ginther, 2001; Ginther et al., 1996). In monotocous species such as cattle, only one follicle dominates the wave and reaches preovulatory status (Hunter et al., 2004). Any of the follicles in the cohort have the capacity to become dominant (Gibbons et al., 1997), as evident by aspiration of the largest diameter follicle early in the follicular wave. The future dominant follicle has been reported to have a slight developmental advantage (Wiltbank et al., 2000) such as larger diameter, greater intraovarian steroids, and possibly greater gonadotropin receptor content. Deviation of the follicle is marked by a difference in growth rates between the two largest follicles (Ginther et al., 1996). Growth of all follicles from the cohort, other than the dominant follicle, ceases at different times of the follicular wave and undergoes atresia, as marked by appearance of pycnotic nuclei in granulosa and later in theca cells (Hunter et al., 2004; Spicer and Echternkamp, 1986, 1995; Wiltbank et al., 2000). In addition, if a preovulatory surge of LH is suppressed by the presence of high levels of progesterone (presence of a corpus luteum), the dominant follicle regresses and is replaced by another growing follicle (Ginther, 1970; Ginther et al., 2003; Wiltbank et al., 2000). Ginther et al. (1996) reported that follicular growth and differentiation around the time of selection was promoted by the acquisition of LH receptors by granulosa cells. Around the time of deviation, an increase in granulosa (Bao and Garverick, 1998; Xu et al., 1995) and theca (Bodensteiner et al., 1996; Ginther et al., 1996) cell LH receptors was reported in some but not in all studies (Evans and Fortune, 1997; Wiltbank et al., 2000). After deviation, low intrafollicular and plasma FSH levels

further allowed the dominant follicle to exclusively grow, regardless of the constant level of FSH receptor (Bodensteiner et al., 1996) and FSH receptor mRNA (Xu et al., 1995) in the largest follicles. Intrafollicular IGF (Mihm et al., 2000; Spicer and Echternkamp, 1995) and estradiol (Spicer, 2005) enhance responsiveness of the dominant follicle to gonadotropins at the time of follicle selection (Austin et al., 2001; Ginther et al., 2003; Mihm et al., 2000; Spicer, 2005; Spicer et al., 2000). The effect of the IGF system in controlling follicular development will be discussed in the following section.

2. The IGF system and its role in follicular development.

In the ovary, the IGF system is composed of two ligands (IGF1 and IGF2), two IGF type 1 and 2 receptors (IGF1R and IGF2R), six IGF binding proteins (IGFBP-1 through 6) and some IGFBP proteases (i.e. PAPP-A and KLKs) (LeRoith, 1991; LeRoith and Raizada, 1989; Raizada and LeRoith, 1991). IGF1 and IGF2, first studied in 1976, are structurally and functionally related to insulin (Rinderknecht and Humbel, 1976, 1978), have highly conserved amino acid sequences across species and are locally produced in many tissues, including ovarian granulosa and theca cells (Spicer and Echternkamp, 1995). IGFBPs are IGF specific carrier proteins that bind IGFs with a higher affinity than their receptors and increase their half-life or contribute to their degradation (Jones and Clemmons, 1995; LeRoith et al., 2003; Spicer and Echternkamp, 1995). Insulin receptor (INSR) and IGF1R are plasma membrane-bound high affinity receptors to insulin and IGF1, respectively, and both receptors share extensive structural homology (Frattali and Pessin, 1993). Both receptors are a heterotetrameric complex of 2

α and 2 β subunits, where the α-subunit is responsible for the high affinity recognition and binding of the ligand, whereas the β subunit is responsible for the intracellular tyrosine-specific protein kinase domain, as shown in figure 1.1. Both IGF1R and INSR contains the ATP binding sites and several phosphotyrosine acceptor sites (for review, see: Frattali and Pessin, 1993; LeRoith and Raizada, 1989; LeRoith et al., 2003). In addition, the presence of insulin/IGF1 heterotetrameric hybrid complexes (Soos and Siddle, 1989; Soos et al., 1990) suggest cross-reactivity of receptors with either insulin or IGF1 (for review, see: Frattali and Pessin, 1993; LeRoith and Raizada, 1989; Lowe, 1991). Signaling of insulin, IGF1 or IGF2 occurs through the tyrosine kinase receptor, IGF1R with different affinities (Jones and Clemmons, 1995; Werner et al., 1991) to control for example DNA synthesis in human fibroblasts (Werner et al., 1991), and through the insulin receptor to control for example glucose oxidation in rat adipocytes (Lowe, 1991).

On the other hand, IGF2R is a 215 KDa cation-independent mannose 6-phosphate transmembrane protein receptor with 15 contiguous repeats as shown in figure 1.1 (Lobel et al., 1987; Werner et al., 1991) that recognizes IGF1 and IGF2, but not insulin (Rechler and Nissley, 1985; Rechler et al., 1980) with a greater affinity for IGF2 than IGF1, and with a cross-reactivity of 95 % versus 10 %, respectively (LeRoith, 1991). IGF2R binds IGF2 and contributes to its degradation in rat adipocytes (Oka and Czech, 1986; Oka et al., 1985), but with no intracellular signaling role (LeRoith, 1991; Spicer et al., 2004), suggesting that IGF2R may act as a membrane bound IGFBP inactivating IGF2 (Spicer et al., 2004).

Within the ovary, IGF1 and IGF2 serve as paracrine, autocrine and endocrine regulators of ovarian function stimulating mitogenesis of granulosa cells (Monniaux et al., 1997; Spicer et al., 1993; Spicer and Chamberlain, 2002), synergizing with FSH to increase granulosa cell estradiol production via an increase in aromatase activity (Spicer et al., 2002a; Spicer and Echternkamp, 1995), and stimulating bovine theca cell proliferation and androgen production (Stewart et al., 1995).

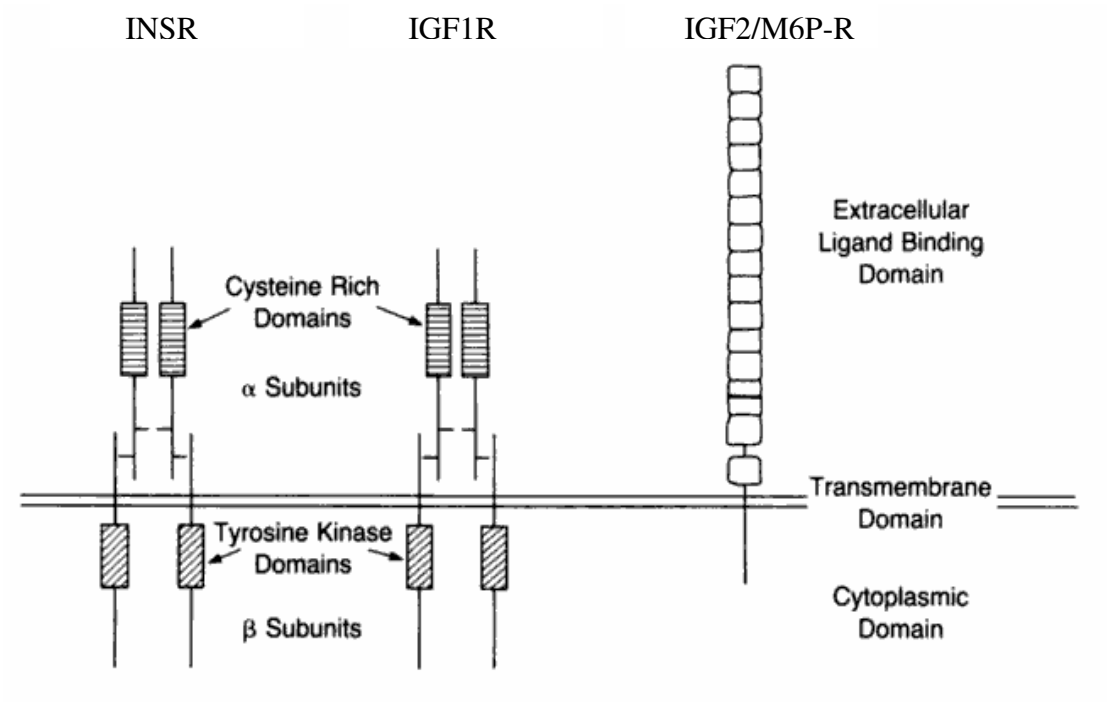

Figure 1.1: Comparison of insulin, IGF1 and IGF2 receptors (Adopted from: Werner et al., 1991). Both insulin and IGF type 1 receptor share structural and molecular signaling. IGF type 2/Mannose 5-phosphate receptor is a single polypeptide composed of a series of cystein rich repeats.

In cattle, total follicular IGF1 levels remain relatively unchanged during the estrous cycle (de la Sota et al., 1996; Echternkamp et al., 1994; Stewart et al., 1996), however an increase in bioavailable (or free) IGF1 occurs at the emergence of the dominant follicle (Monget and Bondy, 2000; Monget et al., 1998; Spicer and Echternkamp, 1995), concomitant with a decrease in free IGF1 in the subordinate follicle (Beg et al., 2001; Ginther et al., 2003; Monget et al., 1998). The alteration of the free IGF1 levels is modulated by IGFBPs with relatively low molecular weights (namely IGFBP-2, -4, and -5) that disappear from the dominant follicles in cattle (de la Sota et al., 1996; Echternkamp et al., 1994; Mihm et al., 2000; Stewart et al., 1996). However, infusions of IGF1 into the ovary in cattle did not alter IGFBPs (Spicer et al., 2000).

Follicular growth in cattle is gonadotropin-independent and gonadotropin-dependant in pre-antral and antral follicles, respectively (Webb and Campbell, 2007; Webb et al., 2007). Detection of IGFBP-2 and IGF1R in follicles at early pre-antral stages (Armstrong et al., 2002), and of IGF2 at later pre-antral stages (Armstrong et al., 2002; Webb and Campbell, 2007), indicate the importance of the IGFs in the ovary. Extra-ovarian IGF1 controls the ability of the follicles to locally produce IGF2, and potentially both IGF1 and IGF2 increase follicular response and sensitivity to gonadotropins.

Recently, IGF2 was shown to act in an autocrine and/or paracrine manner to affect cellular proliferation and differentiation. Spicer et al. (2004) showed that IGF2 stimulates steroidogenesis and mitogenesis in theca cells. With intrafollicular IGF2 levels being greater than those of IGF1 (Spicer and Echternkamp, 1995; Stewart et al., 1995), IGF2 along with IGF1 likely modulates ovarian folliculogenesis (Gong et al.,

1993; Spicer et al., 1993). Since both IGF1 and IGF2 biological actions are via the IGF type 1 receptor (Adashi et al., 1990), and since IGFBPs modulate the IGF effect by sequestering IGF1 or IGF2 and preventing degradation and signaling of these growth factors, any increase in intrafollicular levels of IGF1 (mainly from plasma, Armstrong et al., 2002; Webb and Campbell, 2007) or IGF2 (locally produced, Armstrong et al., 2002; Armstrong et al., 2000) can have a great impact on follicular growth. However, IGF2R, described in the previous sections as a membrane-bound IGF2 binding protein that modulates intrafollicular levels of bioavailable IGF2, is not well studied in the ovary.

3. Twinning in cattle: update on genetic effects, twinning rate, ovulation rates and folliculogenesis in the twinning herd at the Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska.

Cattle are monotocous species with one follicle developing and ovulating at the end of every follicular cycle. Follicular development in cattle is controlled by intraovarian factors and can be influenced by breed (Alvarez et al., 2000; Spicer et al., 2002b), nutrition and body condition score (Webb et al., 2004; Wettemann and Bossis, 1999), milk production (Wiltbank et al., 2000), season (Echternkamp et al., 1990a) and genetics (Gregory et al., 1997; Gregory et al., 1990), thus affecting the occurrence of monozygotic and dizygotic twins in this species (Komisarek and Dorynek, 2002; Rutledge, 1975). In Holstein herd records from Minnesota Dairy Herd Improvement Association (Silva del Rio et al., 2007), twinning rate increased across years and with parity. In monotocous species such as cattle, the occurrence of fraternal or multiple births is low. Twinning rate is regulated by numerous factors, the most important of

which are age of dam, season of breeding and the genetic makeup of the cow (Wiltbank et al., 2000). Other factors affecting twinning rate include the presence of ovarian cysts, number of days open, peak milk production and parity number (Kinsel et al., 1998; Wiltbank et al., 2000). An increase in twinning rates can be achieved following embryo transfer (Anderson et al., 1979; Boland et al., 1975; Sreenan and Mulvehill, 1975), reproductive hormonal therapy (Echternkamp, 1992; McCaughe and Dow, 1977), or the immunological suppression of hormones (Hillard et al., 1995). In addition, long-term genetic selection over multiple selection cycles (Echternkamp et al., 1990a; Gregory et al., 1990; Van Tassell et al., 1998; Van Vleck et al., 1991b) have proven to increase twinning rates significantly (Echternkamp, 1992; Echternkamp et al., 1990a; Gregory et al., 1990). However, in most cases of multiple ovulations (Echternkamp, 1992; Echternkamp et al., 2007), especially in heifers (Vandeplassche et al., 1979), uterine capacity was limiting to the occurrence of twinning. In fact, uterine capacity for cows selected for twinning was adequate to support two fetuses and diminished for every extra fetus (Echternkamp, 1992; Echternkamp et al., 2007) concomitantly jeopardizing fetal survival.

At the Roman L. Hruska U.S. Meat Animal Research Center (MARC), long-term selection of cattle for increased twinning rate has lead to the identification of inheritance of twinning as a quantitative trait (Gregory et al., 1990) and the identification of multiple quantitative trait loci (Kappes et al., 2000; Komisarek and Dorynek, 2002). With the identification of a strong genetic correlation (0.75-1.0) between twinning and ovulation rate (Van Vleck et al., 1991a), and an estimated heritability for ovulation rates across multiple estrous cycles at about 0.36 (Echternkamp et al., 1990a; Van Vleck et al.,

1991a), ovulation rates were used to indirectly select for twinning rates (Van Vleck and Gregory, 1996a, b; Van Vleck et al., 1991a, b), and thus increase the selection intensity. In the MARC twinning population, an increase in twinning rate from 4% in 1984 to greater than 55% in 2000, indicating a rough yearly increase of 3% (Echternkamp, 2000; Echternkamp et al., 1990a; Echternkamp et al., 1994; Van Vleck et al., 1991b) and the selection of two or more dominant follicles within a follicular wave (Cushman et al., 2005; Cushman et al., 2000; Echternkamp, 2000).

The genetic scanning of the twinning population has identified regions of chromosomes 7 and 23 (Blattman et al., 1996), 5 (Kappes et al., 2000), 13, 15 and 19 (Erickson and Danforth, 1995; Komisarek and Dorynek, 2002) as candidate regions for identification of genes likely involved in increased ovulation rate and the control of follicle development and selection of multiple follicles; genes for IGF1 and GDF-9, important hormones controlling follicular growth, are localized on chromosomes 5 and 7, respectively. However, in these genomic scan studies, only the highest peaks for QTLs were selected (e.g., only 10 out of 40 QTL peaks (Kappes et al., 2000) were further analyzed), thus possibly missing other regions of importance in twinning and ovulation rate such as those involved in controlling twinning in sheep (e.g., BMP15; Davis, 2005), and activin-like kinase 6 (also known as BMPR1B; McNatty et al., 2005; McNatty et al., 2001). Oocyte-expressed genes, such as GDF-9, have been reported to be involved in the control of mitogenesis and steroidogenesis in cattle (Spicer et al., 2006; Spicer et al., 2008), and mice (Mazerbourg and Hsueh, 2003), but their involvement in twinning in cattle requires further investigation.

Findings related to follicular development, circulating and intrafollicular hormone levels such as gonadotropins and growth factors in the twinning MARC population and a multi-breed composite control herd will be discussed in the next section.

4. Control of folliculogenesis in cattle selected for twin ovulations and births: update on the role of IGF.

At MARC, long-term selection of cattle for increased twinning rate has resulted in more total follicles (pre-antral and antral) per ovary, the recruitment of more follicles within the cohort of developing follicles and the selection of two or more dominant follicles within a follicular wave (Cushman et al., 2005; Cushman et al., 2000; Echternkamp, 2000), thus indicating alterations of the regulatory system of ovarian folliculogenesis.

Identification of markers for genetic modification in cattle selected for increased ovulation rate is underway (Kappes et al., 2000). However many (Echternkamp, 2000; Wiltbank et al., 2000) have suggested that the differences in follicular growth and the selection of two dominant follicles (Cushman et al., 2005; Cushman et al., 2000) in Twinner might be regulated locally within the ovary. Observations by Echternkamp (2000) indicate that only few Twinner cows express exclusively twin ovulations support the notion that the selection of multiple ovulatory follicles within the follicular cycle are under endocrine control.

Regardless of the genetic influence on the selection of multiple dominant ovulatory follicles, Twinner cows exhibit a typical ovarian follicular growth pattern of

two or three follicular waves (Erickson and Danforth, 1995; Monniaux et al., 1997; Spicer and Echternkamp, 1986), but recruit more medium-sized follicles into the cohort of growing follicles. Following deviation, two co-dominant ovulatory follicles develop (Cushman et al., 2000; Echternkamp, 2000). In studying the ovarian follicular waves, Echternkamp (2000) used single ovulators within the Twinning population for comparison of single versus multiple ovulations. In this study, the 2nd dominant follicle in Twinner cows in this study showed a smaller follicle and a subsequent smaller corpus luteum (Echternkamp, 2000). Greater secondary and tertiary follicles (but not primary or primordial follicles) were observed between Twinner and contemporary control cows (Cushman et al., 2000; Echternkamp, 2000; Echternkamp et al., 1990a). As discussed earlier (section 1), follicular growth depends on pituitary gonadotropins and other intra-ovarian factors such as IGF1.

Plasma progesterone and testosterone profiles did not differ between Control and Twinner cows after PGF2α (Echternkamp et al., 2004). However, plasma estradiol was greater in Twinner as compared to Control cows 0, 24 and 48 h after PGF2α (Echternkamp et al., 2004). Consistent with elevated plasma estradiol levels, follicular fluid estradiol levels at 0 h after PGF2α were greater in large estrogen-active (Estradiol : Progesterone > 1) follicles from Twinner versus Control cows (Echternkamp et al., 2004). Follicular fluid progesterone levels did not differ between Control and Twinner cows, whereas androstenedione concentrations at 48 h after PGF2α were greater in large estrogen-active follicles from Twinner versus Control cows (Echternkamp et al., 2004). Plasma total cholesterol concentrations were greater in Control versus Twinner cows after PGF2α (Echternkamp et al., 2004).

Neither plasma nor follicular fluid FSH and LH differed between Control and Twinner females (Echternkamp, 2000; Echternkamp and Gregory, 1989, 2002), whereas pre-ovulatory levels of plasma LH and FSH were greater in Twinner versus control 72 h after PGF2α (Echternkamp et al., 2004). In contrast, Wiltbank et al. (2000) detected greater FSH concentrations 8 h before follicles reached 8.5 mm and at the time the largest follicle reached 8.5 mm in heifers with codominant versus single dominant follicles. At MARC, the population of Twinner cows does not develop two codominant follicles at every follicular wave (ovulation rate of 50% - Cushman et al., 2005; Echternkamp, 2000; Echternkamp et al., 1994; Echternkamp et al., 2004), and thus could explain the discrepancy between these studies.

Gonadotropin receptors are expressed in preantral follicles (Wandji et al., 1996), however early stages of antral development are not solely dependant on FSH and LH (Echternkamp, 2000) in cattle. In serum-free cultures of primordial follicles without gonadotropin additions (Braw-Tal and Yossefi, 1997), spontaneous growth of ovarian follicles showed independence from gonadotropin at early stages, dependence established with initiation of antrum formation (Echternkamp, 2000; Spicer and Echternkamp, 1986).

An important and redundant increase in plasma and follicular fluid IGF1 was detected in twinning cows compared with contemporary cows (Echternkamp et al., 2004; Echternkamp et al., 1990b). These findings point to a greater role of the IGF system in the selection of multiple follicles, since IGF1 increases follicular cell proliferation and steroidogenesis (Spicer and Echternkamp, 1995), protects granulosa cells against apoptosis (Chun et al., 1994), and increases receptors for gonadotropins (Spicer and

Echternkamp, 1995). However, the interaction between cattle genotype and the intrafollicular /endocrine factors requires further investigation. The lack of differential gonadotropin and growth hormones in the twinning population as compared to contemporary controls indicate a potential for gonadotropin receptors to be responsible for the increased follicular sensitivity (Echternkamp, 2000; Echternkamp et al., 2004) for the purpose of selecting concomitant dominant follicles. In addition, IGF1 may play a role in modulating gonadotropin receptors, and/or steroidogenic enzymes to bridge the development gap (Wiltbank et al., 2000). The IGF binding proteins are modulated in Twinner cows (Echternkamp et al., 2004) and might contribute to the regulation of the bioavailable IGF1. Very few reports examined the role for IGF2 in the bovine ovary. Spicer et al. (2004) indicated increased steroidogenesis and proliferation in bovine granulosa cells, and thus the contribution of IGF2 to the intraovarian total IGFs (IGF1 and IGF2) might indicate the potential for intrafollicular IGF2 to be implicated in this process. As mentioned earlier, IGF2R acts as a membrane bound binding protein with greater affinity to IGF2 than IGF1. However, despite greater concentrations of intrafollicular IGF2, as compared to IGF1, and a potential for IGF2 to compete with IGF1 for its signaling pathway, the role of IGF2 and IGF2R in modulating bovine follicular growth and/or steroidogenesis is poorly understood.

5. Novel genes controlling folliculogenesis in cattle: The Hedgehog System and its role in the ovary.

In growing follicles, communication between the oocyte and the granulosa and theca cell compartments controls proliferation and differentiation of these cell layers (Nilsson and Skinner, 2001; Wijgerde et al., 2005) and involves signaling pathways essential for development in many other cells (Russell et al., 2007). The hedgehog gene family was identified in 1990 as a family of developmentally regulated morphogens controlling basic embryonic development processes (Ingham and McMahon, 2001). Components of the hedgehog system and its intracellular signaling are shown in figure 1.2 (Wijgerde et al., 2005).

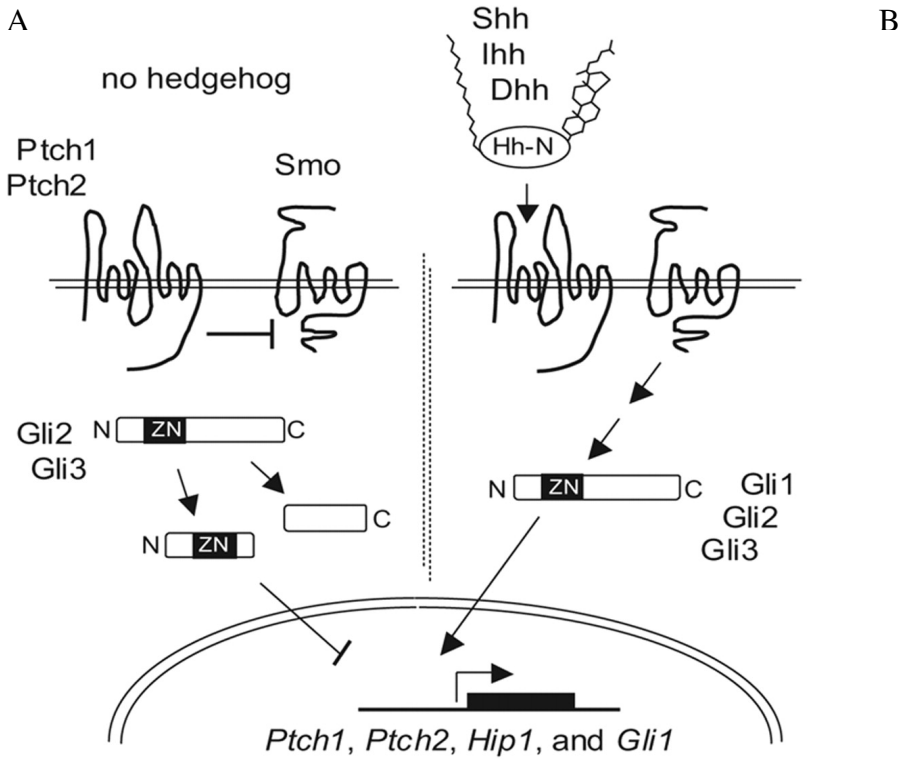

Figure 1.2: The hedgehog system in mammalian cells in the absence (panel A) and presence (panel B) of ligand (Wijgerde et al., 2005). Components of the hedgehog system and pathway: ligands (Shh, Ihh, and Dhh); ligand inhibiting protein (Hip), membrane bound receptors (Ptch1, Ptch2); membrane-

associated signal transducer (Smo); and intracellular transcriptional effectors (Gli1, Gli2, and Gli3). Upon binding the Hh to Ptch, the Ptch-Smo complex undergo conformational changes, altering the state of activity of both components (Alcedo and Noll, 1997) and controlling expression of the various components of the hedgehog system (Wijgerde et al., 2005).

Three secreted glycoproteins, Sonic (Shh), Indian (Ihh) and Desert (Dhh) hedgehogs, have been identified in mammalian cells (Ingham, 1993; Nusslein-Volhard and Wieschaus, 1980; Pangas, 2007), and localized to the mouse ovarian cell layers including granulosa, theca and the oocyte (Russell et al., 2007; Wijgerde et al., 2005). These Hh ligands have highly conserved structure and function (Ingham, 2001; Ingham and McMahon, 2001) and can evoke similar mechanistic intracellular signaling through two membrane-bound receptors Patched (Ptch) 1 and 2 (Burke and Basler, 1997; Pangas, 2007). All hedgehog ligands bind to hedgehog interacting protein 1 (hip1; Pathi et al., 2001), with similar affinity (Wijgerde et al., 2005). Ptch1 and Ptch2 receptors are 12-transmembrane proteins bind Hh ligands, whereas hip1, a membrane-bound glycoprotein, prevents binding of Hh to its receptors (Chuang and McMahon, 1999). Transduction of the Hh signal is mediated by Ptch-Smo complex, where Ptch association with Smo suppresses its constitutive signaling (Alcedo and Noll, 1997) mainly in the absence of Hh (Alcedo et al., 1996; Alcedo and Noll, 1997; Alcedo et al., 2000). Following hedgehog binding to its receptors, the seven transmembrane Smo transduces the Hh intracellular signaling in a G-protein coupled receptor fashion (Alcedo and Noll, 1997; Alcedo et al., 2000) through the intracellular transcriptional effectors Glioma-associated oncogene

homolog (Gli) 1, 2 and 3 (Alcedo and Noll, 1997; Alcedo et al., 2000; Burke and Basler, 1997; Pangas, 2007; Wijgerde et al., 2005).

The hedgehog system was first identified in Drosophila as responsible for the segment polarity (Nusslein-Volhard and Wieschaus, 1980; Tabata et al., 1992), and later, three highly conserved (Wijgerde et al., 2005) ligands (Shh, Dhh and Ihh) were detected in the ovary as responsible for stem cell development. Indeed, excessive Hh signal transduction may lead to division of the somatic stem cell into two daughter stem cells (Zhang and Kalderon, 2000, 2001) and stimulation of germ cell (King et al., 2001) proliferation. In addition, the ligands Ihh, Dhh (Russell et al., 2007; Wijgerde et al., 2005) and Shh (Russell et al., 2007), and their receptors (Ptch1 and Ptch2) mRNA and proteins were detected in immature and adult ovaries, and murine granulosa cells (Russell et al., 2007; Wijgerde et al., 2005).

As mentioned previously, the hedgehog signaling is involved in regulating cell proliferation and survival, determining cell fate, differentiation, and polarity of embryonic cells (Ingham and Kim, 2005; Ingham and Placzek, 2006). In mouse atretic follicles, expression of Ihh, Dhh, and Ptch1 was rapidly lost (Wijgerde et al., 2005), further supporting the hypothesis that Hh affects cell fate in mammals and Drosophila (Forbes et al., 1996; Zhang and Kalderon, 2000, 2001). Russell at al. (2007) detected Hh ligands in murine granulosa cells of antral and small follicles in variable amounts, and found that Ihh stimulates mitogenesis of murine granulosa cells. Shh increased murine follicle diameter above control after 4 days of culture (Russell et al., 2007), suggesting a control of Ihh during follicular development. Hh controlled cell fate in ovarian

Drosophila cells (Forbes et al., 1996; Zhang and Kalderon, 2000), further indicating a possible involvement of hedgehog proteins and Ptch1 in atresia.

Recently, uterine expression and signaling patterns of the hedgehog system were shown to be modulated by progesterone in mouse (Lee et al., 2006; Takamoto et al., 2002), and hamster (Khatua et al., 2006) uteri. However, no current reports have studied the hedgehog system, or have identified how the hedgehog is involved in the regulation of ovarian function in mammalian cells.

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CHAPTER III

IGF Type II Receptor Gene Expression in Granulosa And Theca Cells of Cattle Selected for Twin Ovulations And Births[1]

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Running Title: Granulosa and Theca Cell IGF2R in Twinner cattle

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ABSTRACT

Regulation of multiple ovulations in monotocous species such as cattle is not well understood. The IGF1 and IGF2 play a major role in controlling ovarian mitogenesis and steroidogenesis, however the role of the IGF type 2 receptor and the gonadotropin receptors and their involvement in ovarian follicle selection is not well understood. Therefore, gene expression of the IGF type II receptor (IGF2R) and LH receptor (LHR) in granulosa (GC) and theca (TC) cells as well as estradiol (E2) and progesterone (P4) levels in follicular fluid (FF) were quantitated in cows selected (Twinner) and unselected (Control) for multiple ovulations and twin births. Cows were slaughtered at day 3 (D3) and day 5 (D5) of an estrous cycle, and ovaries collected. Individual follicles were snap frozen in liquid nitrogen, and FF, GC and TC separated and collected. Total RNA was extracted from GC and TC, and LHR, FSHR, CYP17A1 and IGF2R mRNA were quantified using multiplex real-time RT-PCR and expressed as relative mRNA abundance normalized to constitutively expressed 18S ribosomal RNA. Follicles were classified as Estrogen-Active (E-A; E_2:$P_4 \geq 1$) and Estrogen-Inactive (E-I; E_2:$P_4 < 1$), and the largest three follicles (F1, F2, and F3) based on their follicle diameter (Di) and estrogenic profile were identified and compared. E-A follicles had greater E_2 (164.6 ± 13.8 vs. 3.7 ± 11.2 ng/mL) and lower P_4 levels (27.0 ± 18.1 vs 90.8 ± 14.1 ng/mL) than E-I follicles, and expression of granulosa FSHR mRNA was greater ($P < 0.05$) of D3 versus D5 healthy follicles, and in Control versus Twinner cows at D3. Levels of granulosa or thecal LHR mRNA in E-A follicles did not differ ($P > 0.10$) between Control versus Twinner cows. Granulosa IGF2R mRNA was lower ($P > 0.10$) in E-A

versus E-I follicles, and lower ($P < 0.05$) in GC at D5 of an estrous cycle. Within the largest three follicles, Di was greater in Twinner versus Control cows and F2 in Twinner cows had the same ($P > 0.10$) Di as F1 in Control cows. Granulosa and thecal LHR mRNA did not differ ($P > 0.10$) between F1 and F2, but granulosa LHR mRNA was lower in F3 of Twinner than Control cows. Granulosa IGF2R mRNA was greater in Control than Twinner cows at D3 and D5 of an estrous cycle; F2 had greater ($P<0.05$) thecal IGF2R mRNA than F1 and F3 of Control and F1, F2 and F3 of Twinner cows. Because IGF2R sequesters free IGF2 and, thus, reduces its binding to the IGF type I receptor, we hypothesize that reduced thecal IGF2R levels in follicles of Twinner cows may increase the amount of free or bioavailable IGF2, which may act in an autocrine or paracrine fashion to stimulate development of a second dominant follicle.

INTRODUCTION

In the ovary, follicular development is governed by a series of changes in endocrine and paracrine factors, among which insulin-like growth factor-1 (IGF1) plays a major role. The IGF system is composed of two ligands (IGF1 and -2), two receptors (IGF type 1 and 2 receptor), six binding proteins (IGFBP-1 through -6) and several IGFBP proteases (i.e., PAPP-A and KLKs) (LeRoith, 1991; LeRoith and Raizada, 1989; Raizada and LeRoith, 1991). IGF1 stimulates both steroidogenesis and mitogenesis within the bovine follicle (Spicer et al., 1993; Spicer and Chamberlain, 1998). Intrafollicular IGF2 levels are greater than those of IGF1 (Spicer and Echternkamp, 1995; Spicer et al., 2004; Stewart et al., 1995), and thus IGF2 along with IGF1 modulates

ovarian folliculogenesis (Gong et al., 1993; Spicer et al., 1993). Since biological action of both IGF1 and IGF2 are mediated via the IGF type 1 receptor (IGF1R) (Adashi et al., 1990), and since IGFBPs modulate the IGF effect by sequestering IGF1 or -2 and preventing degradation and signaling of these growth factors, any increase in intrafollicular levels of IGF1 (mainly from plasma) or IGF2 (locally produced) can have a great impact on follicular growth. The Mannose-6 phosphate / IGF type 2 receptor (IGF2R), described as a membrane-bound IGF2 binding protein (LeRoith, 1991), acting as an IGFBP and modulating intrafollicular IGF2 levels, is not well studied in the ovary. Furthermore, the involvement of IGF2R in the control of long-term selection for twin ovulations is not yet clear.

At the Meat and Animal Research Center (MARC), long term genetic selection of beef cattle for multiple ovulations and twin births (Gregory et al., 1990) resulted in an 60 % increase in ovulation rate (Echternkamp et al., 1990a; Echternkamp et al., 1994; Echternkamp et al., 2007; Van Vleck et al., 1991) and the selection of two or more dominant follicles within a follicular wave (Echternkamp, 2000). The MARC dizygotic population shows the same gonadotropin levels as the multibreed composite Control population (Echternkamp et al., 1994) and, thus, the selection of multiple follicles within an estrous cycle is likely due to differential expression of other factors, such as gonadotropin receptors. In these cattle, Echternkamp et al. (1990b) identified IGF1 to be greater in follicular fluid and plasma of cattle selected for dizygotic twin births and later found that twinning cattle have greater intrafollicular IGFBP-5 and lower IGFBP-4 levels (Echternkamp et al., 2004), thus indicating that IGFBPs may contribute to the regulation of intrafollicular IGF levels. The role of IGF2R in modulating IGF2 levels, subsequently

regulating follicular growth, atresia and selection of a dominant follicle has not been elucidated. In addition, the study of the IGF2R in the dizygotic twinning model at the MARC may help further decipher the involvement of IGF2R as well as IGF2 in the selection of two, rather than one follicle in the estrous cycle of Twinner cows. The objective of the present study was to evaluate whether long term selection for dizygotic ovulations altered the gonadotropins (FSH and LH) receptors and (or) the IGF type 2 receptor in granulosa and theca cells.

MATERIALS AND METHODS

1. Reagents, Instruments and Consumables

Reagents

Reagents used for sample collection, RNA extraction and quantification were: RNA*later* reagent from Ambion Inc. (Austin, TX); TRIzol reagent from Invitrogen (Carlsbad, CA); Chloroform from Sigma Chemical Co. (St. Louis, MO); Isopropyl alcohol (Pierce Chemical Company (Rockford, IL); TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 7.0) and RiboGreen® RNA Quantitation Reagent and Kit from Molecular Probes (Eugene, OR); TaqMan® One-Step RT-PCR Master Mix Reagents Kit and TaqMan® Ribosomal RNA Control Reagents from Applied Biosystems Inc. (Foster City, CA).

Instruments

Instruments used were: table top eppendorf centrifuge 5417C (Brinkmann Instruments, Westbury, NY), Omni TH tissue homogenizer (Omni International Inc., Marietta, GA) with disposable probes for tissue homogenization; NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and fluorescent plate reader Wallac 1420 (Perkin-Elmer, Boston, MA) for RNA quantitation; and ABI 7500 real-time PCR machine (Applied Biosystems Inc., Foster City, CA) for quantitative RT-PCR gene expression.

Consumables

Consumables included: Omni Tip™ disposable generator probes from Omni International Inc. (Marietta, GA), eppendorf tubes, 96-well microplates (Proxiplate™-96F, Packard Bioscience BV, Meridian, CT), and MicroAmp™ optical 96-well reaction plate with barcode and optical adhesive films (Applied Biosystems Inc., Foster City, CA).

2. Experimental Design

Ovaries of 15 MARC I, II and III (Control) cows and 15 cows with the propensity for twin births (Twinner) were evaluated transrectally by real-time ultrasonography using a 7.5 MHz linear-array probe, Aloka 500 instrument(Corometrics Medical Systems, Wallingford, CT) to determine functional corpora lutea (CL) and to record the follicular population. Cows with a corpus luteum were injected with PGF2α (30 mg), and ultrasounded 2 days prior to slaughter to confirm ovulation and record follicular

populations. Cows were slaughtered (no more than 6 cows per day) at follicle recruitment (day 3-4; D3) or deviation (day 5-6; D5) of the estrous cycle. Ovaries were immediately recovered and transported on ice to the laboratory. Up to 10 of the largest antral follicles (>4 mm in diameter) per pair of ovaries were excised from the ovaries and individually snap frozen and stored in liquid nitrogen. Frozen follicles were processed as described in the next section.

3. Ovarian Cell Collection

Each follicle was cut into two halves while still frozen; the first half was kept frozen and stored at -80 °C, while the other half was used for granulosa and theca cell isolation. The outer follicular wall was slightly thawed in order for the frozen follicular fluid and granulosa cells to be removed as described previously (Murdoch et al., 1981). Follicular fluid was then thawed by incubation at 37 °C for 5 min then centrifuged at 1000 x g at 4 °C for 5 min. The follicular fluid supernatant was transferred into clean eppendorf tubes and stored at -20 °C until hormonal assays. The granulosa cell pellet was lysed in 0.5 mL TRIzol reagent (Invitrogen, Carlsbad, CA), vortexed and incubated for 5 min at 37 °C, then stored at -80 °C until RNA extraction. Theca cells were peeled out from the thawed hemi-follicle and suspended in 0.5 mL of RNA*later* at 4 °C overnight, then stored at -80 °C until RNA extraction (See below).

4. Hormonal Assays

Estradiol (E_2) and progesterone (P_4) levels in follicular fluid were determined by radioimmunoassay (RIA) as previously described (Spicer and Enright, 1991; Stewart et al., 1996). The intraassay coefficient of variation was 10% for the progesterone RIA, and 12% for the E_2 RIA. The E_2: P_4 ratio in follicular fluid was calculated by dividing E_2 concentrations by P_4 concentrations. This ratio was used to assess the health status of the follicles (Ireland and Roche, 1982, 1983a, b), where follicles with E_2: P_4 >1 were considered healthy estrogen active (E-A) follicles where granulosa cells had few pycnotic cells and follicles with E_2: P_4 < 1 were considered atretic estrogen inactive (E-I) follicles (Ireland and Roche, 1983a). Two Control cows had no E-A follicles, and one other had no functional CL and therefore were removed from all subsequent analyses.

5. RNA Extraction and Quantitation

RNA extraction from granulosa cells. RNA from granulosa cell samples was extracted in 13 batches with an average of 13 samples per extraction batch. Each batch consisted of an equivalent number of samples from each treatment group, and a similar number of different size follicles. Granulosa cells (in 0.5 mL of TRIzol and stored at -80 °C) were thawed, and vortexed (15 sec) after adding 0.10 mL chloroform to each sample. After a 2 to 3 min incubation at 25 °C, samples were centrifuged (3500 x g) at 4 °C for 30 min using table top eppendorf centrifuge 5417C (Brinkmann Instruments, Westbury, NY). The upper aqueous phase was transferred to a new eppendorf tube and RNA was

precipitated with 0.25 mL of isopropanol and samples gently mixed by inversion. RNA was pelleted after a 10 min incubation followed by a centrifugation at (3500 x g) at 4 °C for 10 min. The RNA pellet was washed twice with 0.5 mL of 70% ethanol and allowed to dry for 6-10 min at room temperature. The RNA pellet was dissolved in 0.03 mL of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 7.0) and stored at -80 °C until RNA analysis.

RNA extraction from theca cells. Theca cells were extracted in 8 batches, with an average of 20 samples per extraction batch. Each batch consisted of an equivalent number of samples from each treatment group, and a similar number of different size follicles. Theca cells stored in 0.5 mL of RNA*later* (Ambion, Inc., Austin, TX) were transferred into 0.75 mL of TRIzol reagent. Samples were homogenized for 2-3 min on ice using the Omni TH tissue homogenizer (Omni International Inc., Marietta, GA) with Omni Tip™ disposable generator probes to prevent sample carryover and cross contamination between treatments. Samples were then treated as described above for RNA extraction using 0.15 mL of chloroform and 0.375 mL of isopropanol. The RNA pellet was washed twice with 0.75 mL of 70% ethanol, dissolved in 0.03 mL of TE buffer, and stored at -80 °C until RNA analysis.

RNA quantitation. RNA was quantitated by spectrophotometry at 260 nm using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and via ultra-sensitive fluorescent nucleic acid staining using RiboGreen® following manufacturer's specifications with modifications. Aliquots of RNA and RNA standard (1000 μg/mL) were diluted 50 fold with 1x TE buffer (supplied in the Kit) and the RiboGreen® RNA

quantitation reagent was diluted 200-fold. A standard dilution curve was then generated from the 50-fold diluted RNA standard to the following final concentrations: 1000, 750, 500, 250, 100, 75, 25, 10, and 0 ng/mL. The RNA sample and standard were pipetted into 100 μL 96-well black microplates (Proxiplate™-96F, Packard Bioscience BV, Meridian, CT), followed by the aqueous working solution of RiboGreen® RNA quantitation reagent. After 5 min dark incubation at 25 °C, RiboGreen-bound RNA fluorescence was determined via top reading using a Wallac 1420 (Perkin-Elmer, Boston, MA) at 500 nm maximum excitation and 525 nm maximum emission. The fluorescence value of the reagent blank (0 ng/mL RNA) was subtracted from each of the samples. Fluorescence of the standard RNA was plotted against its corresponding concentrations. Given the adjusted fluorescence value of the samples, RNA concentrations were determined. The intra-assay coefficient of variation was 10%.

6. Primers and Probe Design

Primers and probes for quantitative RT-PCR were designed using Primer Express™ software with the following manufacturer's restrictions: primers melting temperature (Tm) was set to 50 to 60°C, whereas the probe's Tm was at least 10°C higher. The GC base pair content for the primers and probe was between 20 % to 80 % to avoid runs of identical nucleotides. The length of the strand was between 20 and 30 nucleotides. Sequences of the target genes (TG) primers and probes are listed in Table 3.1, along with the corresponding accession numbers, melting temperatures and transcript size. Also the design of an exon spanning primer and/or probe is designated by E-E for

exon-exon junction. All designed probes were synthesized with a 5' FAM reporter dye and a 3' TAMRA quencher dye (TaqMan® TAMRA™, Applied Biosystems Inc., Foster City, CA), except the probe for the internal Control (TaqMan® Ribosomal RNA Control Reagents with a supplied VIC™ Probe; Applied Biosystems Inc., Foster City, CA). A no template Control and a no reverse transcriptase Control were included to insure the lack of contaminants in the master mix and the absence of any genomic DNA contamination, respectively.

A "short, nearly exact matches" BLAST query search (http://www.ncbi.nlm.nih.gov/BLAST) was also conducted to insure the specificity of the designed primers and probes and to assure that they were not designed from any homologous regions, coding for other genes. Furthermore, the RT-PCR product generated as described in the next section was ran on a 3% agarose gel (AMRESCO Inc., Solon Industrial, OH) with 0.5 μg/mL ethidium bromide and a lane with PCR markers (Promega, Madison, WI) in a DNA size standard of 50 bp to 1000 bp. The observed imaging verified the length and size of the target gene. The PCR product from each gene was cleaned from the agarose gel using the Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA) following the manufacturer's instructions. Briefly, the bands on the agarose gel were excised, excess agarose gel discarded, bands were incubated at 50°C for 5 min dissolved in 0.3 mL of agarose-dissolving buffer, and transferred to Zymo Spin I column collection tubes. Following centrifugation at 15000 x g for 30 sec, the flow-through was discarded and the column washed twice with 200 μL of wash buffer-ethanol mix and flow-through discarded again. The PCR products caught in the column were then eluted with water after spinning the column in a 1.5 mL eppendorf tube at 15000 x g

for 1 min. These purified PCR products were quantitated using the ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE), and then sequenced via "BigDye™"-terminated reactions analyzed on an ABI DNA Analyzer (Applied Biosystems Inc., Model 3700) to validate the amplified genes.

7. Multiplex Real-Time RT-PCR for mRNA Quantification

The differential expression of target genes (TG) in granulosa or theca cells was quantified using the multiplex one-step real-time RT-PCR (mRT-PCR) for Taqman ® Gold RT-PCR Kit (PE Biosystems, Foster City, CA). Ribosomal 18S RNA (18S rRNA) Control kit (PE Biosystems, Foster City, CA) was used as internal Control to normalize samples for the variation in amounts of RNA loaded. The large number of samples required the use of real-time mRT-PCR. A main advantage of real-time mRT-PCR was a more accurate correction for any loading error, mainly due to the elimination of preparing a different sample and an extra pipetting of the internal Control. The amplification of each TG was validated separately. The combinations of primers for each TG and the 18S were tested to identify the concentrations that would give optimal amplifications (Bustin, 2004) in the real-time PCR reaction. In addition, 50 or 100 ng of total RNA was tested to verify the validity of the amplification with variable amounts of RNA in our multiplex system. Both concentrations of RNA resulted in similar amplification patterns for all concentrations of TG:18S primer combinations.

For the multiplex optimization, five TG to 18S ratios were considered for both the forward and reverse primers as following: 1:1/2, 1:1/4, 1:1/10, 1:1/20, and 1:1/200; i.e.

concentrations of TG to 18S primers of 200:100 nM, 200:50 nM, 200:20 nM, 200:10 nM and 200:1 nM, respectively. Coincidently, the optimal combination of TG: 18S for all measured genes in this chapter was 200:20 nM of TG to 18 S primers respectively; probes were used at 100 nM for both TG and 18S. A 50 or 100 ng of total sample RNA in each mRT-PCR reaction showed consistent amplification patterns that follow the typical sigmoid logarithmic amplification of PCR (Bustin, 2004), and therefore were selected for the mRT-PCR of experimental samples. Furthermore, separate amplification reactions of both the TG (primers at 200 nM and probe at 100 nM) and 18S rRNA (primers at 100 nM and probe at 100 nM) verified that the amplifications of both TGs and 18S rRNA were not being compromised or affected by their co-amplification in real-time mRT-PCR. The first time an RNA sample was amplified, a no reverse transcriptase reaction on a pool of maximum 6 samples confirmed the absence of genomic DNA amplification; in addition, a no template control confirmed the absence of reagents contamination every time these reagents were used.

A serial dilution of RNA samples ranging from 500 ng to 1 ng total RNA were amplified with the real-time mRT-PCR optimized reaction conditions to further validate the use of 18S as internal Control. Two master mixes were used to test optimal reaction conditions of 200:20 nM TG to internal Control primers concentrations. Those master mixes consisted of 200 nM of TG-FWD, 200 nM of TG-REV, 100 nM of TG-Probe, 20 nM of rRNA-FWD, 20 nM of rRNA-REV, 100 nM of rRNA-Probe with 12.5 μL of TaqMan Master Mix without uracil N-glycosylase, and 1U Multiscribe with RNase inhibitor (Applied Biosystems Inc., Foster City, CA). The total reaction volume was 25 μL, including 5 μL of RNA (at 20 or 10 ng/μL), and DEPC-treated water. Thermal

cycling conditions were set to 30 min at 48.8ºC for reverse transcription, 95ºC for 10 min for AmpliTaq Gold activation, and finished with at least 50 cycles at 95ºC for 15 sec for denaturing and 60ºC for 1 min for annealing and extension. When the cycle threshold (Ct) values were plotted against the normal logarithm (Ln) of the RNA concentrations, regression lines across TG Ct and 18S Ct were parallel for the chosen concentrations and reaction conditions, further verifying the chosen mRT-PCR conditions were optimal for real-time mRT-PCR reaction.

For experimental samples 50 or 100 ng of total RNA was amplified in a total reaction volume of 25 μL consisting of: 200 nM forward primer, 200 nM reverse primer, 200 nM FAM/TAMRA fluorescent labeled probe for the target gene, 20 nM of the supplied 18S forward and reverse primers, 100 nM of the 18S probe labeled with VIC/TAMRA dyes, 12.5 μL of TaqMan Master Mix without uracil N-glycosylase, and 1U Multiscribe with RNase inhibitor (Applied Biosystems Inc., Foster City, CA); the volume was added up with DEPC-treated water. Thermal cycling conditions were set to 30 min at 48.8ºC for reverse transcription, 95ºC for 10 min for AmpliTaq Gold activation, and finished with greater than 45 cycles at 95 ºC for 15 sec for denaturing and 60 ºC for 1 min for annealing and extension. Because the total number of samples was greater than the 96 well-plate capacity, samples on each plate were blocked by genotype and cycle, with all follicles from one individual cow on the same plate; all individual samples were run in duplicate.

Quantification of gene expression was done by setting an arbitrary threshold (Ct) on the FAM or VIC curves in the geometric portion of the RT-PCR amplification plot

after examining the log view. Relative quantification of target gene mRNA was expressed using the comparative threshold cycle method (Livak and Schmittgen, 2001; Spicer and Aad, 2007; Voge et al., 2004a; b). Briefly, the ΔCt was determined by subtracting the 18S Ct value from the target unknown value. Within each experiment, the ΔΔCt was determined by subtracting the higher ΔCt (the least expressed unknown) from all other ΔCt values. Relative adundance in mRNA expression (Fold change) was calculated as being equal to $2^{-\Delta\Delta C_t}$ (Bustin, 2004; Voge et al., 2004a; b).

8. Statistical Analysis

Data were analyzed as a completely randomized design with a 2x2x2 factorial treatment structure. Main effects were genotype (Control or Twinner), day of cycle (D3, or D5), and follicle status (E-A: estrogen active E_2:$P_4 \geq 1$, or E-I: estrogen inactive E_2:$P_4 < 1$). The treatment effects on the dependent variables (e.g., E_2 concentration, TG mRNA levels) were determined using ANOVA and the mixed model procedure of SAS for Windows (version 8.02, SAS Institute Inc., Cary-NC, USA, 1999-2001). Outliers were detected according to the procedure described by Grubbs (1950). To correct for heterogeneity of variance, steroids and TG mRNA were analyzed after transformation natural ln (x + 1). Differences in main effects were considered only when the 2- and 3-way interactions were not significant ($P > 0.05$). Mean differences were determined by Fisher's protected least significant differences test, if significant treatment effects in ANOVA were detected. The slice option in the LSMeans statement of SAS was used to separate the mean differences when interactions were significant ($P < 0.05$). Results are

presented as the least square means (LSMeans) ± SEM. Main effects means are presented in tables and significant differences are indicated only when there are no interactions. All graphs show the LSMeans of the 2x2x2 treatment combinations whether interactions are significant ($P \leq 0.05$) or not ($P > 0.05$).

To observe the correlations between the different parameters analyzed and to determine their role and how they affect the follicular cycle, all parameters were correlated against each other using the correlation (Pearson) procedure of SAS. Correlations were considered significant when $p < 0.05$.

Finally, to determine the relationships among follicle diameter and other variables measured (E_2, P_4, TG mRNA), data for each of these parameters were regressed against Diameter (Di) and slopes tested for deviation from "zero" (x axis) for each genotype using regression analysis procedure of SAS and regression equations were generated. The regression equation slope and intercept deviation from zero were tested by assigning a dummy variable to the genotype levels in the regression procedure of SAS.

To further determine the characteristics of follicle dominance, follicles were classified by diameter (Di) and E_2:P_4 ratio and assigned numbers 1, 2, or 3 based on their theoretical dominance, first by estradiol concentration, followed by follicular size, given that FFL progesterone concentration did not exceed 390 ng/mL. In both cattle populations, F1 was always E-A, F2 and F3 in Control cows were mostly E-I whereas F2 and/or F3 in Twinner cows were predominantly E-A. Data from the largest three follicles were analyzed using the mixed procedure of SAS with genotype, cycle, and follicle as main effects, and cow included as a random effect; the model included all 2- and 3-way

interactions. As mentioned earlier, main effects were only observed when the interactions were not significant. Correlation and regression analysis were conducted as described earlier and results confirmed the original analysis that included all follicles from individual cows.

RESULTS

1. Classification of Follicles by their Estrogenic/Progestagenic Profile

Ratio of estradiol to progesterone, reported earlier to determine the health status of the follicle (Ireland and Roche, 1983b), was significantly ($P < 0.0001$) different between classified estrogen-active (E-A) and estrogen-inactive (E-I) follicles. Average E_2:P_4 ratio was 0.12 ± 0.33 in E-I versus 6.62 ± 0.41 in E-A follicles. The E_2:P_4 ratio was not affected ($P > 0.1$) by genotype, day of cycle, or any of the analyzed 2- or 3-way interactions.

2. Diameter, Estradiol and Progesterone in Follicles Collected at Day 3 or 5 Following PGF2α from Control Cows or Cows Selected for Twin Ovulations and Births

Follicle diameter. There was a significant ($P < 0.01$) status by day of cycle interaction ($P < 0.01$) on follicular size (Table 3.2), where E-A follicles had a larger

diameter ($P < 0.001$) than E-I follicles at both D3 and D5 (Fig 3.1). No differences in diameter were observed ($P > 0.1$) between Control and Twinner cows (Table 3.2).

Estradiol. Follicular fluid E_2 levels (Table 3.2) did not differ ($P > 0.1$) between Twinner and Control cows or between follicles at D3 or D5 of an estrous cycle and averaged 84.13 ± 13.6 ng/mL however, E-A follicles had 45-fold greater estradiol level than E-I follicles.

Progesterone. Follicular fluid P_4 levels were 3.4-fold greater ($P < 0.01$) in E-I than E-A follicles, with no observed differences ($P > 0.5$) in P_4 levels between Twinner and Control cows or between follicles at D3 or D5 of an estrous cycle (Table 3.2).

3. Gene Expression for FSHR, LHR and IGF2R in Granulosa Cells

Multiplex real-time RT-PCR (mRT-PCR) was used to amplify, in granulosa cells, genes for FSH receptor (FSHR), LH receptor (LHR) and IGF type 2 receptor (IGF2R) (Table 3.3).

Granulosa cell FSHR gene expression. There was a genotype by day of cycle ($P < 0.01$) and a day of cycle by status ($P < 0.01$) interactions on FSHR gene expression in granulosa cells (Table 3.3). FSHR mRNA was greater ($P < 0.05$) in Control cows at D3 than D5 of an estrous cycle, whereas there was no difference ($P > 0.8$) in FSHR mRNA in granulosa cells of Twinner cows between D3 and D5 (Fig. 3.2A). Further, FSHR mRNA was greater in Control than Twinner cows mainly a D3 of an estrous cycle (Fig

3.2A). In addition, FSHR mRNA in E-I follicles did not differ between follicles at D3 or D5, whereas E-A follicles had greater FSHR mRNA than E-I follicles (Fig. 3.2B).

Granulosa cell LHR gene expression. There was a significant ($P < 0.05$) genotype by status by day of cycle interaction on LHR gene expression in granulosa cells (Table 3.3). Using the slice option of SAS, no difference existed between Twinner and Control Cows within E-A follicles at D3, whereas within Control cows, E-A follicles had lower ($P < 0.05$) GC LHR mRNA than E-I follicles (Fig. 3.3). Within Twinner cows, there was no change in GC LHR mRNA abundance from D3 to D5, and no difference between E-I and E-A follicles (Fig. 3.3).

Granulosa cell IGF2R gene expression. IGF2R mRNA in theca cells did not differ ($P > 0.10$) in granulosa cells between Twinner and Control cows (Table 3.3), but E-I follicles had 1.2-fold greater ($P < 0.05$) abundance of IGF2R mRNA than E-A follicles (Fig. 3.4 A), whereas D3 follicles had 1.6-fold greater ($P < 0.01$) IGF2R mRNA abundance than D5 follicles (Fig. 3.4 B).

4. Gene Expression for LHR, IGF2R and CYP17A1 in Theca Cells

Multiplex real-time RT-PCR (mRT-PCR) was used to amplify the theca cell genes for LH receptor (LHR), IGF type 2 receptor (IGF2R) and 17 alpha hydroxylase/17-20 lyase (CYP17A1) gene expression (Table 3.4).

Theca cell LHR gene expression. In theca cells, LHR mRNA abundance did not differ ($P > 0.6$) between Twinner and Control cows (Table 4). Also, no difference ($P > 0.8$) in LHR mRNA abundance was observed between follicles at either D3 or D5 of the estrous cycle, or between E-I and E-A follicles ($P > 0.25$) (Table 3.4).

Theca cell IGF2R gene expression. In theca cells, IGF2R mRNA abundance did not differ ($P > 0.15$) between Twinner and Control cows (Table 4). Also, no difference ($P > 0.71$) in IGF2R mRNA abundance was observed between follicles at either D3 or D5 of the estrous cycle, or between E-I and E-A follicles ($P > 0.43$) (Table 3.4).

Theca cell CYP17A1 gene expression. In theca cells, CYP17A1 mRNA abundance did not differ ($P > 0.6$) between Twinner and Control cows (Table 4). Also, no difference ($P > 0.6$) in CYP17A1 mRNA abundance was observed between follicles at either D3 or D5 of the estrous cycle ($P > 0.6$), or between E-I and E-A follicles ($P > 0.1$) (Table 3.4).

5. Regression Analysis of Follicular Variables

Follicular fluid E_2 (Fig. 3.5) and P_4 (Fig. 3.6) concentrations relative to follicular diameter did not differ ($P > 0.10$) in Control cows as compared to Twinner cows. Within both genotypes, concentrations of E_2 increased ($P < 0.05$) but P_4 was not associated ($P > 0.10$) with follicle diameter. Granulosa FSHR mRNA abundance (Fig. 3.7) relative to follicular diameter differed ($P < 0.05$) in Control cows as compared to Twinner cows. In Control cows, granulosa FSHR mRNA abundance increased ($P < 0.05$) with an increase

in follicle diameter whereas in Twinner cows, granulosa FSHR mRNA was not related ($P > 0.10$) to follicle diameter. Abundance of granulosa LHR mRNA (Fig. 3.8) and IGF2R mRNA (Fig. 3.9) relative to follicular diameter were similar ($P > 0.10$) for Control and Twinner cows. The regression between abundance of granulosa LHR mRNA (Fig. 3.8) and follicle diameter was not significant ($P > 0.10$), whereas abundance of granulosa IGF2R mRNA (Fig. 3.9) relative to follicular diameter decreased ($P < 0.01$) with increased follicle diameter.

Abundance of thecal LHR mRNA (Fig. 3.10) relative to follicular diameter did not differ ($P > 0.01$) in Control cows as compared to Twinner cows. Abundance of theca LHR mRNA did not change with ($P > 0.10$) with increased follicle diameter. In contrast, thecal IGF2R mRNA abundance (Fig. 3.11) relative to follicular diameter was greater ($P < 0.01$) in Control cows as compared to Twinner cows. Thecal CYP17A1 mRNA abundance (Fig. 3.12) relative to follicular diameter differed ($P > 0.01$) in Control cows as compared to Twinner cows. In Control cows, abundance of thecal CYP17A1 mRNA decreased ($P < 0.05$) with increased follicle diameter, whereas in Twinner cows, CYP17A1 mRNA abundance increased ($P < 0.05$) with increased follicle diameter (Fig. 12).

6. Pearson's Correlations

Pearson correlations were used to observe the relationships between the herein measured parameters (Table 3.5). E_2 and P_4 concentrations were positively correlated (r

= 0.69 and r = 0.24 respectively; $P < 0.001$) to follicular diameter. In granulosa cells, FSHR mRNA abundance was correlated positively (r = 0.38; $P < 0.001$) to E_2, negatively to P_4 (r = -0.21, $P < 0.05$), and positively (r = 0.27; $P < 0.001$) to health of the follicle (E_2:P_4 ratio). Granulosa LHR mRNA abundance was positively correlated (r = 0.18; $P < 0.05$) to E_2:P_4 ratio whereas thecal LHR mRNA was positively correlated to abundance of thecal IGF2R mRNA and CYP17A1 mRNA (r = 0.64 and r = 0.38 respectively; $P < 0.001$). Granulosa IGF2R mRNA abundance was negatively correlated to Di, E_2 and E_2:P_4 ratio (Table 3.5).

7. Size, Estradiol, Progesterone and Gene Expression for FSHR, LHR, IGF2R and /or CYP17A1 in the Largest Three Follicles

Diameter. We identified the largest three follicles (F1, F2, and F3) within each Control or Twinner cow based on their estrogenic profile, their size and lower than 390 ng/mL P4 levels. Twinner cows had greater ($P < 0.05$) follicles than Controls (Table 3.6), with F1 diameter greater ($P < 0.05$) than F2 which in turn was greater than F3 (Fig. 3.13) within both Control and Twinner cows. Diameter in F2 in Twinner cows did not differ from F1 in Control cows (Fig. 3.13), thus indicating the capacity of F2 in Twinner to be selected and become dominant.

Steroids. Estradiol levels in follicular fluid showed significant genotype ($P < 0.05$) and follicle ($P < 0.001$) main effects (Table 3.6). In both Twinner and Control cows, E_2 levels in F1 were greater than F2, which in tern had greater E_2 levels than F3,

with Twinner cows having 1.6-fold greater E_2 in F1 than Control cows (Fig. 3.14). In the largest three follicles, P_4 levels did not differ ($P > 0.50$) between Control and Twinner cows, and P4 did not differ ($P > 0.10$) between follicles at D3 or D5 of an estrous cycle (Table 3.6). However F3 tended ($P=0.053$) to have greater P_4 levels than F2 (Table 3.6), Androstenedione was measured in follicular fluid of the largest two follicles and showed no genotype, day of cycle or follicle significant ($P > 0.05$) effect (Table 3.6), however large variability between the follicles could have accounted for the lack of significance (Fig 3.15).

Receptor mRNA. Granulosa cell FSHR mRNA abundance did not differ ($P > 0.10$) between Control or Twinner cows (Table 3.7) but was greater ($P < 0.05$) in F1 as compared to F3 (Fig. 3.1). Within these largest three follicles, GC FSHR mRNA abundance was greater ($P < 0.01$) at recruitment (D3) than at deviation (D5) (Fig. 3.17). Granulosa LHR mRNA abundance was not affected ($P > 0.45$) by genotype, day of cycle or follicle (Table 3.7), but theca cell LHR mRNA abundance showed a genotype by follicle significant ($P < 0.05$) interaction. Thecal LHR was greater in F3 of Control as compared to F3 of Twinners (Fig. 3.17). In granulosa cells, IGF2R mRNA abundance, was 3.3-fold greater ($P < 0.05$) in Control than Twinner cows, and 1.4-fold greater ($P = 0.053$) at D3 than at D5 of an estrous cycle (Figure 3.18). The largest 3 follicles had the same ($P > 0.10$) granulosa IGF2R mRNA abunadance (Table 3.7). In a similar manner, Twinner cows had 2.5-fold greater ($P = 0.058$) thecal IGF2R mRNA than control cows, theca cells but did not show a difference ($P > 0.9$) in IGF2R mRNA between D3 and D5 (Table 3.8). On the other hand, F2 had 10-fold greater ($P < 0.05$) thecal IGF2R than F1 (Table 3.8). Interestingly, within Twinner cows, the largest three follicles had the same

level of IGF2R mRNA expression in granulosa cells (Fig. 3.19), whereas F2 of Control cows had 13.5 and 8-fold greater IGF2R mRNA than F1 or F2 of Twinner cows (Fig. 3.19),

CYP17A1 mRNA. Thecal CYP17A1 mRNA abundance did not differ ($P > 0.40$) among the largest 3 follicles, between Control and Twinner cows or at D3 or D5 of an estrous cycle.

DISCUSSION AND CONCLUSION

In this study, we characterize the expression of LHR, FSHR and IGF2R in granulosa and theca cells of follicles from cattle not selected (Control) or selected for multiple ovulations and twin births (Twinner) using sensitive quantitative multiplex RT-PCR at two different stages of the follicular cycle. Further we compared the relationships of follicle diameter and with follicular variables between Control and Twinner cows, and evaluated differences between the largest three follicles (F1, F2, and F3).

For the first time in bovine follicles, we found that IGF type 2 receptor (IGF2R), expressed in both granulosa and theca cells, is downregulated in Twinner versus Control cows, and that IGF2R decreases with increased follicular diameter in granulosa but not theca cells in both Control and Twinner cows. Further, the correlations between IGF2R and the follicular variables denote a potential for IGF2R to be regulated during follicular growth from recruitment to deviation. This study further supports the hypothesis that IGF2R is modulated during bovine antral follicular growth, and that IGF2R may be

involved in the selection of two rather than one follicle in cattle selected for multiple ovulations and Twin Births.

Estrogen-active (E-A)/ healthy follicles from Control cows at D5 had a greater diameter (Di) than their D3 counterparts, but estrogen-inactive (E-I; atretic) follicles were smaller in diameter than estrogen-active (E-A; healthy) follicles. In addition, within the largest three follicles, Di was greater in Twinner versus Control and F2 in Twinner had the same Di as F1 in Control cows. Previously, follicles with E_2:P_4 ratio lower than 1 (E-I) showed a majority of pycnotic granulosa cells , indicative of apoptosis and regression of the follicle, and our current results are consistent with this finding because E-I follicles had no increase in diameter from D3 to D5. On the other hand, healthy (E-A) follicles grow at a rate of about 1 mm per day (Spicer and Echternkamp, 1986; Spicer and Stewart, 1996), and our results indicating an increase in the diameter of E-A follicles between D3 and D5 is consistent with these previous studies. In other studies, follicular diameter was not different among dominant follicles between Control and Twinner cows (Echternkamp et al., 1990a; Spicer and Echternkamp, 1995). The largest follicle in Twinner cows had a size advantage as compared to both the 2nd largest follicle in Twinner cows and the largest follicle in Control cows. Further, our findings that Di of F2 of Twinner cows matches F1 of Control cows may indicate that the F2 follicle is co-dominant in Twinner Cows. Some studies (Wiltbank et al., 2000) indicated that when a follicle in cattle shows a growth advantage to its cohort counterparts, as depicted by an increased size, the follicle becomes dominant and suppresses the growth of the subordinate follicles. In this study, we considered 6 to 10 follicles per cow and our findings suggest that follicular size alone might not be an indicator of follicle dominance

in twinning, rather, follicular size and follicular fluid estradiol concentrations together should be considered when ranking follicular dominance in cattle.

Intrafollicular E_2 level was greater and P_4 level was lower in E-A follicles as compared to E-I follicles, with no difference in the levels of E_2 or P_4 levels between D3 and D5 of a follicular cycle, or between Control and Twinner cows. Similar results were previously observed in this same population when compared to contemporary Controls (Echternkamp, 2000; Echternkamp et al., 1990b) or monovulating Twinner cows. In fact, during the follicular cycle, growing follicles (E-A) produce E_2 in order to increase sensitivity of the follicle to gonadotropins (Ireland and Roche, 1983a). The production of these hormones is a sign of follicular activity and influences the responsiveness of the follicle to intrafollicular stimuli. The greater progesterone levels detected in E-I follicles as compared to E-A follicles, is consistent with previous reports (Wiiltbank et al., 2000).

In this study, we characterized for the first time the expression of receptors for FSH and LH in granulosa cells using multiplex real-time RT-PCR in cattle selected for increased ovulation rate. At recruitment, FSH-stimulated E_2 production depends on the expression of FSHR in the follicle, which sensitizes the follicle and stimulates the production of LHR in granulosa and theca cells (Ginther et al., 2001). With the progression of the estrous cycle, a follicle deviates from the rest of the recruited cohort. What exactly controls the selection of a specific follicle is not very clear, but may depend on how ahead of the cohort in size and responsiveness it is (Wiltbank et al., 2000). Previously, expression of FSH receptor mRNA was detected within secondary follicles (2-3 layers of GC), and increased with increasing follicular size in granulosa cells of healthy follicles (4 to 17 mm) (Bao et al., 1997). In both Control and Twinner cows,

FSHR mRNA decreased from recruitment to deviation, and was greater in F1 as compared to F2 or F3, thus indicating that F1 acquires the ability to respond to FSH earlier than all other follicles, thus contributing to its dominance. However, lower levels of FSHR mRNA at D3 in follicles of Twinner than Control cows at D3 indicate that in Twinner cows, the gap between the two largest follicles and their ability to respond to decreased FSH might be bridged, and thus the increased incidence of selection of two follicles in Twinner cows rather than one follicle in Control cows. Previously, FSHR mRNA expression changed with the stage of the first follicular wave and expression of FSHR mRNA in granulosa cells of atretic follicles was lower than in granulosa cells of healthy follicles (Bao et al., 1997). As previously reported, E-A follicles had greater FSHR than E-I follicles (Ireland and Roche, 1983a) and E2 may enhance FSH-stimulated LHR mRNA expression in rat granulosa cells (Richards et al., 1987), but whether greater E_2 production is a result of or a cause of elevated FSHR will require further study.

FSH plays a pivotal role in the control of follicular development (Baird and McNeilly, 1981; Baird et al., 1981), and the increased ability of a follicle to withstand the decrease in FSH after recruitment (Donadeu and Ginther, 2001; Fortune et al., 2001; Mihm and Austin, 2002; Mihm et al., 2006) is mainly attributed to an increased FSHR in the dominant but not subordinate (Ndiaye et al., 2005) follicle and a switch in follicle gonadotropic dependence from FSH to LH (Campbell, 2003; Campbell et al., 1995). Decreased FSHR mRNA in GC was observed following the gonadotropin preovulatory surge and further increased FSH decreased FSHR mRNA in cultured bovine GC (Ndiaye et al., 2005). However, the physiological processes that allow morphologically similar follicles to differentially respond to the same level and pattern of gonadotropic

stimulation are not well understood (Marsters et al., 2003). In the current study, granulosa cell FSHR mRNA was lower in Twinner than in Control cows at D3 of the follicular cycle, and may indicate a difference in the mechanism of control of follicle development between Control and Twinner cows. Previously, in bovine dominant follicles, FSHR mRNA did not differ between day 2, 4, 6 and 10 of an estrous cycle (Bodensteiner et al., 1996; Xu et al., 1995), and was not changed following E_2-induced atresia of follicles (Burke et al., 2007), but was decreased following the preovulatory LH/hCG surge (Ndiaye et al., 2005). What is regulating the change in FSHR in cattle is unclear, but androgens and not E_2 increased FSHR mRNA in 5 mm follicles cultured in vitro, with no apparent regulation of FSHR mRNA expression by FSH or E2 (Luo and Wiltbank, 2006).

The switch in follicle gonadotropin dependency at selection may be due to an increased expression of LHR, and a decreased expression of FSHR in growing follicles (Donadeu and Ginther, 2001; Fortune et al., 2001; Mihm and Austin, 2002; Mihm et al., 2006). In the dominant follicle, granulosa LHR mRNA did not differ between day 2, 4, 6 and 10 of an estrous cycle (Bodensteiner et al., 1996) and was not changed following E_2-induced atresia of follicles (Burke et al., 2007). Whether the selection of the dominant follicle or expression of LHR occurs first is not very clear, but LHR mRNA was detected in one healthy follicle with a Di of greater than 8 mm in diameter per cow (Bao et al., 1997; Xu et al., 1995). Bao and Gavernick (1998) suggested that LHR mRNA expression is limited to granulosa cells of healthy follicles that express 3β-HSD. In this study, we detected LHR mRNA in healthy and atretic follicles in cattle, expression that did not differ in either granulosa or theca cells with follicular status or day of cycle. Also, in the

current study, granulosa and theca cell LHR mRNA did not differ between control and Twinner cows, but LHR mRNA was greater in GC of atretic follicles after D5. Similarly, LHR mRNA in granulosa were less in atretic versus non-atretic follicles (Ireland and Roche, 1982). Further, in the largest 3 follicles, theca cell LHR mRNA in F3 of Twinner was lower than either F1 or F2 in Twinner or all 3 follilces in Control. Echternkamp et al. (2000) suggested that follicles in Twinners were at a more advanced stage of the cycle than Control cows. In support of this suggestion, lower GC FSHR mRNA and TC LHR mRNA abundance were detected in follicles of Twinner cows in the present study. However, follicles collected at day 1-2 and day 7-8 of an estrous cycle might further clarify the sequence of events as pertaining to follicle development in the Twinner population.

In the current study, thecal CYP17A1 mRNA did not differ between Control or Twinner cows or change between D3 or D5 of an estrous cycle. However, regression analysis revealed that thecal CYP17A1 mRNA decreased with increased follicular diameter in Control, but not Twinner cows. Thecal CYP17A1 mRNA in the largest follicle (F1) in Control cows was lower than in F1 of Twinner and the other follicles (F2 and F3), indicating the lag in follicular development between Control and Twinner cows and mirroring the increase in A_4 levels in F1 of Twinner versus Control cows, however the variablility of the measured CYP17A1 mRNA and A4 levels revealed no significant differences. Previously, CYP17A1 mRNA increased in bovine follicles at day 2 and 4 of an estrous cycle following LH microinjections (Manikkam et al., 2001). Similarly, Stewart et al. (Stewart et al., 1996) reported that A4 levels increased between day 5 and 10 of an estrous cycle. CYP17A1 in small-follicle theca cells was increased by IGF1 and

LH but not by GDF9 in vitro (Spicer et al., 2008). In addition, E_2-induced atresia, accelerated loss of CYP17A1 mRNA (Burke et al., 2007). Increased local bioavailable IGF1 in Twinner cows might account for the increased CYP17A1 mRNA abundance in F1 and the greater FFL A_4 levels in this population.

In the MARC twinning population, specific genes responsible for controlling ovulation rate in cattle have not yet been identified. QTL analysis of this population showed regions likely to include genes involved in the regulation of increased ovulation rate and the potential control of follicular development and recruitment. These candidate regions were localized to multiple chromosomes including chromosomes 5 (Kappes et al., 2000), 7 (Blattman et al., 1996), 13, 15, 19 (Erickson and Danforth, 1995; Komisarek and Dorynek, 2002), and 23 (Blattman et al., 1996); genes involved in the regulation of follicular development such as IGF1 and GDF-9 (Spicer et al., 2006; Spicer et al., 2008; Spicer et al., 1993; Spicer and Echternkamp, 1986) are localized, in cattle, on chromosomes 5 and 7, respectively. However, in these genomic scan studies, only the highest peaks for QTLs were selected (Kappes et al., 2000), thus possibly missing other regions of importance in twinning and ovulation rate such as those involved in controlling twinning in sheep (e.g., BMP15; Davis, 2005); activin-like kinase 6 or BMPR1B (McNatty et al., 2005; McNatty et al., 2001). However, the involvement of such genes in twinning in cattle requires further investigation.

Consistent findings by Echternkamp and colleagues (Echternkamp, 2000; Echternkamp et al., 2004; Echternkamp et al., 1990b) point to the importance of the IGF system and its involvement in increased ovulation rate. Heritability of IGF1 has been documented in mice (Blair et al., 1989) and cattle (Davis and Simmen, 2006).

Inheritance of the components of the IGF system and the insulin signaling pathway include imprinted genes such as IGF2 and IGF2R as well as non-imprinted genes such as IGF1 and IGF1R (Feil, 2001; Xing et al., 2007). Imprinting is an epigenic mechanism where gene expression exclusively occurs from only one of the parents' genome (for review, see Dean et al., 1998; Feil, 2001). Imprinting of IGF2R (Feil and Khosla, 1999; Yang et al., 2005), or the loss of (Feil, 2001) has been linked to various diseases and lower survivability of embryos generated by assisted reproductive techniques (Feil, 2001; Feil and Khosla, 1999). The IGF2 gene is a paternally imprinted gene in murine (Barlow et al., 1991) and cattle (Killian et al., 2001) whereas the IGF2R is a maternally imprinted gene (Killian et al., 2000), thus the importance of the findings of the current study. Lower IGF2R in Twinner cows is transmitted maternally to the offspring where the characteristics of Twinning are conserved.

IGF type 2 receptor was measured in both granulosa and theca cells from Twinner and Control cows using multiplex real-time RT-PCR. IGF2R was greater at recruitment in Control versus Twinner cows, and was downregulated in theca cells of Twinner cows. The decrease in granulosa IGF2R mRNA abundance in F2 of Twinner cows further strengthens the theory that increased bioavailability of IGF2 in Twinner cows may be driving an increased sensitivity of the 2nd largest follicle in Twinner, so that two, rather than one follicle are selected at every estrous cycle. In chapter 4, we characterized IGF2R in granulosa cells and hypothesized that IGF2R may act as a type of decoy receptor via binding and inactivating IGF2. This latter suggestion was made for bovine theca cells (Spicer et al., 2004) and is further supported by studies of others (Adashi et al., 1990) indicating that the granulosa cell IGF2R does not participate in transmembrane IGF

signaling. In bovine corpora lutea, IGF2R mRNA levels significantly increased during mid- and late-cycle (Hastie and Haresign, 2006b; Neuvians et al., 2003). In ewes, follicular IGF2R mRNA levels measured using in situ hybridization were greater in atretic than healthy follicles and greater in small than large follicles (Hastie and Haresign, 2006a). Further, the lack of regulation of IGF1R mRNA in granulosa cells of healthy follicles in cattle (Stewart et al., 1996; Sudo et al., 2007) strengthens the importance of the differential regulation of IGF2R expression during the follicular cycle.

Because IGF2R sequesters free IGF2 and, thus, reduces its binding to the IGF type I receptor, we hypothesize that reduced thecal IGF2R levels in follicles of Twinner cows may increase the amount of free or bioavailable IGF2, which may act in an autocrine or paracrine fashion to regulate follicular development.

FIGURES

Figure 3.1: Follicular diameter in cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations were collected between D3-4 (D3; recruitment) or D5-6 (D5; deviation) of an estrous cycle, follicles classified as E-A (E_2:$P_4 \geq 1$; healthy) or E-I (E_2:$P_4 < 1$; atretic). * Within day of cycle, mean differs ($P < 0.05$) from its atretic counterpart.

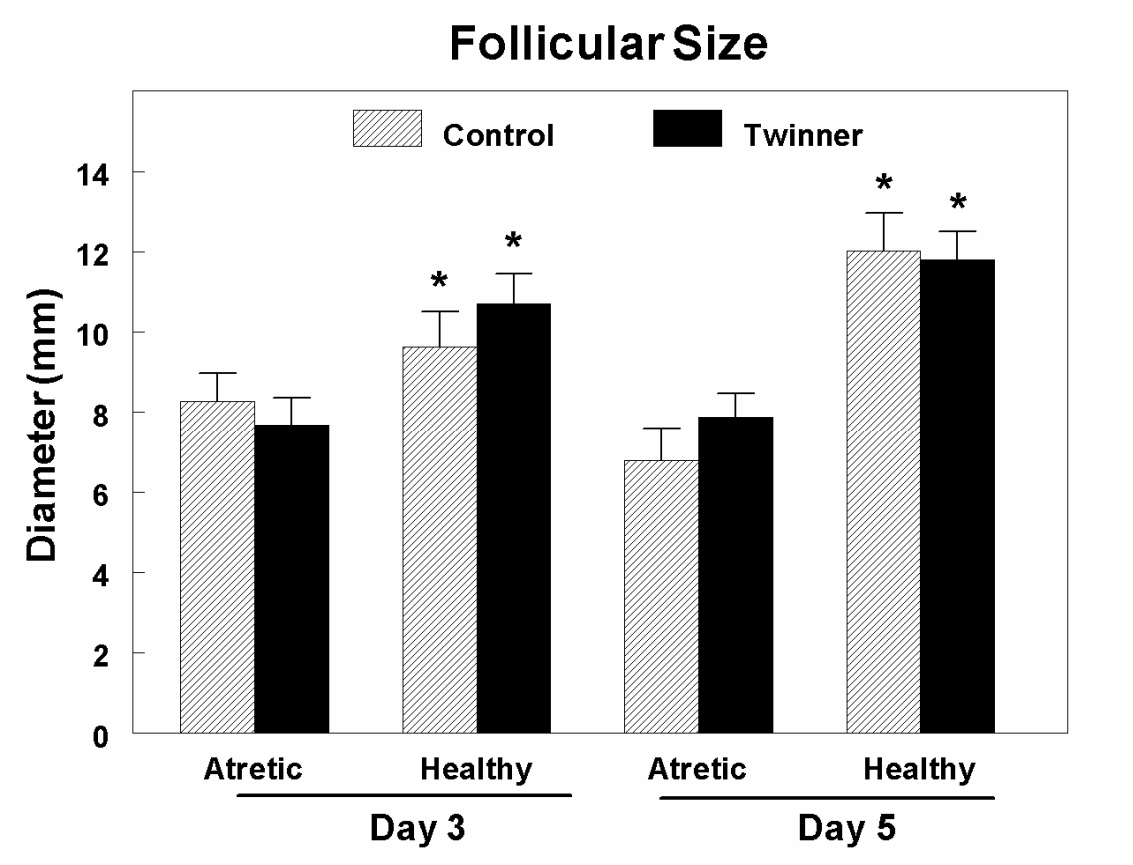

Follicular Size
Control
Twinner
Diameter (mm)
0
2
4
6
8
10
12
14
*
*
*
*
Atretic
Healthy
Atretic
Healthy
Day 3
Day 5

Figure 3.2: FSH receptor gene expression in granulosa cells of cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations were collected between D3-4 (D3; recruitment) or D5-6 (D5; deviation) of an estrous cycle, follicles were classified as E-A (E_2:$P_4 \geq 1$; healthy) or E-I (E_2:$P_4 < 1$; atretic). Follicles were bisected, granulosa cells isolated and RNA extracted. FSHR mRNA quantitated using multiplex real-time RT-PCR, and means expressed as relative abundance to 18 S rRNA. Panel A: Genotype by day of cycle interaction on FSHR mRNA. [a, b] Means without a common superscript differ ($P < 0.05$). Panel B: Day of cycle by status interaction on FSHR mRNA). [a, b, c] Means without a common superscript differ ($P < 0.06$).

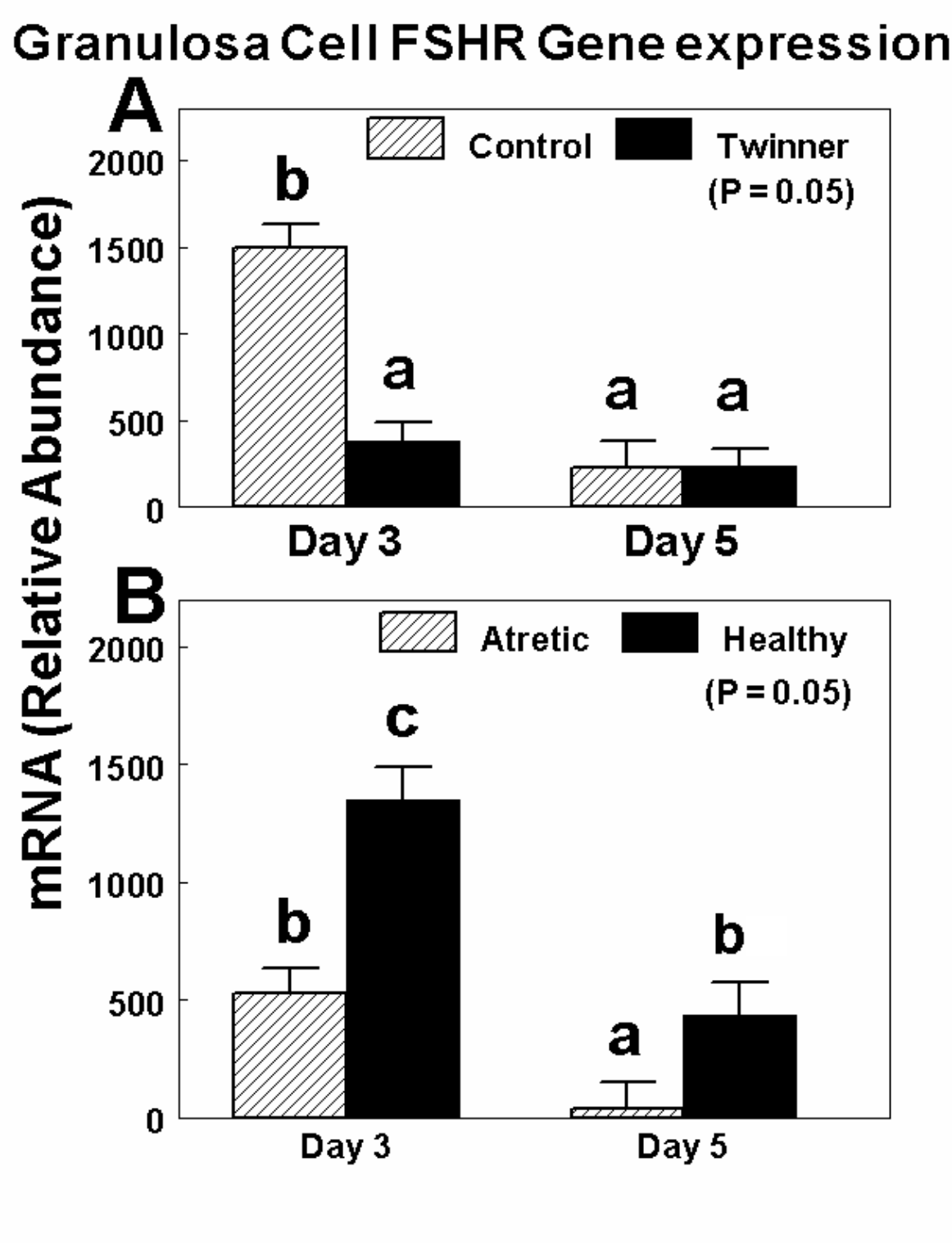

Granulosa Cell FSHR Gene expression
A
Control
Twinner
(P = 0.05)
2000
1500
1000
500
0
b
a
a
a
Day 3
Day 5
B
Atretic
Healthy
(P = 0.05)
mRNA (Relative Abundance)
2000
1500
1000
500
0
c
b
b
a
Day 3
Day 5

Figure 3.3: LH receptor gene expression in granulosa cells of cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations were collected between D3-4 (D3; recruitment) or D5-6 (D5; deviation) of an estrous cycle, follicles were classified as E-A (E_2:$P_4 \geq 1$; healthy) or E-I (E_2:$P_4 < 1$; atretic). Follicles were bisected, granulosa cells isolated and RNA extracted. LHR mRNA quantitated using multiplex real-time RT-PCR, and means expressed as relative abundance to 18 S rRNA. [a, b, c] Means without a common superscript differ ($P < 0.05$).

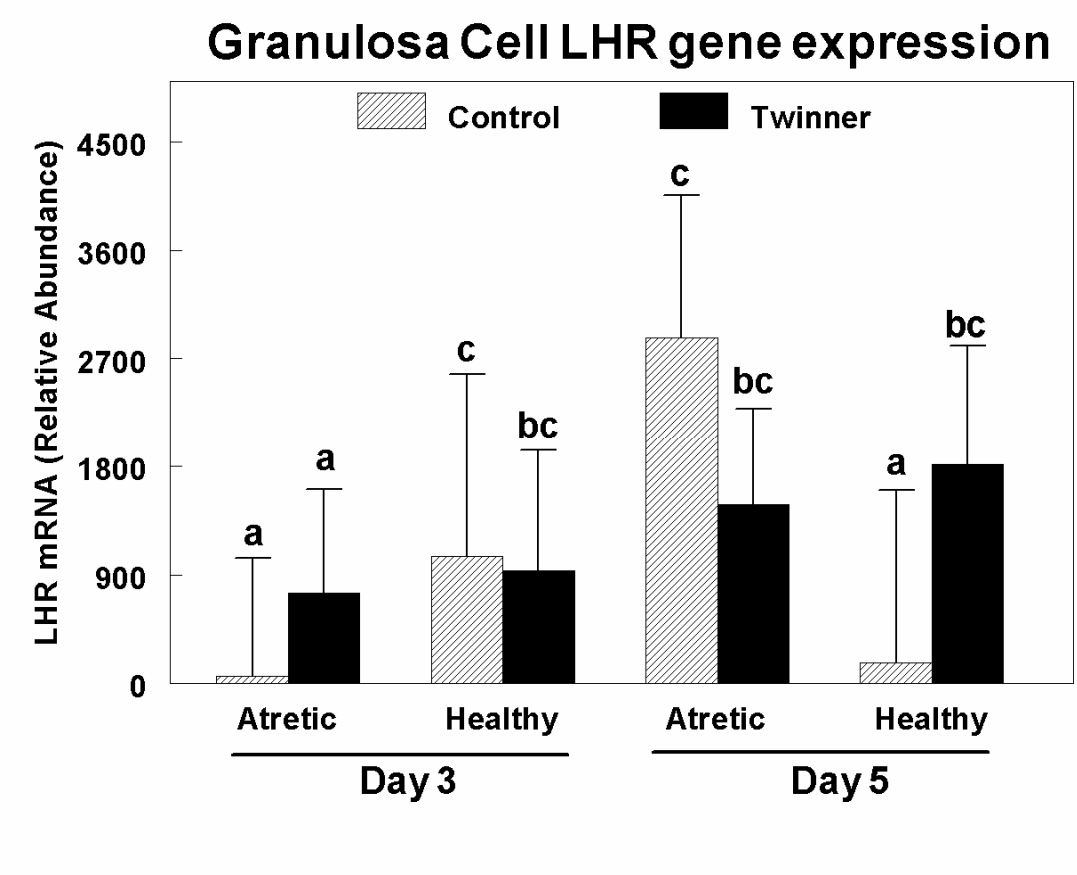

Granulosa Cell LHR gene expression
Control
Twinner
LHR mRNA (Relative Abundance)
4500
3600
2700
1800
900
0
a
a
c
bc
c
bc
a
bc
Atretic
Healthy
Atretic
Healthy
Day 3
Day 5

Figure 3.4: IGF type 2 receptor gene expression in granulosa cells of cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations were collected between D3-4 (D3; recruitment) or D5-6 (D5; deviation) of an estrous cycle, follicles were classified as E-A (E_2:$P_4 \geq 1$; healthy) or E-I (E_2:$P_4 < 1$; atretic). Follicles were bisected, granulosa cells isolated and RNA extracted. IGF2R mRNA quantitated using multiplex real-time RT-PCR, and means expressed as relative abundance to 18 S rRNA. Panel A: Follicle status significant main effect ($P < 0.05$). Panel B: Day of cycle main effect ($P = 0.06$). [a, b] Within each panel, means without a common superscript differ ($P < 0.06$).

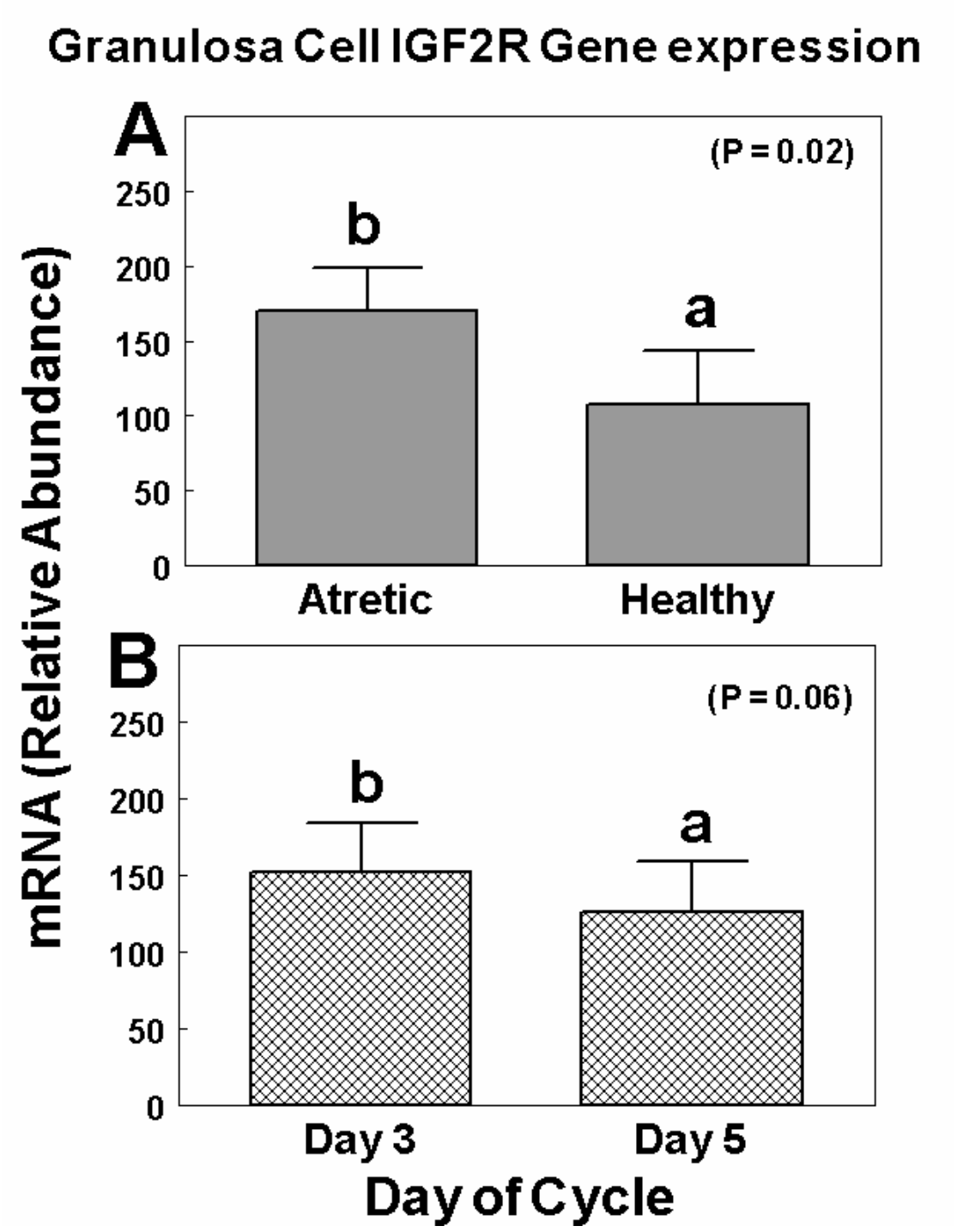

Granulosa Cell IGF2R Gene expression
A
(P = 0.02)
b
a
250
200
150
100
50
0
Atretic
Healthy
B
(P = 0.06)
b
a
250
200
150
100
50
0
Day 3
Day 5
mRNA (Relative Abundance)
Day of Cycle

Figure 3.5: Linear regression analysis of estradiol concentration in follicular fluid of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. Estradiol concentrations in follicles of Control cows (—) is represented by the line $y = 0.48x-1.68$; Estradiol concentrations in follicles of Twinner cows (- - -) is represented by the line $y = 0.50 x -1.95$.

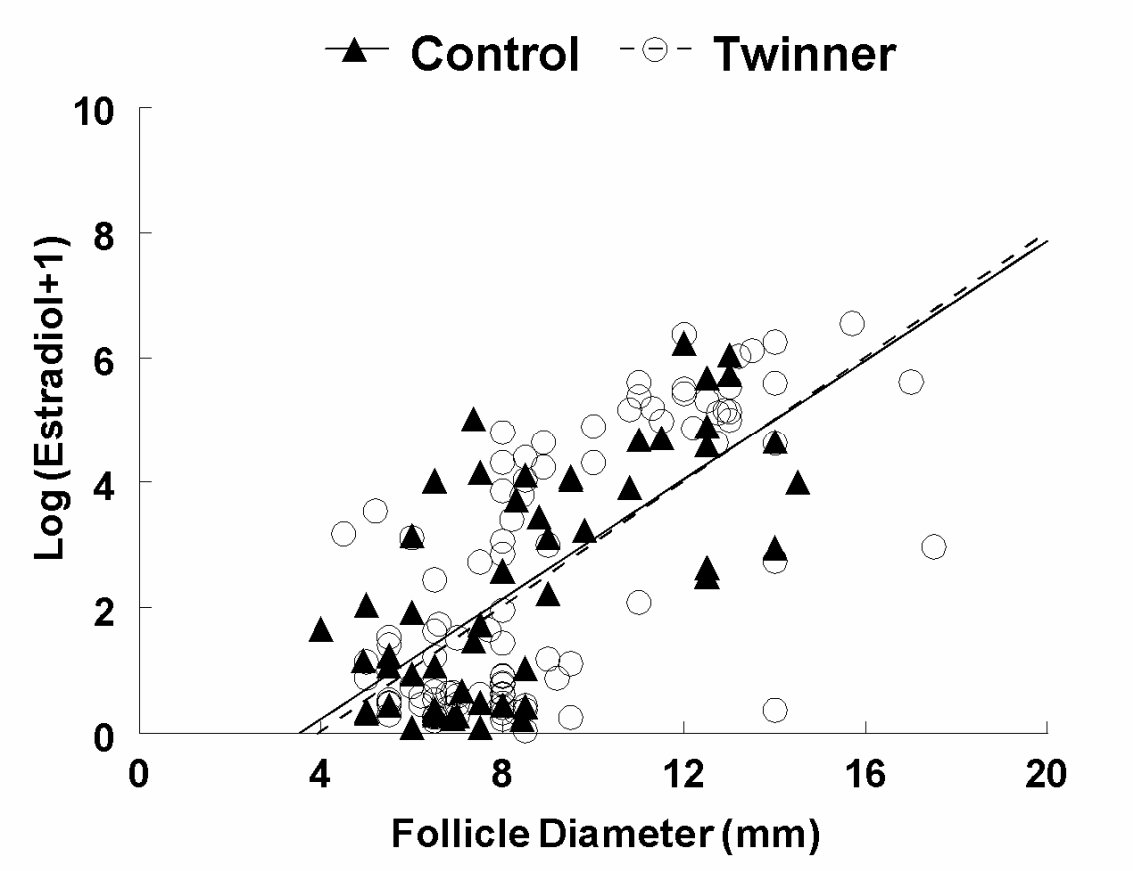

Control
Twinner
Log (Estradiol+1)
10
8
6
4
2
0
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 3.6: Linear regression analysis of progesterone concentration in follicular fluid of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. Progesterone concentrations in follicles of Control cows (—) is represented by the line $y = 0.084\ x + 2.70$; progesterone concentrations in follicles of Twinner cows (- - -) is represented by the line $y = 0.07\ x + 2.90$.

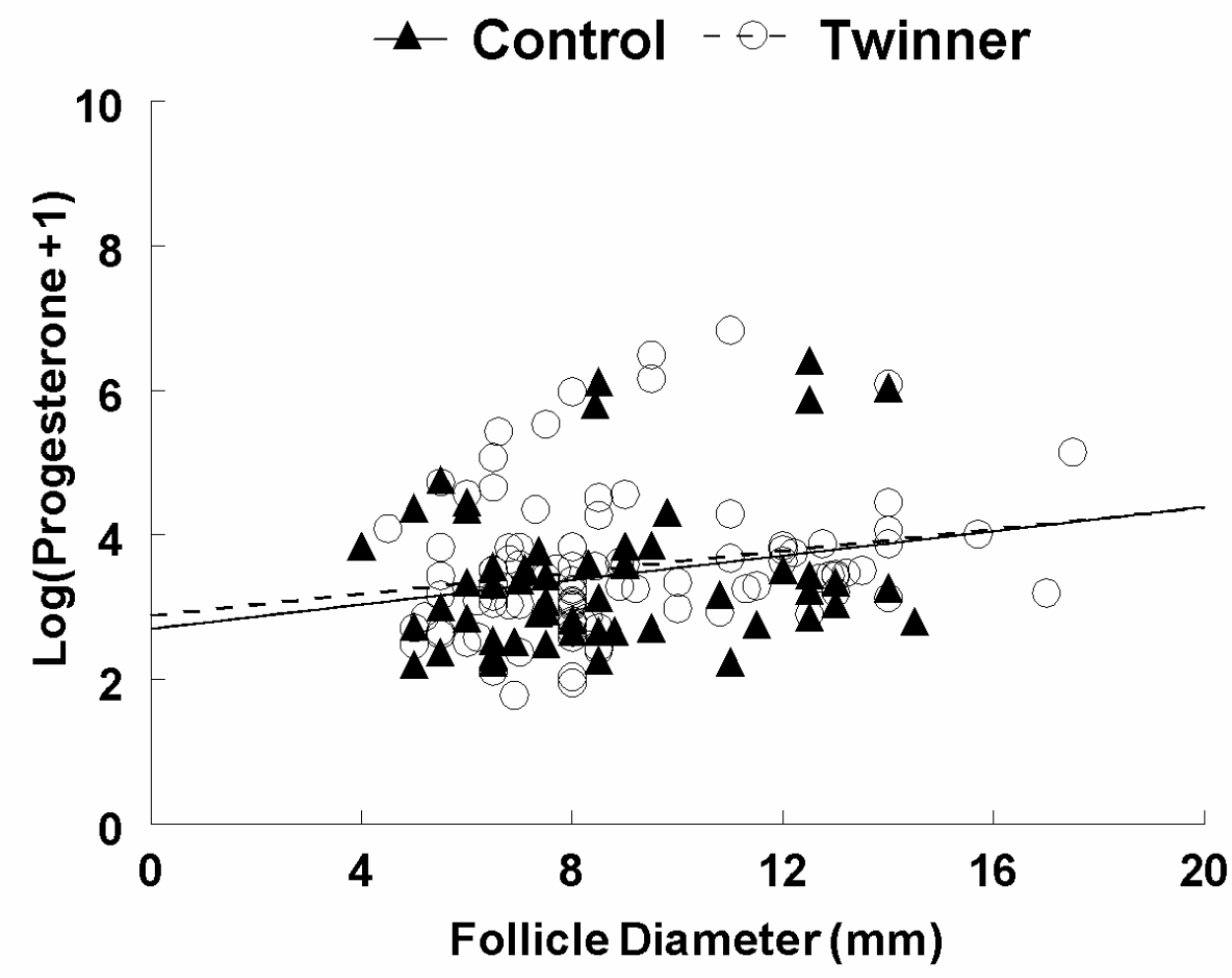

Control
Twinner
Log(Progesterone +1)
10
8
6
4
2
0
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 3.7: Linear regression analysis of FSH receptor mRNA in granulosa cells of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. FSHR mRNA levels in granulosa cells of follicles from Control cows (—) is represented by the line $y = 0.08\ x + 4.15$; FSHR mRNA levels in granulosa cells of follicles from Twinner cows (- - -) is represented by the line $y = -0.01\ x + 4.12$.

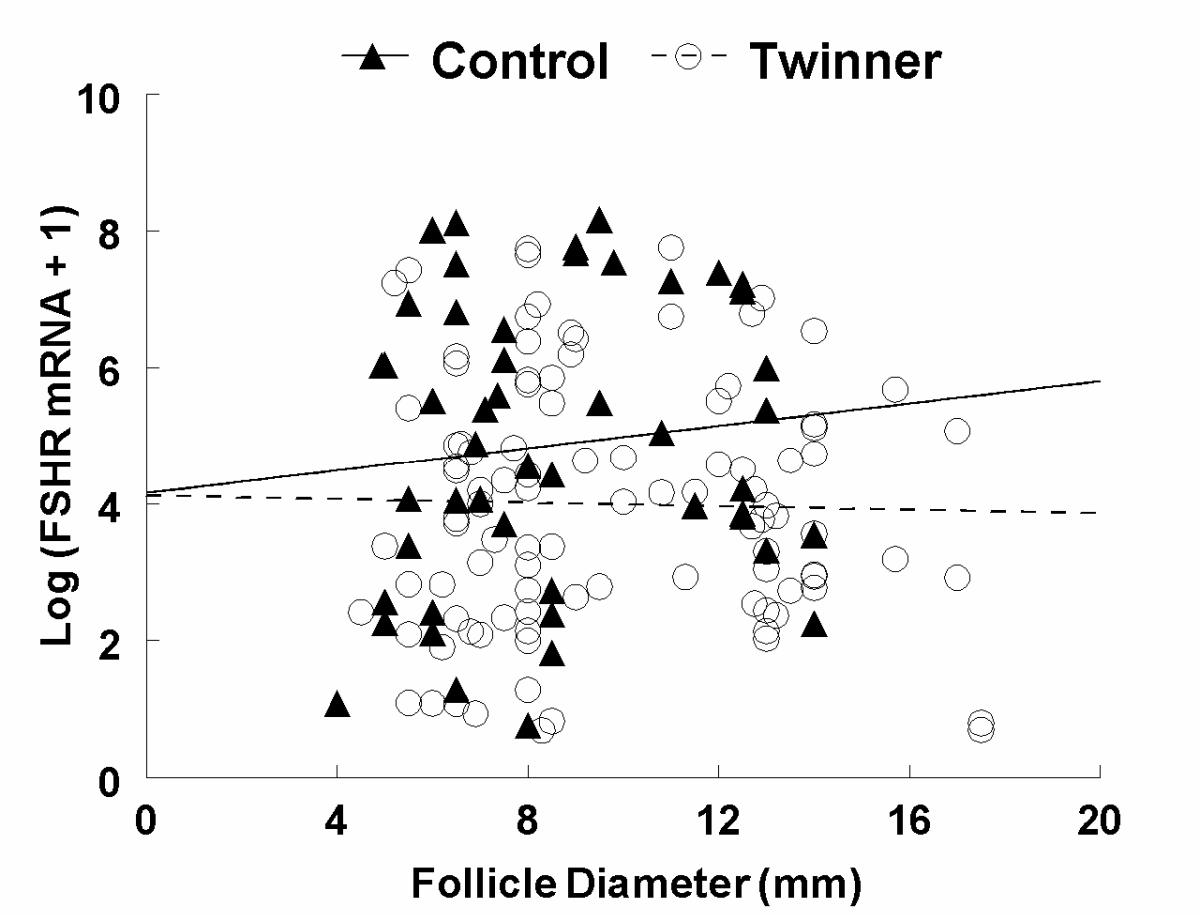

Control
Twinner
Log (FSHR mRNA + 1)
Follicle Diameter (mm)
0
2
4
6
8
10
0
4
8
12
16
20

Figure 3.8: Linear regression analysis of LH receptor mRNA in granulosa cells of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. LHR mRNA levels in granulosa cells of follicles from Control cows (—) is represented by the line $y = - 0.03 x + 4.62$; LHR mRNA levels in granulosa cells of follicles from Twinner cows (- - -) is represented by the line $y = 0.03 x + 3.96$.

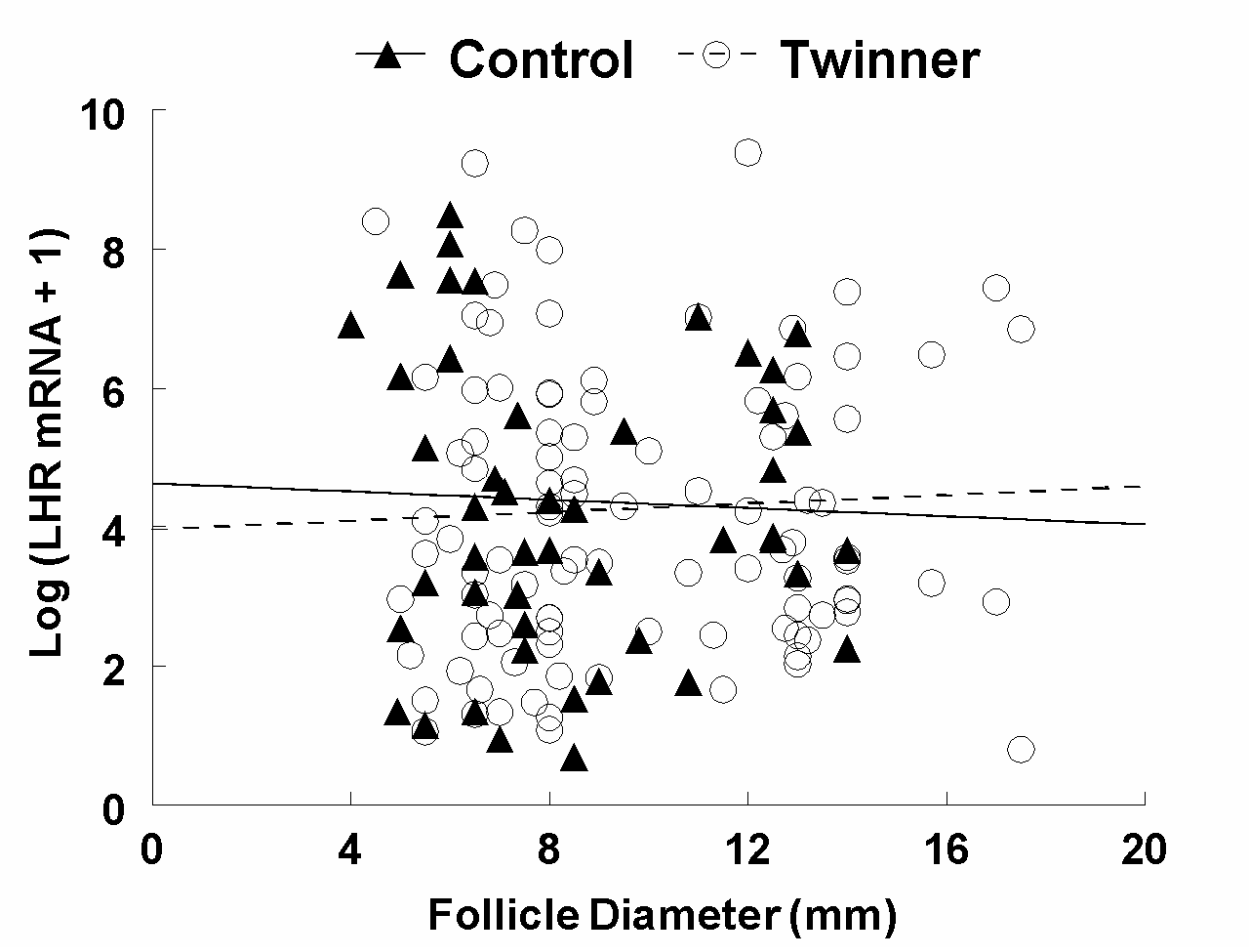

Control
Twinner
Log (LHR mRNA + 1)
10
8
6
4
2
0
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 3.9: Linear regression analysis of IGF type 2 receptor mRNA in granulosa cells of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. IGF2R mRNA levels in granulosa cells of follicles from Control cows (—) is represented by the line $y = - 0.23 x + 6.11$; levels in granulosa cells of follicles from Twinner cows (- - -) is represented by the line $y = - 0.15 x + 5.22$.

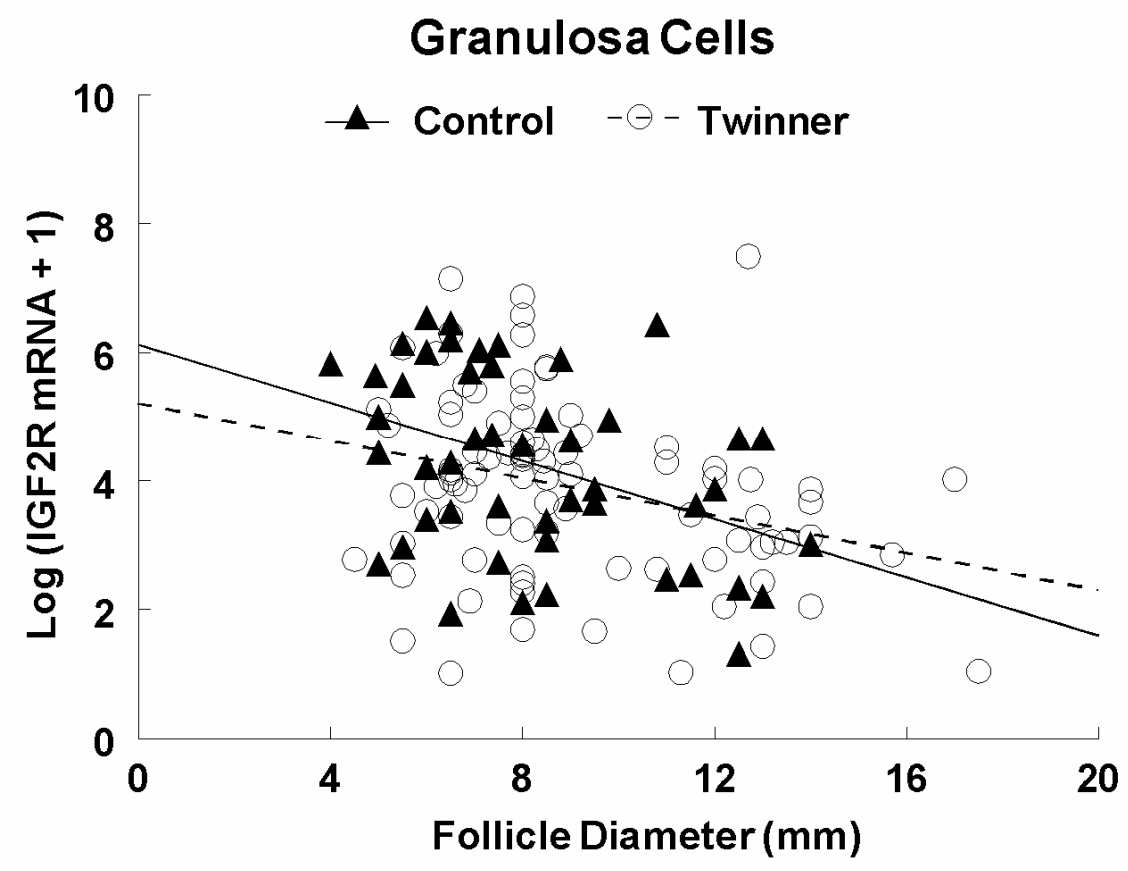

Granulosa Cells
Control
Twinner
Log (IGF2R mRNA + 1)
10
8
6
4
2
0
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 3.10: Linear regression analysis of LH receptor mRNA in theca cells of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. LHR mRNA levels in theca cells of follicles from Control cows (—) is represented by the line y = 0.042 x + 3.59; LHR mRNA levels in theca cells of follicles from Twinner cows (- - -) is represented by the line y = 0.065 x + 2.94.

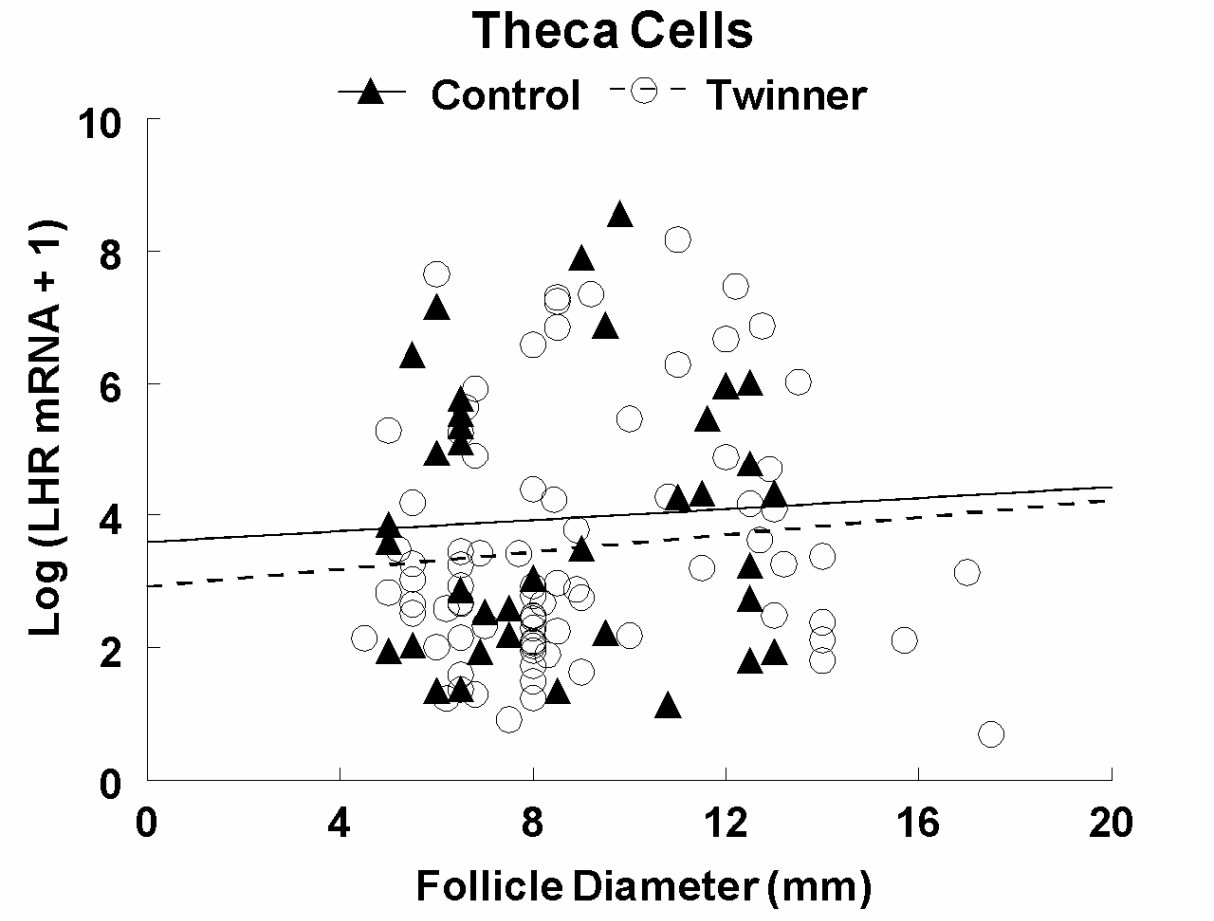

Theca Cells
Control
Twinner
Log (LHR mRNA + 1)
10
8
6
4
2
0
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 3.11: Linear regression analysis of IGF type 2 receptor mRNA in theca cells of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. IGF2R mRNA levels in theca cells of follicles from Control cows (—) is represented by the line $y = - 0.01\ x + 5.35$; IGF2R mRNA levels in theca cells of follicles from Twinner cows (- - -) is represented by the line $y = - 0.01\ x + 4.55$.

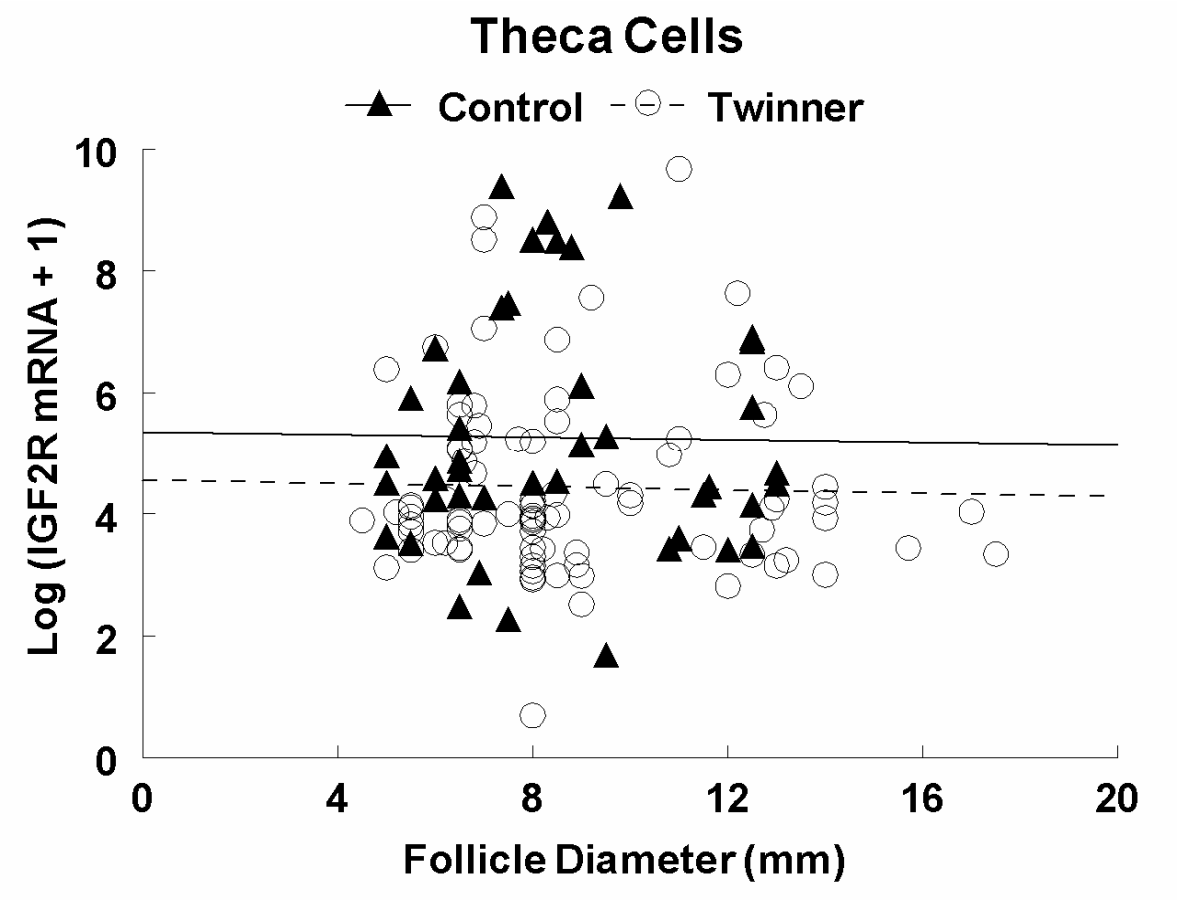

Theca Cells
Control
Twinner
Log (IGF2R mRNA + 1)
0
2
4
6
8
10
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 3.12: Linear regression analysis of CYP17A1 mRNA in theca cells of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. CYP17A1 mRNA levels in theca cells of follicles from Control cows (—) is represented by the line $y = - 0.14 x + 6.68$; CYP17A1 mRNA levels in theca cells of follicles from Twinner cows (- - -) is represented by the line $y = 0.04 x + 5.51$.

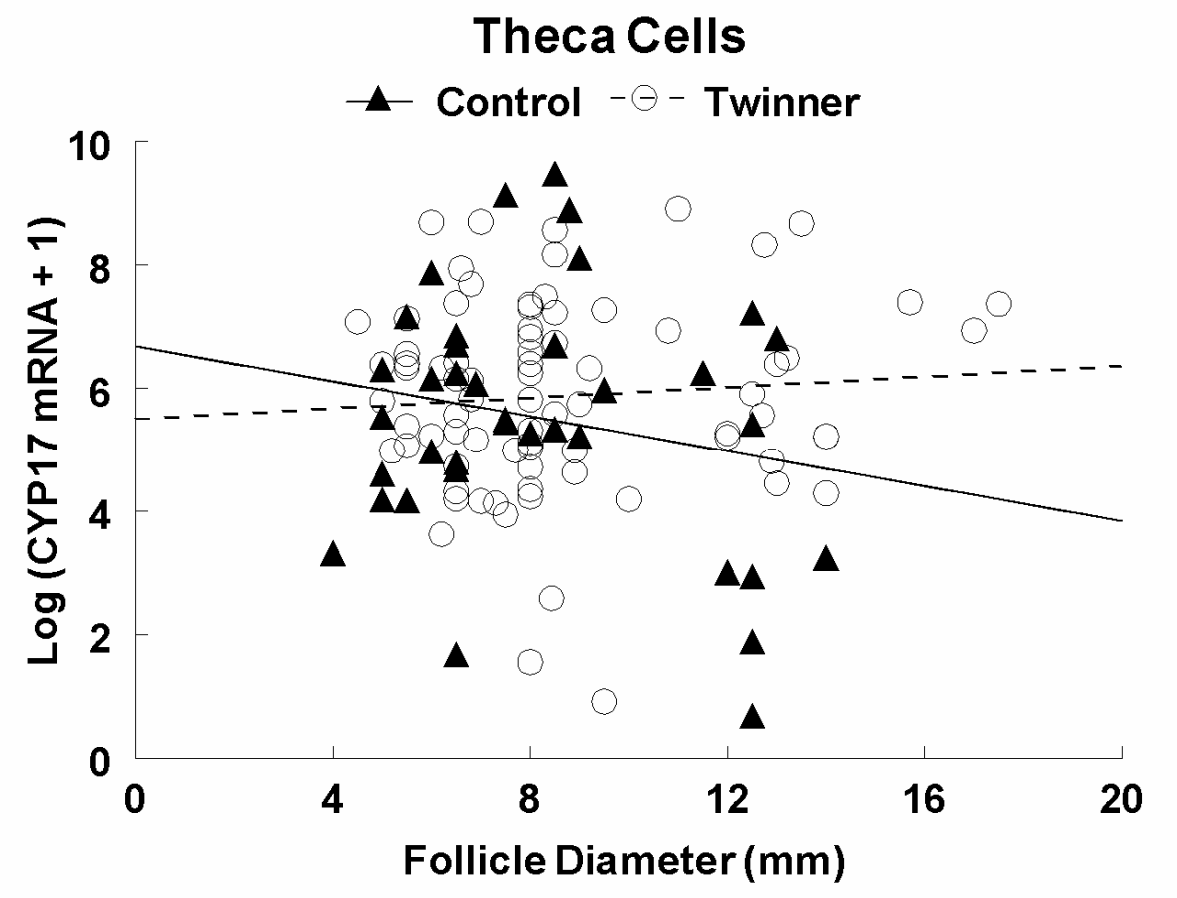

Theca Cells
Control
Twinner
Log (CYP17 mRNA + 1)
0
2
4
6
8
10
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 3.13: Follicular diameter of the largest three follicles in cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations, collected between D3-4 (D3) or D5-6 (D5) of an estrous cycle, the largest three follicles (F1, F2, or F3) were identified based on their diameter and estrogenic profile. $^{a, b}$ and ^{x,y} Within genotype, means without a common superscript differ ($P < 0.05$).

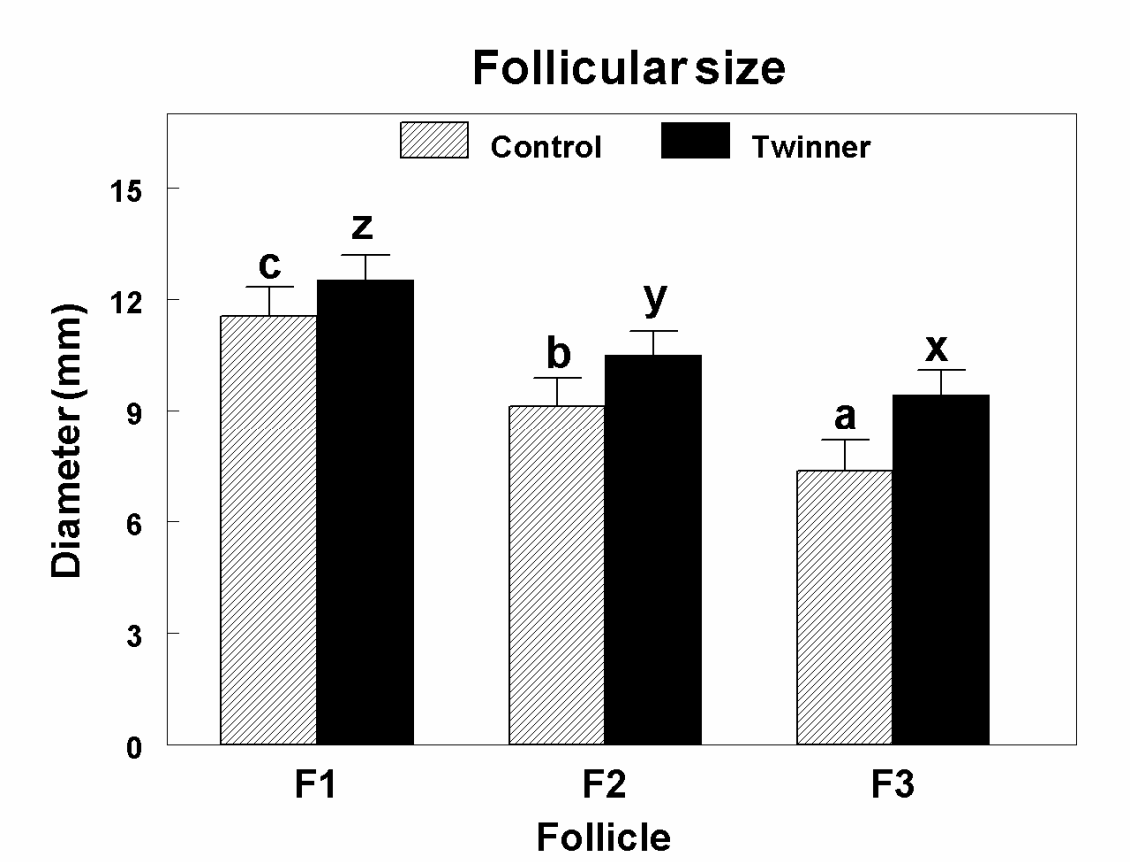

Follicular size
Control
Twinner
15
12
9
6
3
0
Diameter (mm)
c
z
b
y
a
x
F1
F2
F3
Follicle

Figure 3.14: Follicular fluid estradiol levels in the largest three follicles in cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations, collected between D3-4 (D3) or D5-6 (D5) of an estrous cycle, the largest three follicles (F1, F2, or F3) were identified based on their diameter and estrogenic profile. $^{a, b}$ and $^{x, y}$ Within genotype, means without a common superscript differ ($P < 0.05$).

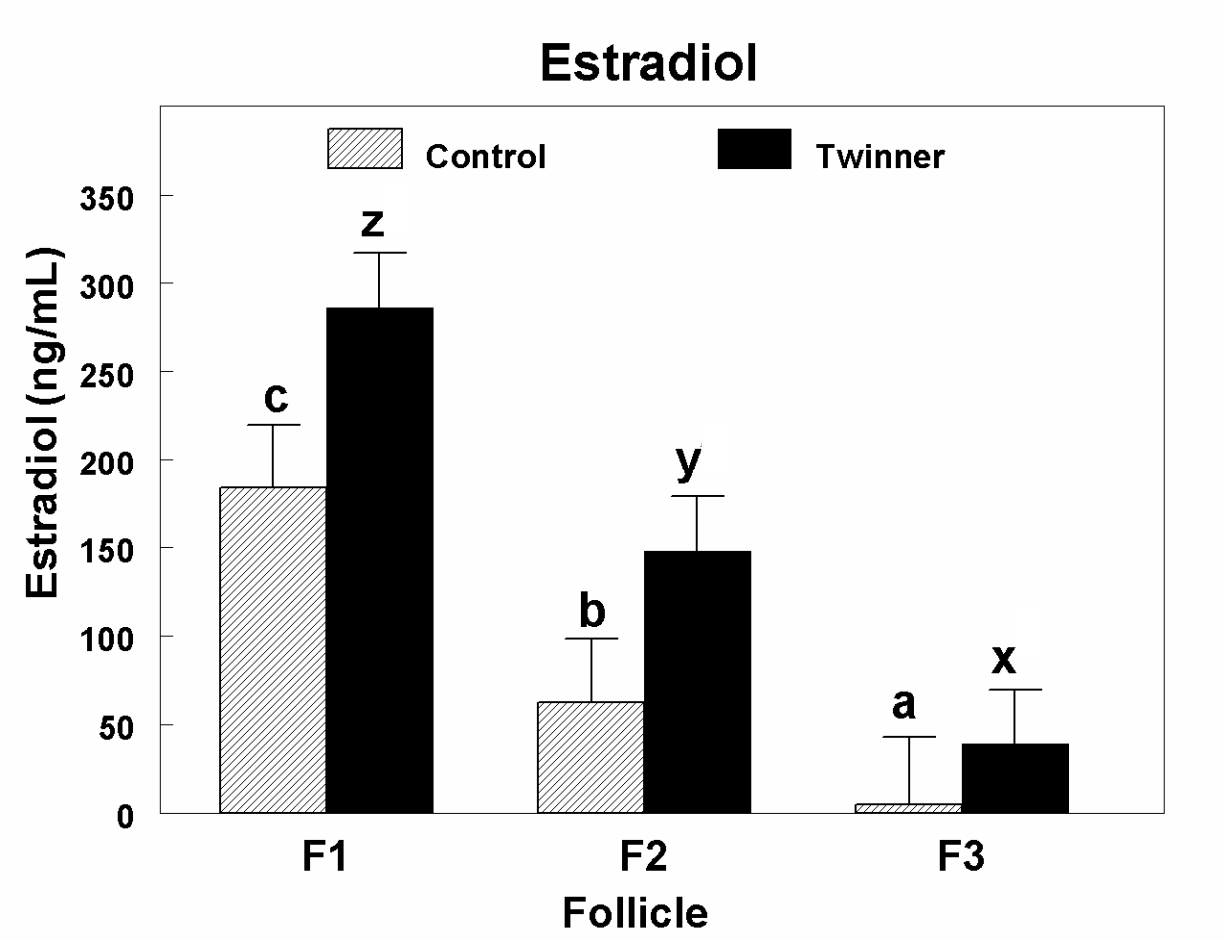

Estradiol
Control
Twinner
Estradiol (ng/mL)
350
300
250
200
150
100
50
0
z
c
y
b
x
a
F1
F2
F3
Follicle

Figure 3.15: Follicular fluid androstenedione levels in the largest two follicles in cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations, collected between D3-4 (Recruitment, D3) or D5-6 (Deviation, D5) of an estrous cycle, the largest 2 follicles (F1 or F2) were identified based on their diameter and estrogenic profile.

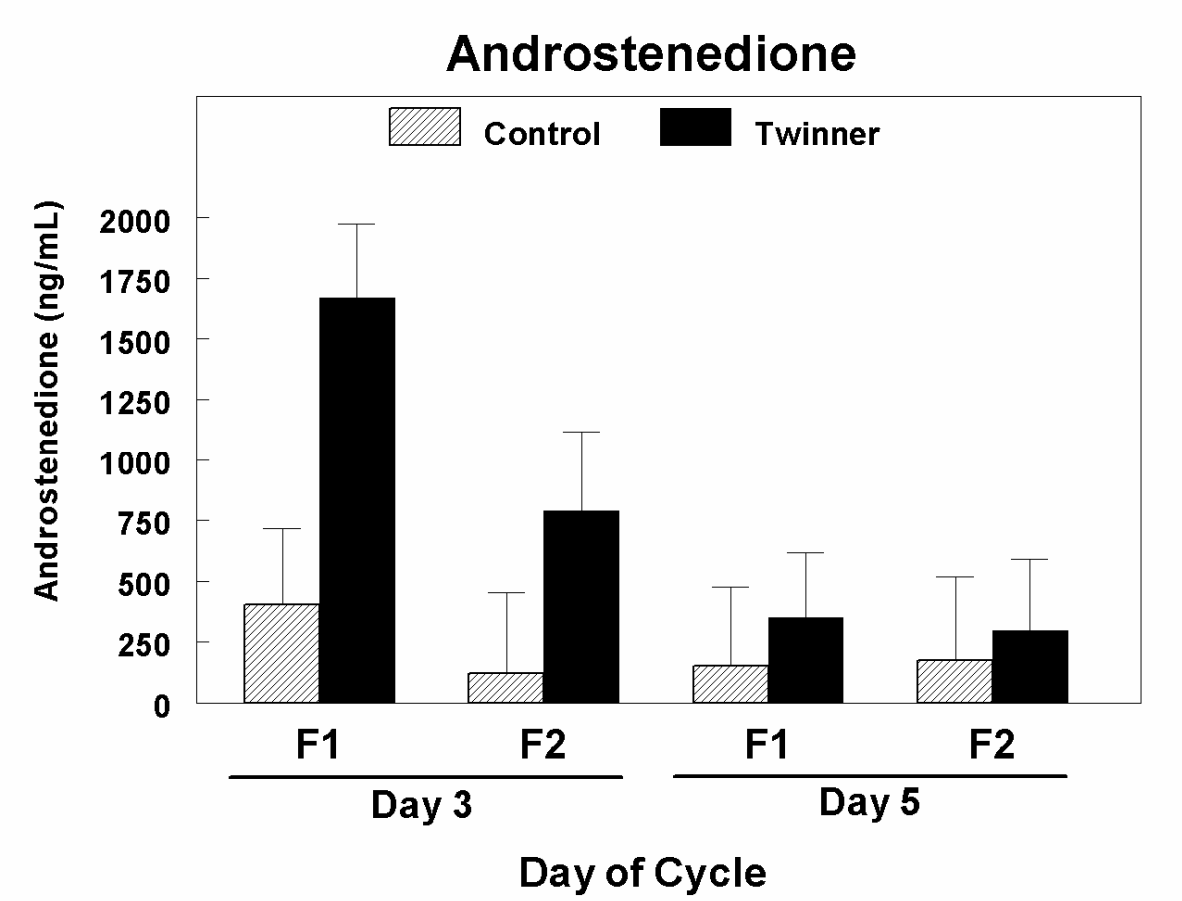

Androstenedione
Control
Twinner
Androstenedione (ng/mL)
2000
1750
1500
1250
1000
750
500
250
0
F1
F2
F1
F2
Day 3
Day 5
Day of Cycle

Figure 3.16: FSH receptor gene expression in granulosa cells of the largest three follicles from cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations, collected between D3-4 (D3, recruitment) or D5-6 (D5, deviation) of an estrous cycle, the largest three follicles (F1, F2, or F3) were identified based on their diameter and estrogenic profile. Follicles were bisected, granulosa cells isolated and RNA extracted. FSHR mRNA quantitated using multiplex real-time RT-PCR, and means expressed as relative abundance to 18 S rRNA.

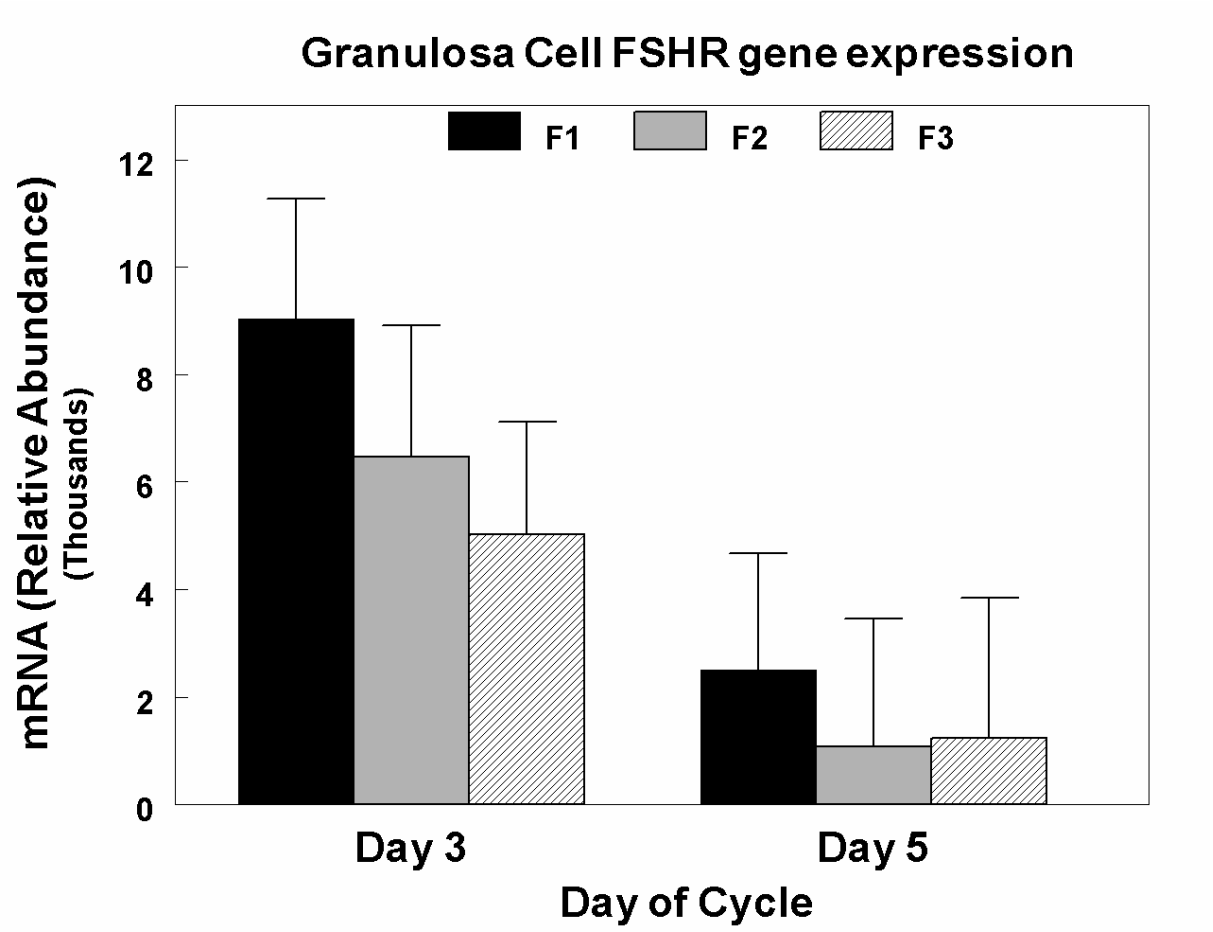

Granulosa Cell FSHR gene expression
F1
F2
F3
mRNA (Relative Abundance)
(Thousands)
0
2
4
6
8
10
12
Day 3
Day 5
Day of Cycle

Figure 3.17: LH receptor gene expression in theca cells of the largest three follicles from cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations, collected between D3-4 (D3, recruitment) or D5-6 (D5, deviation) of an estrous cycle, the largest three follicles (F1, F2, or F3) were identified based on their diameter and estrogenic profile. Follicles were bisected, theca cells isolated and RNA extracted. LHR mRNA quantitated using multiplex real-time RT-PCR, and means expressed as relative abundance to 18 S rRNA. * Within F3, mean differs ($P < 0.05$) from control counterpart.

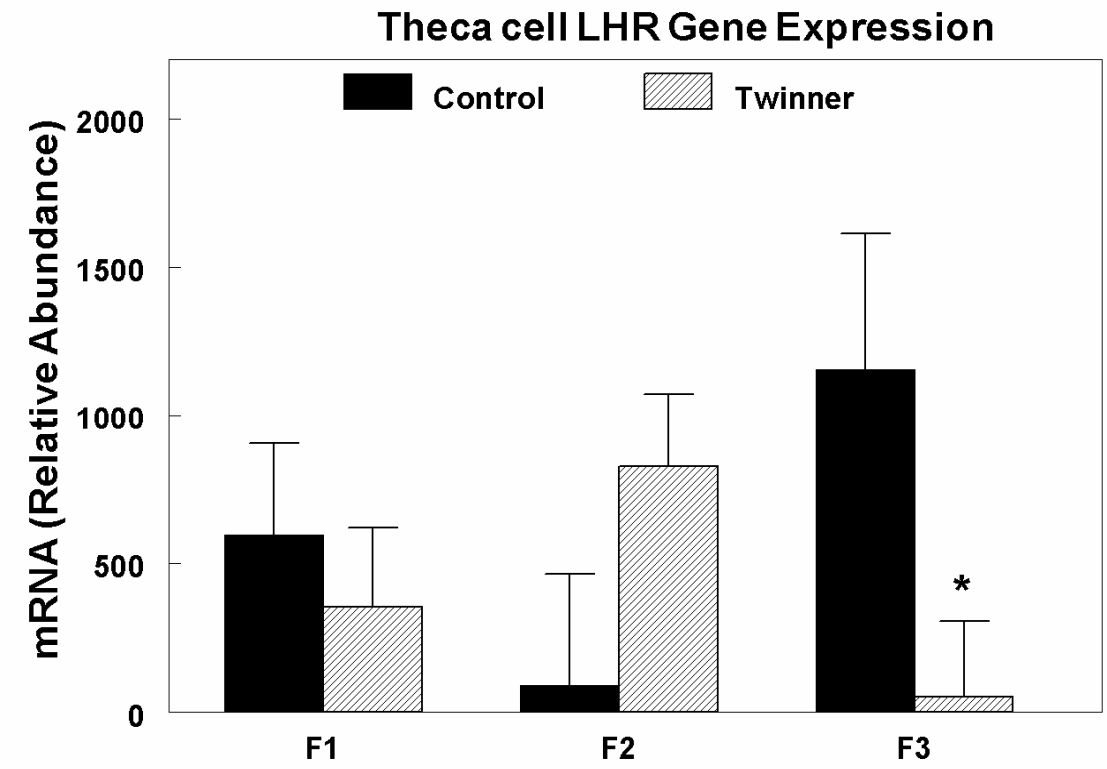

Theca cell LHR Gene Expression
Control
Twinner
mRNA (Relative Abundance)
2000
1500
1000
500
0
*
F1
F2
F3

Figure 3.18: IGF type 2 receptor gene expression in granulosa cells of the largest three follicles from cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations, collected between D3-4 (D3, recruitment) or D5-6 (D5, deviation) of an estrous cycle, the largest three follicles (F1, F2, or F3) were identified based on their diameter and estrogenic profile. Follicles were bisected, granulosa cells isolated and RNA extracted. IGF2R mRNA quantitated using multiplex real-time RT-PCR, and means expressed as relative abundance to 18 S rRNA. a, b Within day of cycle, mean differs ($P < 0.05$) from control means.

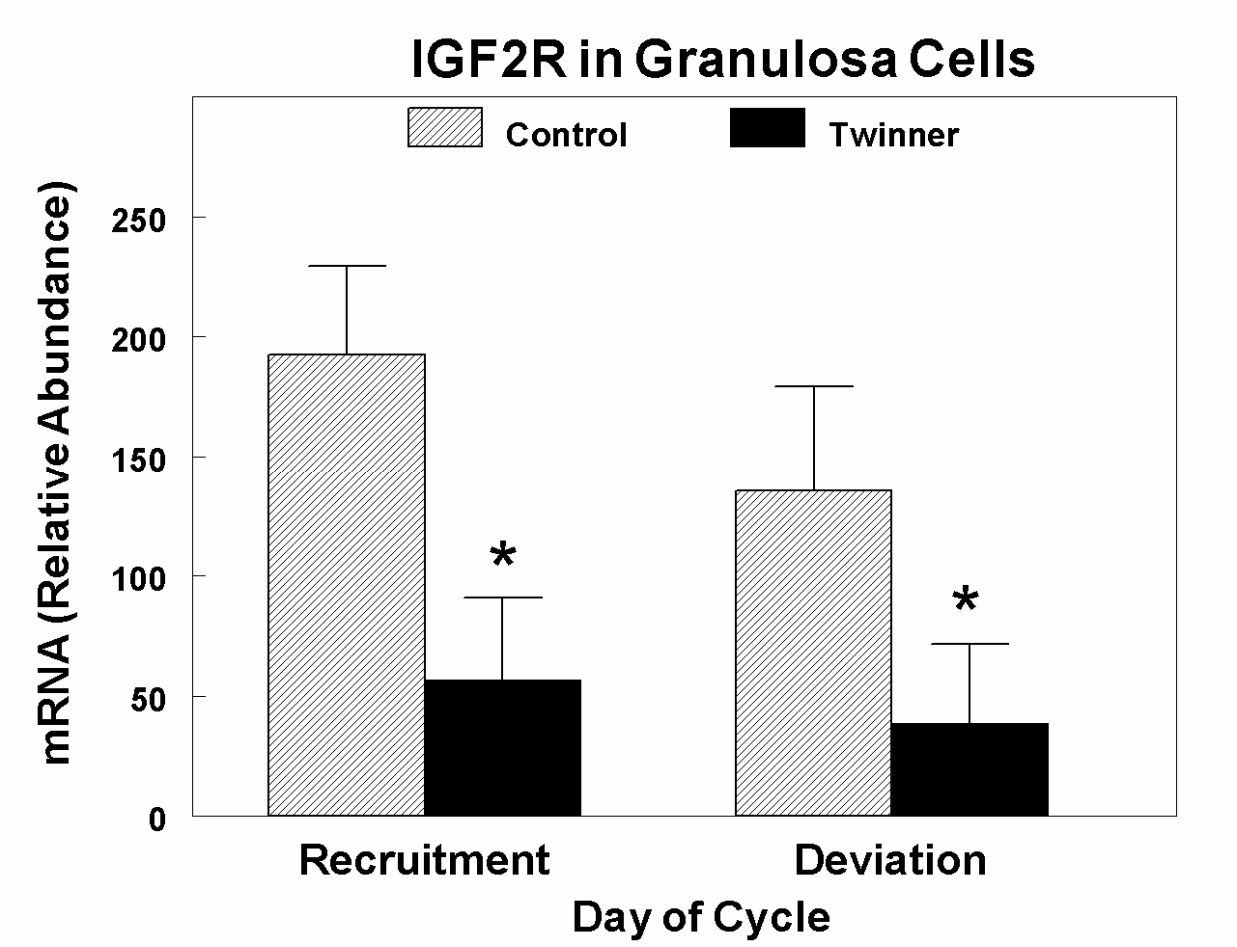
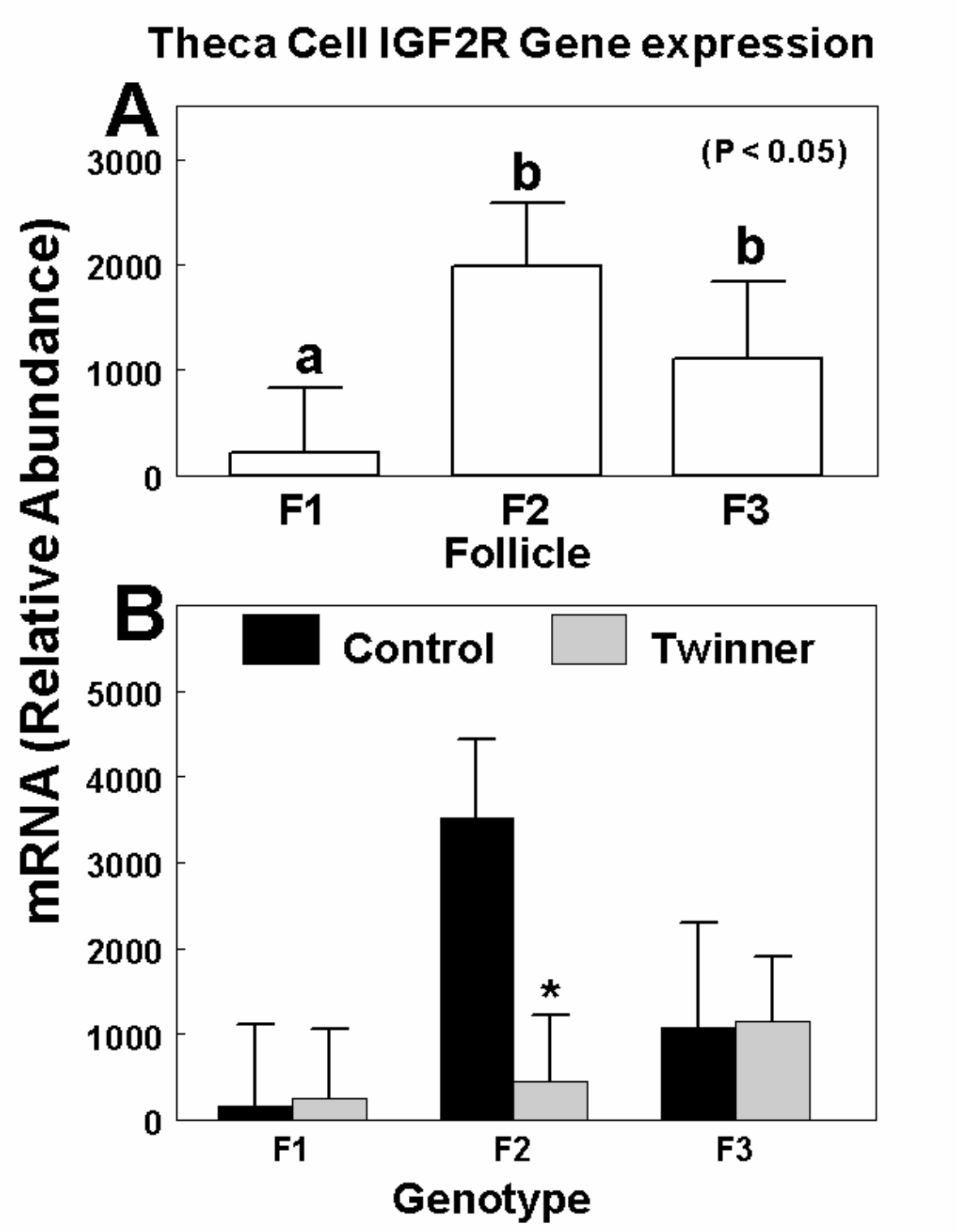

IGF2R in Granulosa Cells
Control
Twinner
mRNA (Relative Abundance)
250
200
150
100
50
0
*
*
Recruitment
Deviation
Day of Cycle

Figure 3.19: IGF type 2 receptor gene expression in theca cells of the largest three follicles from cows unselected (Control) and selected (Twinner) for multiple ovulations. Follicles from unselected (Control) or selected (Twinner) cows for multiple ovulations, collected between D3-4 (D3, recruitment) or D5-6 (D5, deviation) of an estrous cycle, the largest three follicles (F1, F2, or F3) were identified based on their diameter and estrogenic profile. Follicles were bisected, theca layer peeled, homogenized in TRIzol and RNA extracted. IGF2R mRNA quantitated using multiplex real-time RT-PCR, and means expressed as relative abundance to 18 S rRNA. Panel A: IGF2R mRNA in the largest three follicles. a, b Means without a common superscript differ ($P < 0.05$). Panel B: Genotype by Day of Cycle interaction on thecal IGF2R mRNA. * Within F2, mean differs ($P < 0.05$) from its control counterpart.


Theca Cell IGF2R Gene expression
A
(P < 0.05)
a
b
b
3000
2000
1000
0
F1
F2
F3
Follicle
B
Control
Twinner
mRNA (Relative Abundance)
5000
4000
3000
2000
1000
0
*
F1
F2
F3
Genotype

Tables

Table 3.1: Sequences, accession number and melting temperatures of the bovine specific primers and probes for the selected target genes of interest. Accession number is from the national center for biotechnology information (National library of medicine and national institutes of health), available at http://www.ncbi.nlm.nih.gov/.

Gene	Oligo[1]	Sequence	Accession	Anneals between	Tm[1] (°C)
FSH receptor (FSHR)	FWD	AACCTGCTATACATCGACCCTGAT	**L22319**	383-406	59
	REV	GCTTAATACCTGTGTTGGATATTAACAGA		464-436	58
	Probe	CCTTCCAGAACCTTCCCAACCTCCG		408-432	69
LH receptor (LHR)	FWD	TGACTATGGTTTCTGCTTACCCAA	**AF491303**	999-1022	58
	REV	CCATAATGTCTTCACAGGGATTGA		1055-1078	59
	Probe	CTCCAGTGTGCTCCTGAACCAGCTGCT		1026-1053	69
IGF type 2 receptor (IGF2R)	FWD	GCAATGCTAAGCTTTCGTATTACG	**AF342811**	2308-2331	58
	REV	GGTGTACCACCGGAAGTTGTATG		2495-2473	59
	Probe	ACGCCGGAGTGGGTTTCCCC		2428-2447	68
Steroid 17-α-hydroxylase (CYP17A1)	FWD	CCATCAGAGAAGTGCTCCGAAT	**NM_174304**	1113-1134	59
	REV	GCCAATGCTGGAGTCAATGA		1192- 1173	58
	Probe*	CGGCCTGTGGCCCCTACGCT		1136-1155	69

[1]FWD is forward primer, REV is reverse primer. Tm: melting temperature.

* Exon-spanning oligo

Table 3.2: Follicular diameter and estradiol and progesterone levels in follicular fluid of Control and Twinner cows

		N	Di (mm)	SEM		E_2 (ng/ml)	SEM		P_4 (ng/ml)	SEM	
	Control	57	9.17	0.49		70.29	15.07		55.18	18.43	
Genotype	**Twinner**	97	9.51	0.42		97.98	12.13		62.54	13.71	
	P-value*			0.60			0.59			0.45	
	Day 3	78	9.07	0.45		89.69	13.28		66.52	15.64	
Day of cycle	**Day 5**	76	9.61	0.46		78.57	14.07		51.20	16.83	
	P-value*	97		0.41			0.70			0.98	
	E-I	57	7.65	0.35	a	3.68	11.17	a	90.78	14.12	b
Status	**E-A**		11.04	0.41	b	164.59	13.81	b	26.94	18.12	a
	P-value*			<.0001			<.0001			0.008	
Genotype x Cycle	P-value*			0.89			0.86			0.82	
Genotype x Status	P-value*			0.83			0.27			0.51	
Cycle x Status	P-value*			0.005			0.14			0.74	
Genotype x Cycle x Status	P-value*			0.07			0.84			0.65	

[a, b] Within effect, means without a common superscript differ ($P < 0.05$) if interaction is not significant.

* P-value of ANOVA on Log transformed data for E_2 and P_4 only.

Abbreviations: Di: diameter; E_2: estradiol; P_4: progesterone; E-A: estrogen active (E_2: $P_4 \geq 1$); E-I: estrogen inactive (E_2: $P_4 < 1$).

Table 3.3: Granulosa cell gene expression of FSH receptor (FSHR), LH receptor (LHR), and IGF type 2 receptor (IGF2R) in Control and Twinner cows.

		FSHR mRNA			LHR mRNA			IGF2R mRNA			
		N	**LSMean***	**SEM**	**N**	**LSMean***	**SEM**	**N**	**LSMean***	**SEM**	
Genotype	**Control**	46	863.49	100.73	45	1038.7	668.38	49	147.43	36.95	
	Twinner	85	312.81	73.01	86	1246.04	483.42	88	130.94	27.6	
	P-value**		0.03			0.79			0.41		
Day of cycle	**Day 3**	65	940.29	86.83	66	697.39	581.27	70	152.38	31.6	b
	Day 5	66	236	89.1	65	1587.36	585.29	67	125.99	33.59	a
	P-value**					0.076			0.06		
Status	**E-I**	82	283.77	74.95	82	1291.89	482.76	83	170.31	28.86	b
	E-A	49	892.53	97.34	49	992.85	627.7	54	108.06	35.28	a
	P-value**		<.0001			0.24			0.016		
Genotype x Cycle	P-value**		0.03			0.98			0.13		
Genotype x Status	P-value**		0.12			0.58			0.48		
Cycle x Status	P-value**		0.02			0.0006			0.82		
Genotype x Cycle x Status	P-value**		0.36			0.001			0.56		

[a, b] Within effect, means without a common superscript differ ($P < 0.05$) if interaction is not significant.

* LSMean of the mRNA relative abundance ($2^{-\Delta\Delta Ct}$)

** P-value of ANOVA on Log transformed data.

Abbreviations: E-A: estrogen active (E_2: $P_4 \geq 1$); E-I: estrogen inactive (E_2: $P_4 < 1$).

Table 3.4: Theca cell gene expression of LH receptor (LHR), and IGF type 2 receptor (IGF2R), and 17 alpha hydroxylase/17-20 lyase (CYP17A1) in Control and Twinner cows.

		LHR mRNA			IGF2R mRNA			CYP17A1 mRNA		
		N	**LSMean***	**SEM**	**N**	**LSMean***	**SEM**	**N**	**LSMean***	**SEM**
Genotype	**Control**	38	290.56	122.27	39	1178.14	372.62	38	1482.27	424.78
	Twinner	82	225.44	81.15	89	453.9	282.61	80	981.03	326.36
	P-value**		0.49			0.15			0.55	
Day of cycle	**Day 3**	63	297.6	99.76	68	1102.95	315.29	59	1161.83	373.89
	Day 5	57	218.4	107.62	60	529.08	345.41	59	1301.47	383.61
	P-value**		0.79			0.71			0.97	
Status	**E-I**	73	332.66	91.53	76	751.78	287.13	79	822.64	285.86
	E-A	47	183.35	114.71	52	880.25	334.56	39	1640.66	378.78
	P-value**		0.25			0.43			0.26	
Genotype x Cycle	P-value**		0.12			0.17			0.80	
Genotype x Status	P-value**		0.55			0.61			0.35	
Cycle x Status	P-value**		0.56			0.76			0.56	
Genotype x Cycle x Status	P-value**		0.47			0.51			0.28	

[a, b] Within effect, means without a common superscript differ ($P < 0.05$) if interaction is not significant.

* LSMean of the mRNA relative abundance ($2^{-\Delta\Delta Ct}$)

** P-value of ANOVA on Log transformed data.

Abbreviations: E-A: estrogen active (E_2: $P_4 \geq 1$); E-I: estrogen inactive (E_2: $P_4 < 1$).

Table 3.5: Pearson Correlation coefficients among diameter, follicular fluid hormones and follicular cell mRNAs.

			Follicular Fluid		Granulosa cells			Theca Cells		
		E_2:P_4 ratio	**E_2**	**P_4**	***FSHR***	***LHR***	***IGF2R***	***LHR***	***IGF2R***	**CYP17A1**
Diameter		0.55***	0.69***	0.24**	0.1	0.13	-0.44***	-0.02	-0.09	-0.10
E_2: P_4 ratio		-	0.79***	-0.17	0.27**	0.18*	-0.32**	0.16	-0.08	0.06
FFL	**E_2**	-	-	-0.09	0.38**	0.14	-0.39**	0.16	-0.06	0.04
	P_4	-	-	-	-0.21*	0.12	-0.14	-0.12	-0.03	-0.17
GC	***FSHR***	-	-	-	-	-0.08	0.38**	0.16	-0.02	-0.14
	LHR	-	-	-	-	-	-0.06	-0.10	-0.15	-0.01
	IGF2R	-	-	-	-	-	-	-0.02	0.1	-0.13
TC	***LHR***	-	-	-	-	-	-	-	0.64***	0.38***
	IGF2R	-	-	-	-	-	-	-	-	0.30**

* P < 0.05, ** P < 0.001, *** P < 0.0001

Abbreviations: FFL: follicular fluid; GC: granulosa cells; TC: theca cells; E2: estradiol; P4: progesterone; *LHR*: luteinizing hormone receptor mRNA; *FSHR*: Follicle stimulating hormone receptor mRNA; *IGF2R*: IGF type 2 receptor mRNA; CYP17A1: 17 alpha hydroxylase/17-20 lyase mRNA; and n = 112 to 145

Table 3.6: Follicular diameter and hormonal levels in follicular fluid from the largest three follicles of Control and Twinner cows.

		N	Di (mm)	SEM		E_2 (ng/ml)	SEM		P_4 (ng/ml)	SEM		A_4 (ng/ml)	SEM	
Genotype	**Control**	32	9.34	0.56	a	83.54	26.1	a	43.2	8.71		5.93	1.11	
	Twinner	45	10.82	0.47	b	157.72	22	b	35.66	7.29		6.74	0.95	
	P-value*		0.05			0.004			0.5			0.16		
Day of cycle	**Day 3**	39	9.5	0.51		134.12	23.7		28.52	7.83		6.16	1.05	
	Day 5	38	10.65	0.53		107.14	24.6		50.34	8.22		6.51	1.02	
	P-value*		0.12			0.86			0.1			0.92		
Follicle	**F1**	26	12.04	0.51	c	234.83	23.7	c	29.17	9.45	ab	8.28	0.86	b
	F2	26	9.8	0.51	b	105.2	23.7	b	26.33	9.45	a	4.39	0.86	a
	F3	25	8.39	0.53	a	21.86	24.6	a	62.79	9.82	b	-		
	P-value*		<.0001			<.0001			0.05			0.0003		
Genotype x Cycle	P-value*		0.35			0.72			0.3			0.34		
Genotype x Follicle	P-value*		0.69			0.43			0.28			0.55		
Cycle x Follicle	P-value*		0.49			0.95			0.08			0.67		
Genotype x Cycle x Follicle	P-value*		0.83			0.68			0.38			0.32		

[a, b] Within effect, means without a common superscript differ ($P < 0.05$) if interaction is not significant.

* P-value of ANOVA on Log transformed data for E_2 and P_4 only.

Abbreviations: Di: diameter; E_2: estradiol; P_4: progesterone; A_4: Androstenedione

Table 3.7: Granulosa cell gene expression of FSH receptor (FSHR), LH receptor (LHR), and IGF type 2 receptor (IGF2R) from the largest three follicles of Control and Twinner cows.

		FSHR mRNA			LHR mRNA			IGF2R mRNA			
		N	LSMean*	SEM	N	LSMean*	SEM	N	LSMean*	SEM	
Genotype	**Control**	27	4824.48	1493.5	27	1190.4	353.86	29	164.01	28.55	b
	Twinner	42	3621.8	1172.7	40	809.6	284.47	41	47.59	23.74	a
	P-value*		0.1658			0.6791			0.0216		
Day of cycle	**Day 3**	35	6845.79	1305.1 b	34	806.14	312.64	37	124.65	25.08	b
	Day 5	34	1600.5	1379.3 a	33	1193.86	329.23	33	86.95	27.38	a
	P-value*		0.0084			0.1441			0.0533		
Follicle	**F1**	25	5771.82	1547.3 b	23	751.8	377.87	25	46.3	27.38	
	F2	21	3775.42	1703.6 ab	21	782.34	403.62	21	160.64	29.93	
	F3	23	3122.19	1678.3 a	23	1465.86	397.64	24	110.46	29.64	
	P-value*		0.0454			0.4859			0.1114		
Genotype x Cycle	P-value*		0.1253			0.9315			0.2488		
Genotype x Follicle	P-value*		0.6258			0.3124			0.2123		
Cycle x Follicle	P-value*		0.2959			0.5699			0.8983		
Genotype x Cycle x Follicle	P-value*		0.8854			0.8816			0.4803		

[a, b] Within effect, means without a common superscript differ ($P < 0.05$) if interaction is not significant.

* LSMean of the mRNA relative abundance ($2^{-\Delta\Delta Ct}$)

** P-value of ANOVA on Log transformed data.

Table 3.8: Theca cell gene expression of LH receptor (LHR), IGF type 2 receptor (IGF2R) and 17 alpha hydroxylase/17-20 lyase (CYP17A1) from the largest three follicles of Control and Twinner cows.

		LHR mRNA				IGF2R mRNA				CYP17A1 mRNA		
		N	LSMean*	SEM		N	LSMean*	SEM		N	LSMean*	SEM
Genotype	**Control**	19	614.95	221.7		25	1586.52	662	b	20	1882.46	598.37
	Twinner	39	411.51	146.84		41	621.67	507.6	a	33	1022.51	462.8
	P-value*		0.2896				0.0584				0.8878	
Day of cycle	**Day 3**	30	403.25	185.74	b	36	1141.41	549.4		27	1471.78	542.32
	Day 5	28	623.2	190.3	a	30	1066.79	627.7		26	1433.16	527.38
	P-value*		0.9055				0.9325				0.6766	
Follicle	**F1**	21	476.15	202.31	b	22	212.56	619.6	a	18	757.26	656.94
	F2	20	459.32	223.07	ab	24	1985.22	596.9	b	17	2791.95	651.44
	F3	17	604.21	261.57	a	20	1114.51	720.8	b	18	808.24	656.94
	P-value*		0.779				0.0385				0.416	
Genotype x Cycle	P-value*		0.4823				0.507				0.4575	
Genotype x Follicle	P-value*		0.0391				0.1653				0.9095	
Cycle x Follicle	P-value*		0.7584				0.7734				0.557	
Genotype x Cycle x Follicle	P-value*		0.5568				0.5273				0.8462	

[a, b] Within effect, means without a common superscript differ ($P < 0.05$) if interaction is not significant.

* LSMean of the mRNA relative abundance ($2^{-\Delta\Delta Ct}$)

** P-value of ANOVA on Log transformed data.

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CHAPTER IV[1]

Insulin-Like Growth Factor 2 Stimulates Steroidogenesis and Mitosis of Bovine Granulosa Cells through the IGF-1 receptor: Role of Follicle-Stimulating Hormone and IGF2 receptor[2]

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Running Title: Granulosa Cell IGF2R and IGF2 Effects

[1]Published in Biol Reprod 2007, 77: 18-27

[2]Supported by National Research Initiative Competitive Grant no. 2005-35203-15334 from the USDA Cooperative State Research, Education, and Extension Service.

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ABSTRACT

Little is known regarding the role of insulin-like growth factor 2 (IGF2) and regulation of the IGF2 receptor (IGF2R) during follicular development. Granulosa cells were collected from small (1- 5 mm) and large (8 - 22 mm) bovine follicles, treated with IGF2 for 1 to 2 days in serum-free medium, and steroid production, cell proliferation, specific ^{125}I-IGF2 binding, and gene expression quantified. IGF2 increased both estradiol and progesterone production by granulosa cells, and cells from large follicles were more responsive to the effects of IGF2 than those from small follicles. Abundance of aromatase (*CYP19A1*) mRNA was stimulated by both IGF2 and IGF1. The effective dose (ED_{50}) of IGF2 stimulating 50% of the maximal estradiol production was 63 ng/ml for small follicles and 12 ng/ml for large follicles, and these values were not affected by FSH. The ED_{50} of IGF2 for progesterone production was 20 ng/ml for both small and large follicles. IGF2 also increased proliferation of granulosa cells by 2- to 3-fold as determined by increased cell numbers and ^{3}H-thymidine incorporation into DNA. Treatment with IGF1R antibodies reduced the stimulatory effect of IGF2 and IGF1 on estradiol production and cell proliferation. Specific receptors for ^{125}I-IGF2 existed in granulosa cells and two-day treatment with estradiol, FSH or cortisol had no significant effect on specific ^{125}I-IGF2 binding. Also, FSH treatment of small and large follicle granulosa cells had no effect on *IGF2R* mRNA levels whereas IGF1 decreased *IGF2R* mRNA and specific ^{125}I-IGF2 binding. Granulosa cell *IGF2R* mRNA abundance was threefold greater in small than large follicles. These findings support the hypothesis that both IGF2 and its receptor may play a role in granulosa cell function during follicular development. In particular, increased free IGF1 in developing follicles may decrease synthesis of IGF2R thereby

allowing for more IGF2 to be bioavailable (free) for induction of steroidogenesis and mitogenesis via the IGF1R.

(Key Words: Insulin-like Growth Factor 2, IGF 2 Receptor, Granulosa Cells)

INTRODUCTION

Stimulatory effects of insulin and insulin-like growth factor-I (IGF1) on estradiol production by mammalian granulosa cells are well documented [for review see (Hsueh et al., 1984; Adashi et al., 1985; Spicer and Echternkamp, 1995)], and are likely due, in part, to their ability to enhance the action of gonadotropins on ovarian follicular steroidogenesis (Davoren et al., 1986; Spicer et al., 2002). Female IGF1-null mice that survive to adulthood have reduced *FSHR* mRNA levels in granulosa cells, impaired ovarian steroidogenesis, and are infertile (Baker et al., 1996; Nakae et al., 2001) and provide further support for the important role of IGF1 on ovarian function. Although direct effects of IGF2 on granulosa cell function was discovered several years before IGF1 (Veldhuis and Hammond, 1979), little is known about the role of IGF2 and its receptor (IGF2R) in ovarian function. The present study was undertaken because in cattle, IGF2 is present in follicular fluid at concentrations greater than those of IGF1 (Spicer and Echternkamp, 1995; Stewart et al., 1996), thecal cell *IGF2* mRNA levels are greater in dominant than subordinate follicles (Yuan et al., 1998), and both granulosa and theca cells have *IGF2* mRNA (Schams et al., 2002). The interaction between gonadotropins and IGF2 on granulosa cell steroidogenesis, studied to some extent in

bovine theca cells (Spicer et al., 2004) and in granulosa cells of humans (Giudice, 1992; Nahum et al., 1995; Willis et al., 1998) and rats (Hsueh et al., 1984; Adashi et al., 1985), has not been examined in granulosa cells of cattle. IGF2R exist in granulosa and thecal cells of humans (el-Roeiy et al., 1993; el-Roeiy et al., 1994), rats (Hernandez, 1995), and ewes (Teissier et al., 1994), but IGF2 effects on granulosa cell steroidogenesis and mitogenesis are less than that of IGF1 (Teissier et al., 1994; Nahum et al., 1995). Based on previous studies in rat granulosa cells (Davoren et al., 1986) and bovine theca cells (Spicer et al., 2004), it appears that IGF2 acts through the IGF1 receptor (IGF1R) even though specific IGF2R exist in theca and granulosa cells. The function of these IGF2R in ovarian cell function is less clear, but it has been postulated that IGF2R may serve as a membrane bound IGF binding protein, inactivating IGF2 (Spicer et al., 2004). A similar role for the IGF2R during embryonic development has been postulated (Ludwig et al., 1996).

Therefore, to determine the biological function of IGF2 and its receptor in bovine granulosa cells, we set out first to evaluate the dose-response of IGF2 on granulosa cell proliferation and estradiol and progesterone production in the absence and presence of FSH, and then to evaluate the effect of various hormones on the IGF2 responses as well as on specific ^{125}I-IGF2 binding sites and *IGF2R* mRNA in bovine granulosa cells.

MATERIALS AND METHODS

1. Hormones and Reagents

Recombinant human IGF1 and IGF2 and anti-human IGF1R antibody for cell culture were obtained from R & D Systems (Minneapolis, MN); ovine F1913, FSH activity, 15 x NIHFSH-S1 U/mg) was obtained from Scripps Laboratories (San Diego, CA); methyl-^{3}H-thymidine was obtained from GE Healthcare (Arlington Heights, IL), staurosporine was obtained from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA), testosterone from Steraloids (Wilton, NH), and estradiol, cortisol, trypsin, collagenase, DNase, and fetal calf serum (FCS) were obtained from Sigma Chemical Co. (St. Louis, MO).

2. Cell Culture

Ovaries of cattle obtained at slaughter from a nearby abattoir were brought to the laboratory on ice and processed as previously described for obtaining granulosa cells from small (1 to 5 mm) and large (> 8 mm) follicles (Langhout et al., 1991; Spicer and Chamberlain, 1998). These follicle size categories were selected because: 1) previous studies indicate that granulosa cells from small follicles are less responsive to FSH and IGF1 than are cells from large follicles (Spicer and Chamberlain, 1998; Spicer et al., 2002), 2) the observations that follicles 8 mm and greater have much greater E2 concentrations than small follicles (Stewart et al., 1995; Spicer et al., 2001), 3) follicles that are destined to ovulate average 10 + 2 mm surface diameter (Marion et al., 1968),

and 4) selection of the dominant follicle occurs at about 8 mm in diameter (Ginther et al., 2000). Medium was a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 containing gentamicin, glutamine and sodium bicarbonate. Approximately 2 x 10^5 viable cells were seeded in each plastic multiwell containing 1 ml of medium. Prior to plating, granulosa cells were resuspended in medium containing 1.25 mg/ml of collagenase and 0.5 ml of DNase. Using trypan blue exclusion method, granulosa cell viability averaged 77 ± 3% and 67 ± 95 6% for small and large follicles, respectively, at time of plating. Cultures were kept at 38.5°C in a 95% air – 5% CO2 atmosphere, and for all experiments medium was changed every 24 h. This culture system utilizes serum-free medium so that specific effects of growth factors can be ascertained; FSH has little or no effect alone but when concomitantly treated with insulin or IGF1, FSH consistently stimulates aromatase activity (Spicer and Francisco, 1997; Spicer and Chamberlain, 1998). For Experiment 1, cells were cultured in medium containing 10% FCS for the first 48 h, washed twice with 0.5 ml of serum-free medium, and treated for an additional 48 h with various doses (0, 10, 30, or 100 ng/ml) of recombinant human IGF2, ovine FSH (0 or 30 ng/ml), and(or) recombinant human IGF1 (0 or 30 ng/ml) in serum-free medium containing 500 ng/ml of testosterone as an estrogen precursor. For Experiment 2, cells were cultured as in Experiment 1 except that after the first 48 h in culture, cells were treated for 24 h with serum-free medium with no additions, 100 ng/ml of IGF2, or 100 ng/ml of IGF1 after which cellular RNA was collected (see below). For Experiment 3, cells from small follicles were cultured for 48 h in 10% FCS, serum-starved for 24 h by culturing in serum-free medium, medium changed, and then cells cultured for an additional 40 h in serum-free medium with either no treatment, 100 ng/ml of IGF2, 100

ng/ml of IGF1, or 10% FCS in the presence of 1 μCi of ^{3}H-thymidine to assess DNA synthesis as previously described (Spicer and Hammond, 1989). For Experiment 4, granulosa cells were cultured from large follicles as described for Experiment 1 except that treatments were Control (no additions), FSH (30 ng/ml), IGF1 (30 ng/ml) or IGF2 (30 ng/ml), and viability was assessed using the trypan blue exclusion test. For Experiment 5, granulosa cells were cultured for 48 h in the presence of 10% FCS, 115 and then cells were washed and incubated in serum-free medium for 24 h with 0 or 25 ng/ml of IGF2 or IGF1 with 30 ng/ml of FSH and 500 ng/ml of testosterone. After 24 h, medium was replaced with medium containing FSH, testosterone, and 0 or 2.5 μg of anti-IGF1R antibody without or with 0 or 25 ng/ml of IGF2 or IGF1 for an additional 24 h. The cells were exposed to the anti-IGF1R antibody for 1 h before addition of IGF2 or IGF1. For Experiment 6, granulosa cells were cultured from small follicles as described for Experiment 1 except that treatments were FSH (30 ng/ml), IGF1 (100 ng/ml) or IGF2 (100 ng/ml), and the protein kinase inhibitor, staurosporine (0 or 10 nM). For Experiment 1, 4 and 6, medium was collected and stored at -20°C until radioimmunoassays previously validated in our laboratory (Langhout et al., 1991; Spicer and Chamberlain, 1998) were conducted to quantify progesterone and estradiol concentrations. Numbers of cells in the same wells that medium was collected were determined using a Coulter counter as previously described (Langhout et al., 1991; Stewart et al., 1995), and used to calculate steroid production on a ng or pg per cell basis. For Experiment 7, cells were maintained in 10% FCS for a total of 3 days; at the end of the culture period, ^{125}I-IGF2 binding assays were conducted as previously described (Spicer et al., 1994; Spicer and Stewart, 1996a; Spicer et al., 2004). For Experiment 8, granulosa cells were cultured for

48 h in the presence of 10% FCS, and then cells were washed and treated in serum-free medium for an additional 48 h with either estradiol (300 ng/ml), FSH (30 ng/ml) or cortisol (30 ng/ml) in the absence or presence of 30 ng/ml of IGF1 after which binding assays were conducted with 250,000 cpm of ^{125}I-IGF2 or ^{125}I-IGF1 in the absence or presence of 200 ng/well of IGF2 as previously described (Spicer et al., 1994; Spicer, 2001; Spicer et al., 2004). Briefly, the ^{125}I-IGF2 and ^{125}I-IGF1 assays were conducted directly in the culture wells with an assay volume of 400 μL. Incubation was for 16 h at 4°C for ^{125}I-IGF2 and ^{125}I-IGF1 and 200 ng/well of unlabeled IGF2 or IGF1 was used for determination of non-specific binding (Spicer et al., 1994; Spicer, 2001; Spicer et al., 2004). For Experiment 9 and 10, granulosa cells were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated for 24 h in serum-free medium with 0 or 30 ng/ml of IGF1 (Exp. 9) or 100 ng/ml of IGF2 with 0 or 30 ng/ml of FSH (Exp. 10). At the end of culture, RNA was collected and real-time RT-PCR was used to quantify *IGF2R* and *FSHR* mRNAs both of which were normalized to constitutively expressed 18S ribosomal RNA (see below). For Experiment 11, aliquots of freshly collected granulosa and theca cells were treated with TRIzol, RNA collected and real-time RT-PCR used to quantify *IGF2R* mRNA which was normalized to constitutively expressed 18S ribosomal RNA (see below).

3. RNA Extraction and RT-PCR

For Experiment 2, 9, 10 and 11, granulosa cells were lysed in 0.5 ml of TRIzol Reagent (Invitrogen, Carlsbad, CA), RNA extracted, and RNA quantity determined

spectrophotometrically at 260 nm as previously described (Voge et al., 2004a; Voge et al., 2004b) The target gene primers (forward, reverse) and probe sequences for *CYP19A1* (Accession NM_174305) were TGCCAAGAATGTTCCTTACAGGTA, CACCATGGCGATGTACTTTCC, CATTTGGCTTTGGGCCCCGG, respectively; for *CYP11A1* (Accession NM_176644) were ACAGGGAGAAGCTTGGCAATT, GTAGGATCCCTCGAACTTGAAGA, and AGTTTATATCATTCACCCTGAAGACGTGGCCC, respectively; for *IGF2R* (Accession 155 AF342811) were GCAATGCTAAGCTTTCGTATTACG, GGTGTACCACCGGAAGTTGTATG, and ACGCCGGAGTGGGTTTCCCC, respectively; and for *FSHR* (Accession L22319) were AACCTGCTATACATCGACCCTGAT, GCTTAATACCTGTGTTGGATATTAACAGA, and CCTTCCAGAACCTTCCCAACCTCCG, respectively. A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) was also conducted to insure the specificity of the designed primers and probe and to 160 assure that they were not designed from any homologous regions, coding for other genes. Furthermore, the RT-PCR product was run on an agarose gel to verify the length and size of the expected target gene. Also, 6 µl of the same RT-PCR cDNA sample was treated with 0.5 µl of shrimp alkaline phosphatase (Amersham Biosciences, Piscataway, NJ) and 0.5 µl of exonuclease I (Amersham Biosciences), incubated at 37ºC for 30 min and further incubated at 85ºC for 15 min before sequencing, to verify the sequence and specificity of the RT-PCR quantification of the desired target gene. The differential expression of target gene mRNA in granulosa cells was quantified using the one-step real-time RT-PCR reaction for Taqman® Gold

RT-PCR Kit (Applied Biosystems, Foster City, CA) as previously described (Voge et al., 2004a; Voge et al., 2004b). Briefly, based on preliminary optimization results, 50 or 100 ng of total RNA was amplified in a total reaction volume of 25 µl consisting of 200 nM forward primer, 200 nM reverse primer and 200 nM fluorescent (FAM/TAMRA) probe for each target gene, 10 nM of 18 S rRNA primers and 100 nM of the 18S rRNA VIC-labeled probe, along with 12.5 µl of TaqMan Master Mix without uracil N-glycosylase, and 1 U Multiscribe with RNase inhibitor (Applied Biosystems). Thermal cycling conditions were set to 30 min at 48.8ºC for reverse transcription, 95ºC for 10 min for AmpliTaq Gold Activations, and finished with 45 cycles at 95ºC for 15 sec for denaturing and 60ºC for 1 min for annealing and extension. The 18S rRNA values were used as internal controls to normalize samples for any variation in amounts of RNA loaded as previously described (Voge et al., 2004a; Voge et al., 2004b). All samples were run in duplicate. Relative quantification of target gene mRNAs were expressed using the comparative threshold cycle method as previously described (Voge et al., 2004a; Voge et al., 2004b; Aad et al., 2006). Briefly, the ΔCt was determined by subtracting the 18S Ct value from the target unknown value. For each target gene and within each experiment, the ΔΔCt was determined by subtracting the higher ΔCt (the least expressed unknown) from all other ΔCt values. Fold changes in target gene mRNA expression were calculated as being equal to $2^{-\Delta\Delta Ct}$.

4. Statistical Analyses

Experimental data are presented as means ± SEM of measurement from replicated

experiments. Each experiment was replicated three or more times, and within each experiment, treatments were applied in triplicate culture wells. Each experiment was conducted on a separate pool of granulosa cells obtained from 8 to 15 cows or heifers. The main effects and their interactions on the variables measured were assessed by general linear models procedure of SAS (SAS, 1996). To correct for heterogeneity of variance, estradiol production, *CYP19A1* mRNA, *IGF2R* mRNA, and ^{125}I-IGF1 binding were analyzed after transformation natural log (x + 1). Specific differences among treatments were tested using Fisher's protected least-significant difference procedure (Ott, 1977). Significance was declared at ($P < 0.05$) unless noted otherwise. Specific binding of ^{125}I-IGF2 and ^{125}I-IGF1 to granulosa cells was expressed as cpm/100 cells or as a percentage of total binding.

RESULTS

Experiment 1 was conducted to compare the dose-response of IGF2 on basal and FSH-induced steroid production by granulosa cells collected from small (Exp. 1A) and large (Exp. 1B) follicles. In Experiment 1A, granulosa cells from small follicles treated with 10 and 30 ng/ml of IGF2 in the absence of FSH had no effect ($P > 0.10$) on estradiol production, whereas 100 ng/ml increased estradiol production by 8-fold (Fig. 4.1A). In the presence of FSH, all doses of IGF2 tested increased ($P < 0.05$) estradiol production with maximal effects observed in small-follicle granulosa cells (I.e., 24-fold increase; Fig. 4.1A) with 100 ng/ml of IGF2, and 10 with 30 ng/ml of IGF2 in granulosa cells from large follicles (I.e., 7-fold; Fig. 4.1B). In small-follicle granulosa cells, 30 and 100 ng/ml

of IGF2 increased progesterone production by 2- to 3- fold regardless of whether FSH was present (Fig. 4.2A). Similarly, granulosa cells from large follicles (Experiment 1B) treated with 30 and 100 ng/ml of IGF2 in the absence of FSH increased ($P < 0.001$) both estradiol (Fig. 4.1A) and progesterone (Fig. 4.2B) production by 2- to 4-fold above controls. The estimated effective dose (ED_{50}) of IGF2 stimulating 50% of the maximal aromatase response (calculated from stimulation curves that were linearized using a semi-log plot) averaged 63 ng/ml and 12 ng/ml for small- and large-follicle granulosa cells, respectively, and these values were not affected by FSH. The estimated effective dose (ED_{50}) of IGF2 stimulating 50% of the maximal progesterone response averaged 20 ng/ml for both small and large-follicle granulosa cells and this value was not affected by FSH. Also, IGF2 increased ($P < 0.05$) cell numbers by 1.4- to 2.2-fold (Table 4.1).

Experiment 2 was conducted to compare the effect IGF2 and IGF1 on *CYP19A1* and *CYP11A1* mRNA abundance in small and large follicle granulosa cells. Treatment of granulosa cells with 100 ng/ml of either IGF2 or IGF1 increased ($P < 0.05$) *CYP19A1* mRNA abundance by threefold in small and large follicle granulosa cells (Fig. 4.3A). Although the increases induced by IGF2 and IGF1 were similar within each cell type, the amount of *CYP19A1* mRNA was 8-fold greater ($P < 0.05$) in granulosa cells from large than small follicles (Fig. 4.3A). Neither IGF2 nor IGF1 affected *CYP11A1* mRNA abundance in granulosa cells (Fig. 4.3B). The amount of *CYP11A1* mRNA was 2.3-fold greater ($P < 0.05$) in granulosa cells of large than small follicles (Fig. 4.3B).

Experiment 3 was conducted to compare the effect IGF2 and IGF1 on DNA synthesis as measured by ^{3}H-thymidine incorporation. Treatment of granulosa cells from small

follicles with 100 ng/ml of either IGF2 or IGF1 increased ($P < 0.05$) ^{3}H-thymidine incorporation similarly (by fourfold) but to a lesser extent than 10% FCS (13-fold increase; Fig. 4.4).

Experiment 4 was conducted to compare the effect FSH, IGF1 and IGF2 on viability of large-follicle granulosa cells. Compared with untreated Controls, 2-day treatment of granulosa cells with 30 ng/ml of FSH, 100 ng/ml of IGF2 or 100 ng/ml of IGF1 had no effect ($P < 0.05$) on cell viability which averaged 63% (Table 4.2). FSH had no effect on cell numbers whereas IGF1 and IGF2 increased ($P < 0.05$) cell numbers 1.8- and 1.6-fold above controls, respectively (Table 4.2). Also, IGF1 and IGF2 increased ($P < 0.05$) basal progesterone production by 7.3- and 5.9-fold above controls and basal estradiol production by 14.5- and 19.5-fold above controls. FSH increased ($P < 0.05$) progesterone production by 52% and tended to increase ($P < 0.08$) estradiol production by 69% (Table 4.2).

Experiment 5 was conducted to determine if blocking the IGF1R reduced the stimulatory effect of IGF2 or IGF1 on granulosa cell steroidogenesis and cell proliferation. Treatment of large-follicle granulosa cells with 2.5 μg/ml of an anti-IGF1R antibody during the second 24 h treatment with IGF2 or IGF1 significantly decreased the stimulatory effect of 25 ng/ml of IGF2 and IGF1 on estradiol production (Fig. 4.5A) and cell proliferation (Fig. 4.5B). Anti-IGF1R antibody had no effect on estradiol production stimulated by FSH and had no effect on basal cell numbers (Fig. 4.5). The stimulatory effect of IGF2 and IGF1 on progesterone production was not significantly altered by concomitant treatment with the anti-IGF1R antibody (data not shown).

Experiment 6 was conducted to compare the effect of a protein kinase inhibitor, staurosporine, on IGF1- and IGF2-induced steroid production and cell numbers. Two-day treatment of small-follicle granulosa cells with 10 nM of staurosporine caused a reduction ($P < 0.05$) in the aromatase (Fig. 4.6A) and cell proliferation (Fig. 4.6B) response to both IGF1 and IGF2. IGF1 increased estradiol and progesterone production to a greater ($P < 0.05$) level than IGF2. Staurosporine had no effect ($P > 0.10$) on progesterone production induced by IGF1 or IGF2 which averaged 156 and 89 + 5 ng/10^5 cells/24 h, respectively.

Experiment 7 was conducted to compare IGF1 and IGF2 competition for ^{125}I-IGF1 and ^{125}I-IGF2 binding sites on granulosa cells. In Experiment 7A, 1 and 100 ng/well of IGF2 inhibited binding of ^{125}I-IGF2 to granulosa cells to a greater extent than the same doses of IGF1 (Fig. 4.6A). In Experiment 7B, 3, 10 and 100 ng/well of IGF1 and IGF2 inhibited granulosa ^{125}I-IGF1 binding whereas 1 ng/well of IGF1 or IGF2 had no effect on ^{125}I-IGF1 binding (Fig. 4.6B). These and additional ligand binding experiments revealed that IGF1 cross-reactivity with granulosa IGF2R averaged 0.3% whereas cross-reactivity of IGF2 with granulosa IGF1R averaged 53%.

Experiment 8 was conducted to determine if various hormones can alter specific ^{125}I-IGF2 binding sites on granulosa cells. Two-day treatment of small-follicle granulosa cells with estradiol (300 ng/ml), FSH (30 ng/ml) or cortisol (30 ng/ml) in the presence or absence of 30 ng/ml of IGF1 had no significant effect on specific binding of ^{125}I-IGF2 to granulosa cells (Fig. 4.7A). However, 2-day treatment with IGF1 decreased ($P < 0.05$) specific ^{125}I-IGF2 binding to granulosa cells (Fig. 4.7A).

Experiment 9 was conducted to determine the effect of IGF1 on *IGF2R* and *FSHR*

mRNA levels in small-follicle granulosa cells. Granulosa cells were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated with 0 or 30 ng/ml of IGF1 for 24 h and RNA was collected. Real-time PCR revealed that levels of *IGF2R* mRNA was decreased ($P < 0.05$) by 29% whereas the level of *FSHR* mRNA was increased ($P < 0.05$) by 3.3- fold with IGF1 treatment (Fig. 4.7B).

Experiment 10 was conducted to determine the effect of FSH on *IGF2R* mRNA and *FSHR* mRNA abundance in granulosa cells. Granulosa cells from small and large follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated in the presence of 30 ng/ml of IGF2 without or with 30 ng/ml of FSH for 24 h. Real-time PCR revealed that FSH had no effect on *FSHR* (Fig. 4.8A) or *IGF2R* (Fig. 4.8B) mRNA levels in granulosa cells collected from small or large follicles.

Experiment 11 was conducted to determine if theca and granulosa cells collected from small and large follicles differed in *IGF2R* mRNA levels. Real-time RT-PCR revealed that granulosa cells from small follicles had three-fold greater ($P < 0.05$) *IGF2R* mRNA abundance than granulosa cells from large follicles (Fig. 4.9). In contrast, theca cells from large follicles had 14-fold greater ($P < 0.01$) *IGF2R* mRNA abundance than theca cells from small follicles (Fig. 4.9).

DISCUSSION AND CONCLUSION

For the first time, developmental and hormonal regulation of IGF2R gene expression and its function in granulosa cells was documented. In particular, results of

the present study revealed that: 1) IGF2 increased both estradiol and progesterone production by granulosa cells, and cells from large follicles were more responsive to the effects of IGF2 than those from small follicles; 2) IGF2 also increased cell numbers and DNA synthesis; 3) IGF2 and IGF1 increased abundance of *CYP19A1* mRNA but did not affect abundance of *CYP11A1* mRNA in granulosa cells; 4) the stimulatory effects of IGF2 and IGF1 on estradiol production and cell proliferation were inhibited by addition of antibodies against IGF1R; 5) IGF2 and IGF1 competed for both IGF1R and IGF2R; 6) 2-day treatment with IGF1 inhibited ^{125}I-IGF2 binding and *IGF2R* mRNA levels, whereas FSH had no effect; and 7) granulosa cell *IGF2R* mRNA levels were greater in small than large follicles, whereas theca cell *IGF2R* mRNA levels were greater in large than small follicles. Thus, IGF2 appears to increase proliferation and stimulate differentiation in granulosa cells via the IGF1R, and the IGF2R may be hormonally regulated and associated with decreased granulosa cell responses via acting as a type of decoy receptor for IGF2.

Previously, little evidence existed that characterized the interaction between FSH and IGF2 on granulosa cell steroidogenesis and mitogenesis in cattle. We observed that aromatase activity in granulosa cells from large follicles was much more sensitive to the effect of IGF2 than small follicles, and that FSH and IGF2 synergized to induce steroidogenesis in bovine granulosa cells. This synergism likely involves IGF-Induced upregulation of the FSHR as shown in the present and previous studies (Zhou et al., 1997), but likely does not involve FSH-induced down-regulation of the IGF2R since FSH did not alter IGF2 specific binding or *IGF2R* mRNA in the present study. Alternatively, differences in cellular responses to IGF2 and FSH may depend on the interactions among

intracellular cascade mechanisms (e.g., protein kinase C and cAMP pathways) that may co-exist within a particular cell type. Such differences for IGF1 signaling exist between small and medium-follicle granulosa cells in pigs (Hylka et al., 1989). Specific intracellular signaling systems that contribute to aromatase and progesterone production in FSH responsive cells include MAP kinase (Amsterdam et al., 2003), phosphatidylinositol 3-kinase (Cunningham et al., 2003), and protein kinase A and B (Cottom et al., 2003; Zeleznik et al., 2003). Inhibition of IGF1- and IGF2-induced aromatase activity by the protein kinase inhibitor, staurosporine, supports the role of protein kinases in both IGF2 and IGF1 signal transduction. However, which specific intracellular protein kinases are activated by IGF1 and IGF2 in granulosa cells will require further study.

Gene expression studies revealed that granulosa cells from large follicles had greater abundance of *CYP19A1* mRNA than those from small follicles, and in both cell types IGF2 and IGF1 induced *CYP19A1* mRNA similarly. The ED_{50} for IGF2-stimulated steroid production by bovine granulosa cells of the present study (i.e., 12 to 63 ng/ml) is within the range of those reported for rat (Adashi et al., 1985) and human (Erickson et al., 1990; Nahum et al., 1995) granulosa cells and bovine theca cells (Spicer et al., 2004). Also, consistent with a previous report (Spicer et al., 2002), estradiol production by small-follicle granulosa cells was less sensitive to the stimulatory effects of FSH on estradiol production than large-follicle granulosa cells. As previously suggested (Spicer, 2004), it is likely that in the presence of elevated FSH, only a small amount of “free” IGF2 or IGF1 is needed to increase estradiol production by the early dominant follicle as the level of IGFBP decreases in follicular fluid. A similar conclusion was made regarding

the role of IGF1 in stimulating aromatase activity of granulosa cells in an elevated FSH environment (Spicer et al., 2002). The present study revealed that the ED_{50} of IGF2 (i.e., 12 to 63 ng/ml) for bovine granulosa cell progesterone and estradiol production is about two-fold greater than that of IGF1 (5 to 36 ng/ml; (Spicer et al., 2002)), and that the maximal effect of IGF2 (i.e., 7- to 24-fold increases) is similar to or slightly less than that of IGF1 (i.e., 13- to 37-fold increases; (Spicer and Chamberlain, 1998; Spicer et al., 2002)).

In the present study, the maximal effect of IGF2 on estradiol production was usually less than IGF1 in granulosa cells of small follicles but estradiol responses were similar for IGF1 and IGF2 in granulosa cells of large follicles. Previous reports using human (Willis et al., 1998) and rat (Davoren et al., 1986; Adashi et al., 1990) granulosa cells and bovine theca cells (Spicer et al., 2004) indicated that the maximal effect of IGF2 on steroidogenesis was less than that of IGF1. Because bovine theca cells from large follicles do not respond to IGF2 as well as granulosa cells and have a greater abundance of *IGF2R* mRNA than granulosa cells, perhaps the relative maximal effects of IGF2 versus IGF1 are dependent on the relative proportions of IGF1R and IGF2R present which may be influenced by cell type and(or) species.

In the present study, IGF2 competed half as well as IGF1 with ^{125}I-IGF1 binding sites and anti-IGF1R antibodies attenuated the stimulatory effect of IGF2 and IGF1 on estradiol production and cell proliferation, and thus, we speculate that the stimulatory effect of IGF2 on granulosa steroidogenesis and cell proliferation is mediated via IGF1R. Although not reported previously for bovine granulosa cells, a similar conclusion was

reached using bovine adrenocortical cells (Weber et al., 1995) and bovine theca cells (Spicer et al., 2004). Previous studies have documented the presence of ^{125}I-IGF1 and ^{125}I-IGF2 binding sites in granulosa cells of cattle (Spicer and Stewart, 1996b; Stewart et al., 1996; Spicer and Chamberlain, 1998; Spicer, 2001) and sheep (Teissier et al., 1994) as well as IGF1R and/or *IGF2R* mRNA in granulosa cells of sheep (Perks et al., 1995; Hastie and Haresign, 2006a), pigs (Zhou et al., 1996), rats (Hernandez et al., 1990; Hernandez, 1995) and humans (Hernandez et al., 1992; el-Roeiy et al., 1993; el-Roeiy et al., 1994; Voutilainen et al., 1996). We further speculate that because IGF2R are present on granulosa cells and the ED_{50} of IGF2 on granulosa cell steroidogenesis is appreciably less than IGF1, IGF2R may act as a type of decoy receptor via binding and inactivating IGF2. This latter suggestion was made for bovine theca cells (Spicer et al., 2004) and is further supported by studies of Adashi et al. (Adashi et al., 1990) that reported the granulosa cell IGF2R does not participate in transmembrane IGF signaling. Similarly, in human MCF-7 breast cancer cells, the IGF2R is thought to operate as an IGF2 antagonist, suppressing IGF2-induced cellular proliferation (Ellis et al., 1996). In the present study, IGF2 receptors (*IGF2R* mRNA and specific ^{125}I-IGF2 binding) were decreased by IGF1 but not affected by FSH, estradiol or cortisol. This suggests that as free IGF1 increases within the growing follicle, it suppresses IGF2R in granulosa cells thereby reducing the attenuating effect that IGF2R may have on IGF2-stimulated differentiation. Consistent with the present study, FSH and estrogen treatment had no effect on ovarian *IGF2R* mRNA levels in rats (Hernandez, 1995). Physiologic changes in IGF2R in granulosa cells have not been studied previously in cattle, but in bovine corpora lutea, *IGF2R* mRNA levels significantly increased during mid- and late-cycle (Neuvians et al., 2003; Hastie

and Haresign, 2006b). In ewes, follicular *IGF2R* mRNA levels measured using in situ hybridization were greater in atretic than healthy follicles and greater in small than large follicles (Hastie and Haresign, 2006a). A greater number of IGF2R in small than large follicles measured in the present study along with a less sensitive steroid response to IGF2 is consistent with the notion that IGF2R reduces the biological effect of IGF2. It should be noted that IGF2R may serve other functions in addition to acting as a negative regulator of IGF2 ligand bioavailability (Braulke, 1999). For example, in other cell types IGF2R can bind urokinase-type plasminogen activator receptor and plasminogen (Kreiling et al., 2003; Veugelers et al., 2006), and may be a receptor for retinoic acid (Kang et al., 1997). Whether these other functions of the IGF2R are operative in ovarian tissue or granulosa cells in particular will require further elucidation. For the first time, a stimulatory effect of IGF2 was observed on bovine granulosa cell proliferation as measured by increased cell numbers and increased ^{3}H-thymidine incorporation in the present study. These observations agree with previous reports in which treatment with IGF2-increased ^{3}H-thymidine incorporation and numbers of porcine granulosa (Kamada et al., 1992; Kolodziejczyk et al., 2001) and human granulosa-lutein (Di Blasio et al., 1994) cells. Moreover, the stimulatory effect of IGF2 on cell numbers was significantly reduced with concomitant treatment with anti-IGF1R antibodies or a protein kinase inhibitor staurosporine. The lack of an effect of IGF1 or IGF2 on cell viability suggest that IGF1 and IGF2 may be targeting cell cycle events more than anti-apoptotic events to induce proliferation of granulosa cells. Collectively, these studies indicate that IGF2 acting through IGF1R may be involved in growth of the granulosa layer within ovarian follicles in addition to its stimulatory effect on steroidogenesis.

Because IGF2-induced increases in estradiol production (I.e., < 2 ng/10^5 cells/24 h) are no more than 10% of the progesterone produced by granulosa cells in the present study, it is likely that IGF2 also acts to enhance de novo steroidogenesis from cholesterol in bovine granulosa cells. In support of this suggestion, inhibition of HMG-CoA reductase, a key enzyme for de novo cholesterol synthesis, dramatically reduces IGF1-induced progesterone production by bovine granulosa cells (Spicer et al., 1996). Although IGF2 stimulates *CYP11A1* mRNA by several-fold in porcine granulosa cells (Garmey et al., 1993), IGF2 had no effect on *CYP11A1* mRNA in bovine granulosa cells in the present study suggesting that species differences may exist in terms of IGF2 responsiveness. Interestingly, the anti-IGF1R antibody inhibited estradiol production but not progesterone production, suggesting that cholesterol stores that can serve as progestin precursors are increased during the first 24 h of treatment with IGF2 and IGF1 so that sufficient progestin precursor existed to maintain progesterone production during the second 24 h treatment period. It is unlikely that the granulosa cells luteinized with time in culture in the present study because: 1) progesterone production remains constant or does not increase with time using this culture paradigm (Langhout et al., 1991; Spicer and Chamberlain, 1998), 2) the morphology of the granulosa cells retain a fibroblastic appearance (Chamberlain and Spicer, 2001), 3) aromatase activity of granulosa cells remains responsive to FSH and IGF1, and their responses increase between days 3 and 4 of culture (Spicer and Chamberlain, 1998; Spicer et al., 2002), and 4) granulosa cells from small and large follicles have little or no progesterone response to LH (Spicer et al., 2002).

In conclusion, this study is first to demonstrate expression and function of IGF2R

in bovine granulosa cells indicating that the IGF2R may serve as a type of decoy receptor to modulate IGF2 action. Moreover, IGF2 and IGF1 may share common intracellular cascade mechanisms in bovine granulosa cells in spite of the fact that specific receptors for each hormone exists in this cell type. Additional studies will be required to further characterize the hormonal regulation of IGF2R in granulosa cells and whether changing levels of granulosa IGF2R are involved in the control of follicle growth.

FIGURES

Figure 4.1. Effect of 2-day treatment of IGF2 on basal and FSH-induced estradiol production by small (Panel A) and large (Panel B) follicle granulosa cells (Experiment 1). Cells were cultured for 48 h in the presence of 10% FCS, and then treated in serum-free medium for an additional 48 h with 0, 10, 30 or 100 ng/ml of IGF2 in the absence (solid circle dashed line) or presence (filled box, solid line) of 30 ng/ml of FSH. Values are means ± SEM of triplicate culture wells replicated in three separate experiments (n = 9). *Mean differs ($P < 0.05$) from its respective 0 ng/ml dose. **Mean differs ($P < 0.05$) from the immediately preceding value.

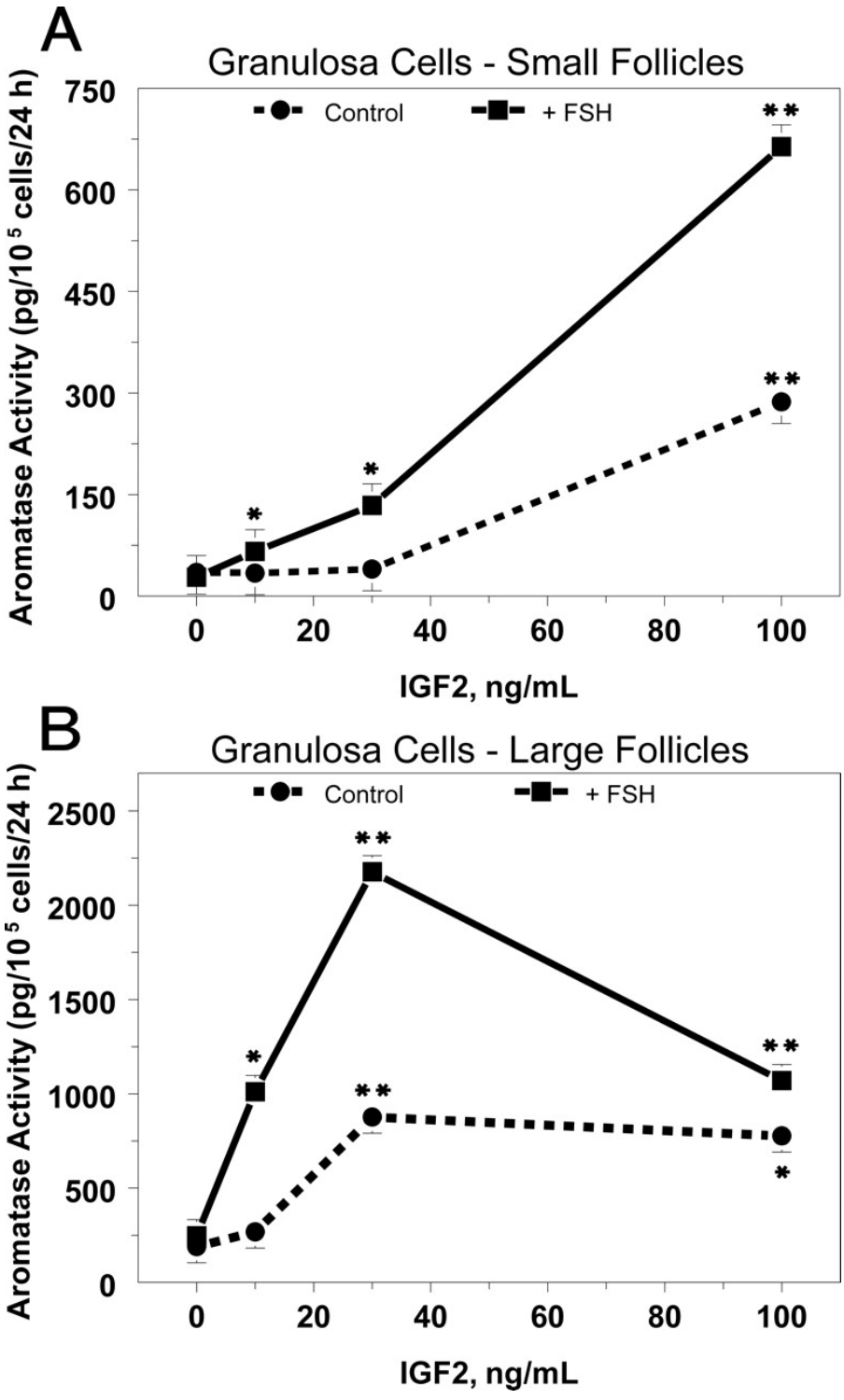

A
Granulosa Cells - Small Follicles
Control
+ FSH
Aromatase Activity (pg/10 5 cells/24 h)
750
600
450
300
150
0
0
20
40
60
80
100
IGF2, ng/mL
B
Granulosa Cells - Large Follicles
Control
+ FSH
Aromatase Activity (pg/10 5 cells/24 h)
2500
2000
1500
1000
500
0
0
20
40
60
80
100
IGF2, ng/mL

Figure 4.2. Effect of 2-day treatment of IGF2 on basal and FSH-induced progesterone production by small (Panel A) and large (Panel B) follicle granulosa cells (Experiment 1). Cells were cultured for 48 h in the presence of 10% FCS, and then treated in serum-free medium for an additional 48 h with 0, 10, 30 or 100 ng/ml of IGF2 in the absence (solid circle dashed line) or presence (filled box, solid line) of 30 ng/ml of FSH. Values are means ± SEM of triplicate culture wells replicated in three separate experiments (n = 9). *Mean differs ($P < 0.05$) from its respective 0 ng/ml dose. **Mean differs ($P < 0.05$) from the immediately preceding value.

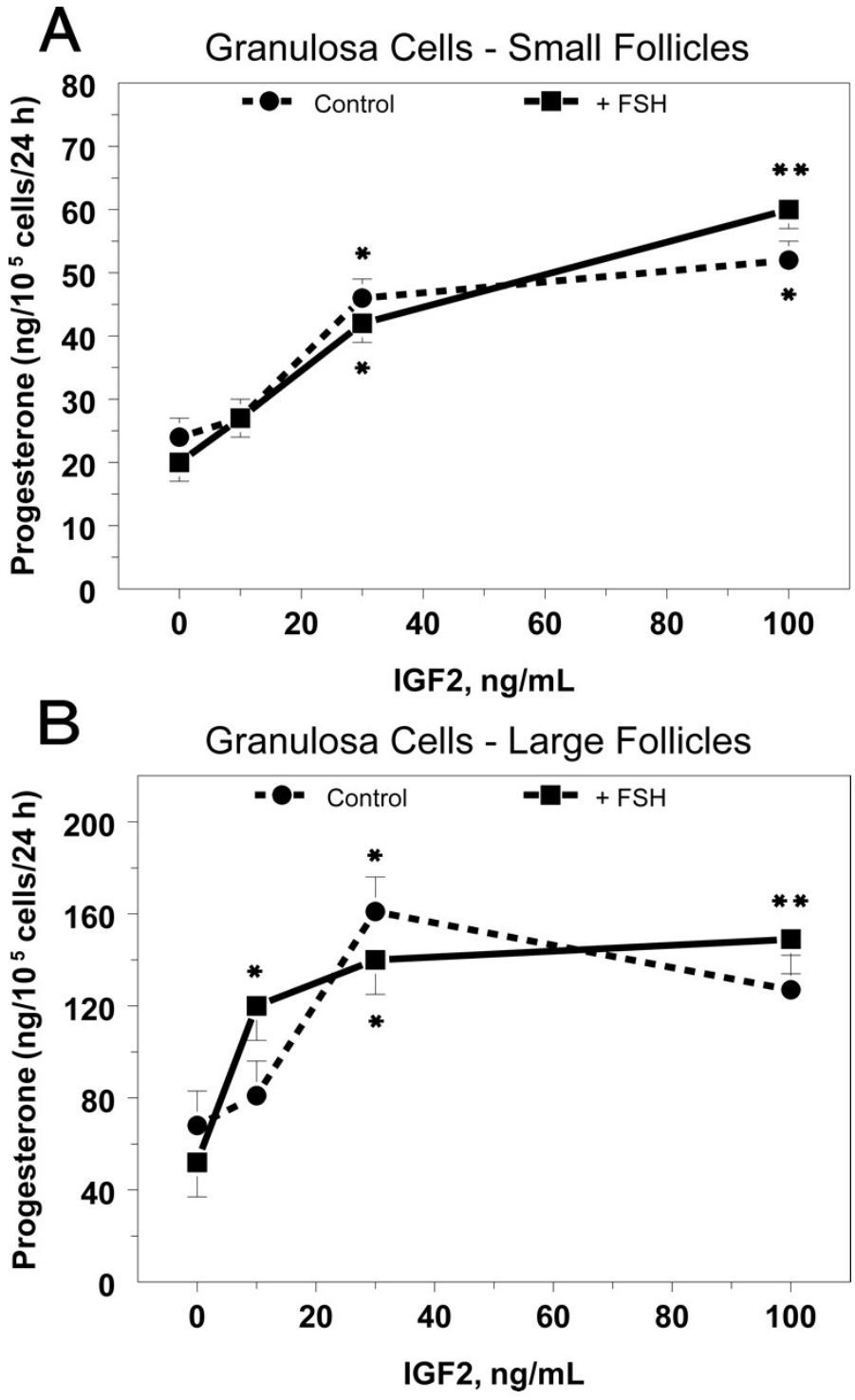

A
Granulosa Cells - Small Follicles
Control
+ FSH
Progesterone (ng/10 5 cells/24 h)
0
10
20
30
40
50
60
70
80
0
20
40
60
80
100
IGF2, ng/mL
B
Granulosa Cells - Large Follicles
Control
+ FSH
Progesterone (ng/10 5 cells/24 h)
0
40
80
120
160
200
0
20
40
60
80
100
IGF2, ng/mL

Figure 4.3. Effect of IGF1 and IGF2 on CYP19A1 (Panel A) and CYP11A1 (Panel B) mRNAs in granulosa cells (Experiment 2). Granulosa cells from small and large follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated in the presence of 100 ng/ml of IGF2 or IGF1 for 24 h. Values are means of three separate experiments (± SEM) and normalized to constitutively expressed 18S ribosomal RNA. a,b,c,d Within a panel, means without a common superscript differ ($P < 0.05$).

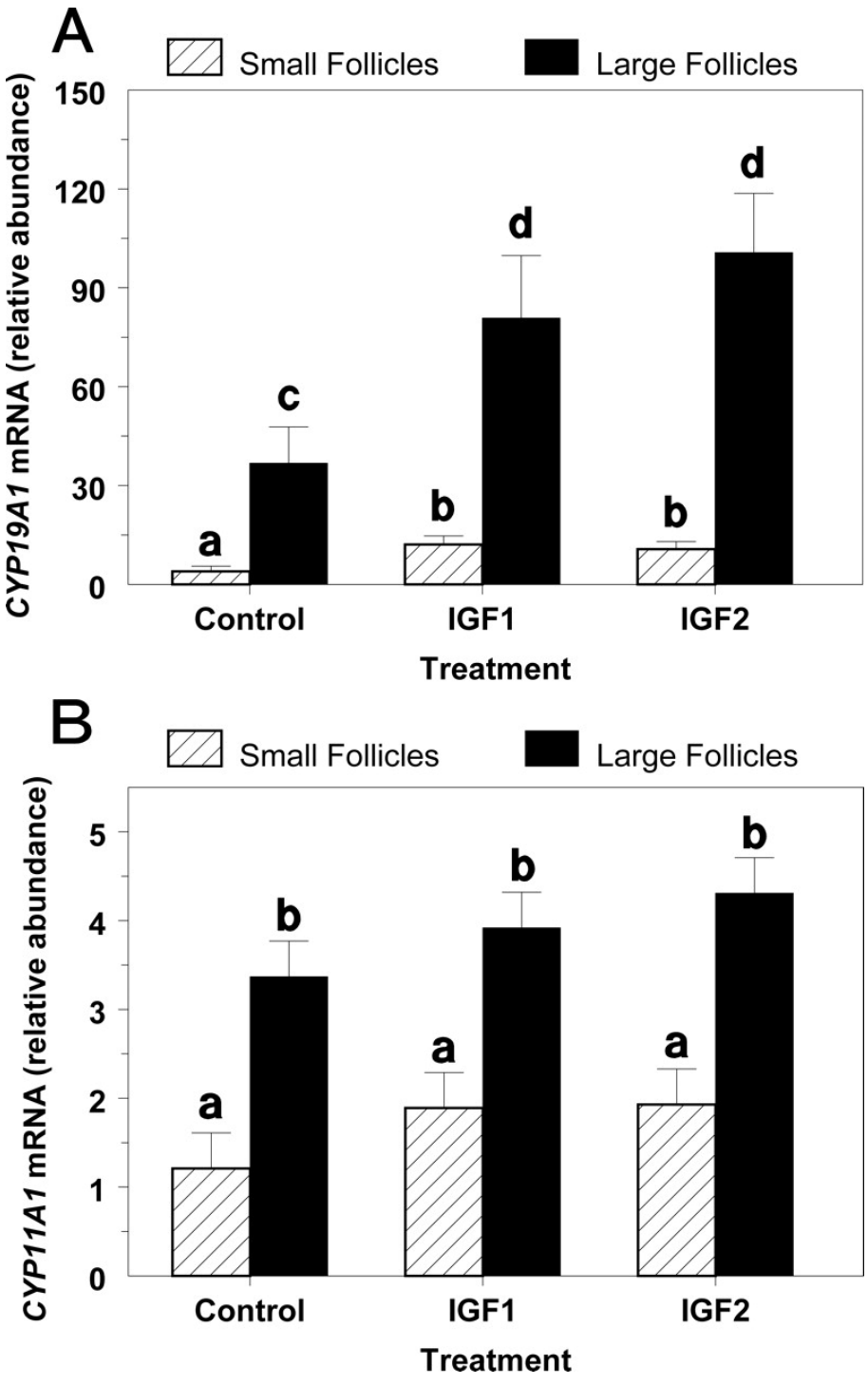

A
Small Follicles
Large Follicles
CYP19A1 mRNA (relative abundance)
150
120
90
60
30
0
a
c
b
d
b
d
Control
IGF1
IGF2
Treatment
B
Small Follicles
Large Follicles
CYP11A1 mRNA (relative abundance)
5
4
3
2
1
0
a
b
a
b
a
b
Control
IGF1
IGF2
Treatment

Figure 4.4. Comparison of the effect of IGF1 and IGF2 on DNA synthesis in bovine granulosa cells (Experiment 3). Granulosa cells from small follicles were cultured for 48 h in 10% FCS, serum-starved for 24 h in serum-free medium, and then cultured with various treatments for 40 h in the presence of 1 μCi of ^{3}H-thymidine. Values are means ± SEM of triplicate culture wells replicated in three separate experiments (n = 9). a,b,c Means without a common superscript differ ($P < 0.05$).

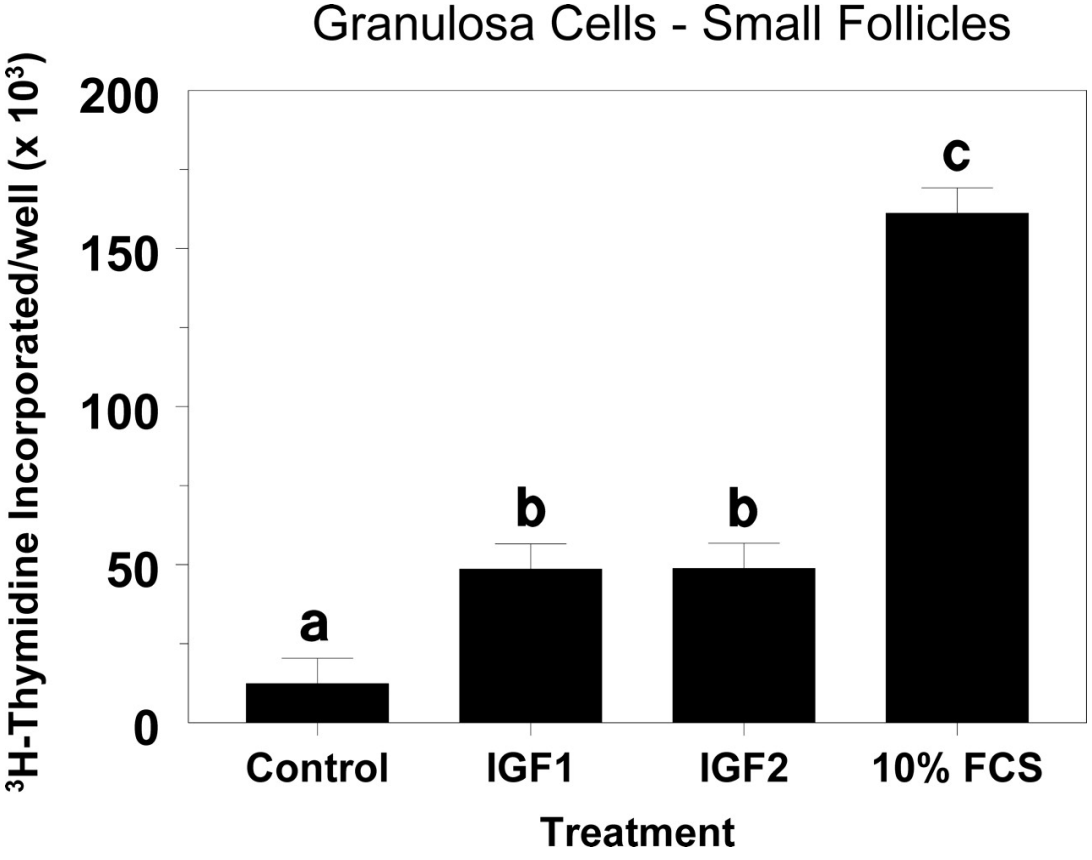

Granulosa Cells - Small Follicles
^{3}H-Thymidine Incorporated/well (x 10^3)
200
150
100
50
0
a
b
b
c
Control
IGF1
IGF2
10% FCS
Treatment

Figure 4.5. Comparison of the effect of an anti-IGF1R antibody on IGF1- and IGF2-induced estradiol production (Panel A) and proliferation (Panel B) of bovine granulosa cells (Experiment 5). Granulosa cells from large follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated in serum-free medium for 24 h with 0 or 25 ng/ml of IGF2 or IGF1 with 30 ng/ml of FSH. After 24 h, medium was replaced with medium containing 0 or 5 μg of anti-IGF1R antibody without or with 0 or 25 ng/ml of IGF1 or IGF2 for an additional 24 h. The cells were exposed to the anti-IGF1R antibody for 1 h before addition of IGF1 or IGF2. Values are means ± SEM of triplicate culture wells replicated in three separate experiments (n = 9). a,b,c,d,e Within a panel, means without a common superscript differ ($P < 0.05$).

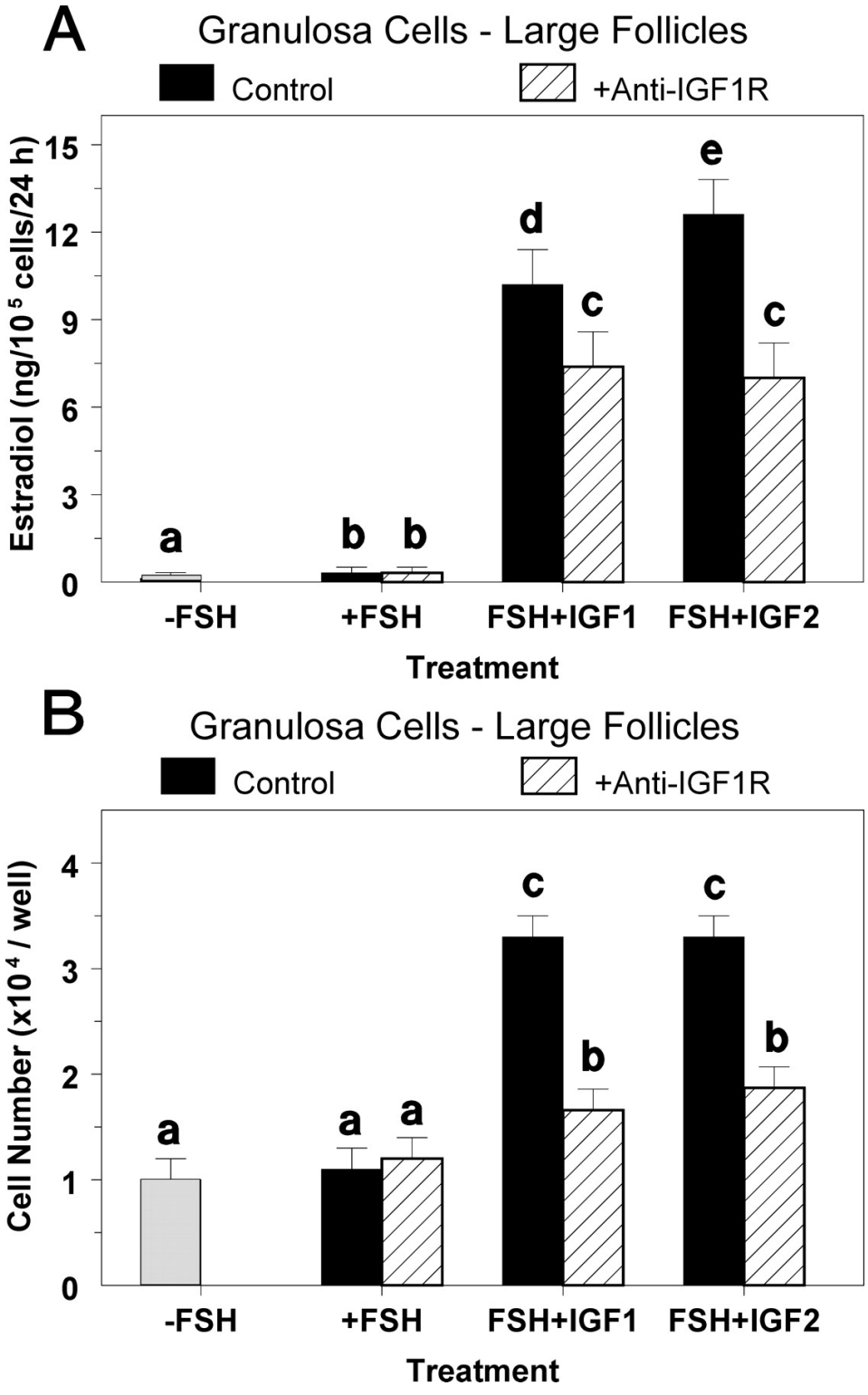
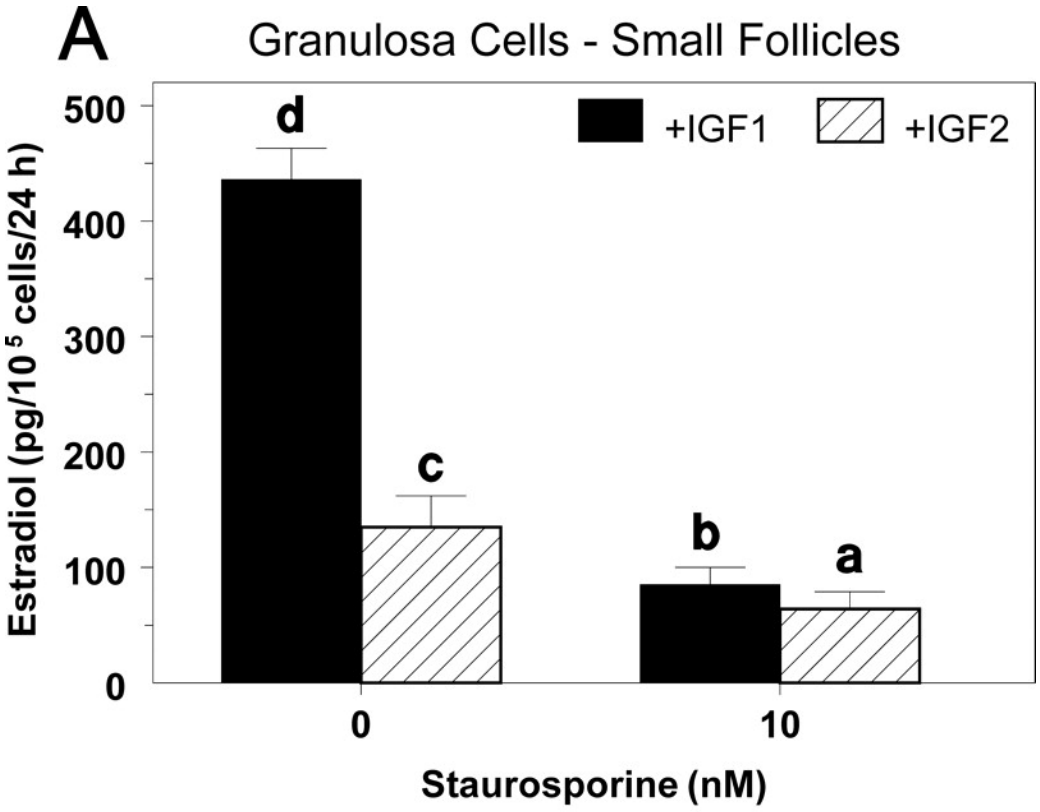

A
Granulosa Cells - Large Follicles
Control
+Anti-IGF1R
Estradiol (ng/10 5 cells/24 h)
0
3
6
9
12
15
a
b b
d
c
e
c
-FSH
+FSH
FSH+IGF1
FSH+IGF2
Treatment
B
Granulosa Cells - Large Follicles
Control
+Anti-IGF1R
Cell Number (x10 4 / well)
0
1
2
3
4
a
a a
c
b
c
b
-FSH
+FSH
FSH+IGF1
FSH+IGF2
Treatment

Figure 4.6. Effect of 2-day treatment of staurosporine on IGF1- and IGF2-induced estradiol (Panel A) and progesterone (Panel B) production by bovine granulosa cells (Experiment 6). Granulosa cells from small follicles were cultured for 48 h in the presence of 10% FCS, and then treated in serum-free medium for an additional 48 h with FSH (30 ng/ml) and either 100 ng/ml of IGF1 or 100 ng/ml of IGF2 in the absence (hatched bar) or presence (solid bar) of 10 nM of staurosporine. Values are means ± SEM of triplicate culture wells replicated in three separate experiments (n = 9). a,b,c,d Within a panel, means without a common superscript differ ($P < 0.05$).


A
Granulosa Cells - Small Follicles
+IGF1
+IGF2
Estradiol (pg/10 5 cells/24 h)
500
400
300
200
100
0
d
c
b
a
0
10
Staurosporine (nM)

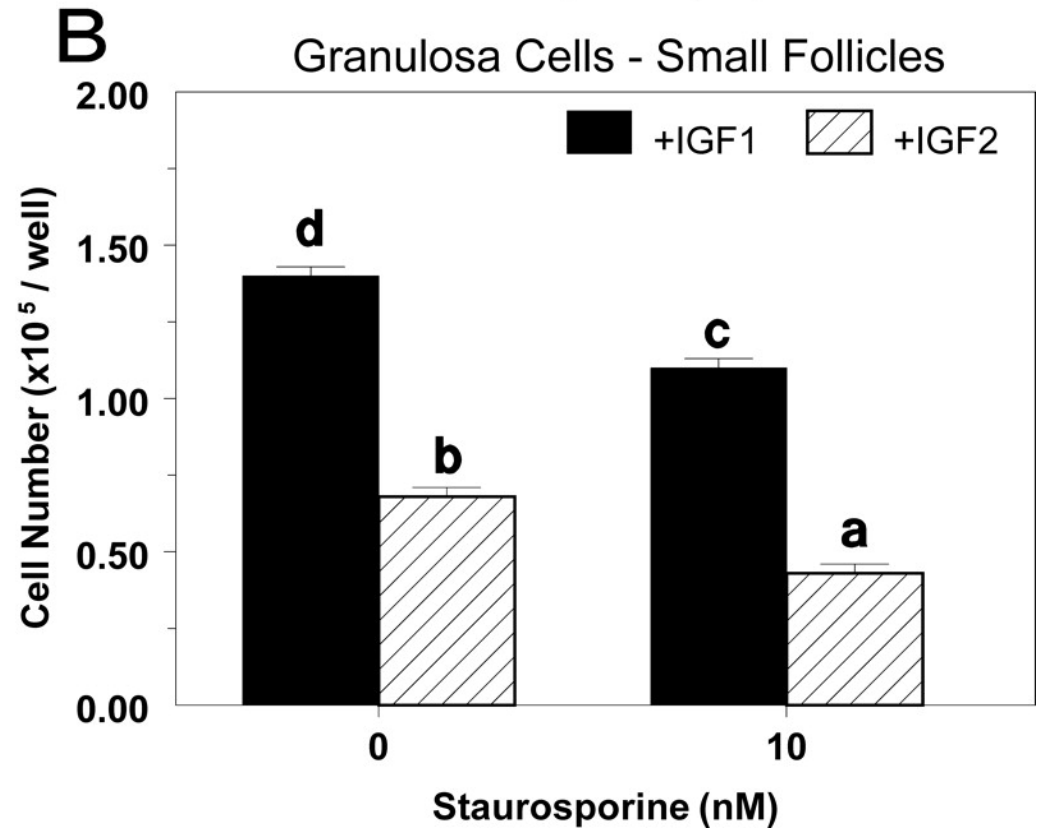

B
Granulosa Cells - Small Follicles
+IGF1
+IGF2
Cell Number (x10 5 / well)
2.00
1.50
1.00
0.50
0.00
d
b
c
a
0
10
Staurosporine (nM)

Figure 4.7. Comparison of IGF1 and IGF2 on competing for 125I-IGF2 (Panel A) and 125I-IGF1 (Panel B) binding by granulosa cells (Experiment 7). Granulosa cells were cultured for 3 days in the presence of 10% FCS, and then cells were washed and incubated with 60,000 dpm of ^{125}I-IGF1 or ^{125}I-IGF2 in the absence or presence of 0, 1, 3, 10 or 100 ng/well of IGF1 or IGF2. Values are means of three to four separate experiments and are expressed as a percentage of control total ^{125}I-IGF2 and ^{125}I-IGF1 binding which averaged 5621 and 6622 cpm/10^5cells, respectively. Panel A and B: *Mean (± SEM) differs ($P < 0.05$) from control (0 ng/well).

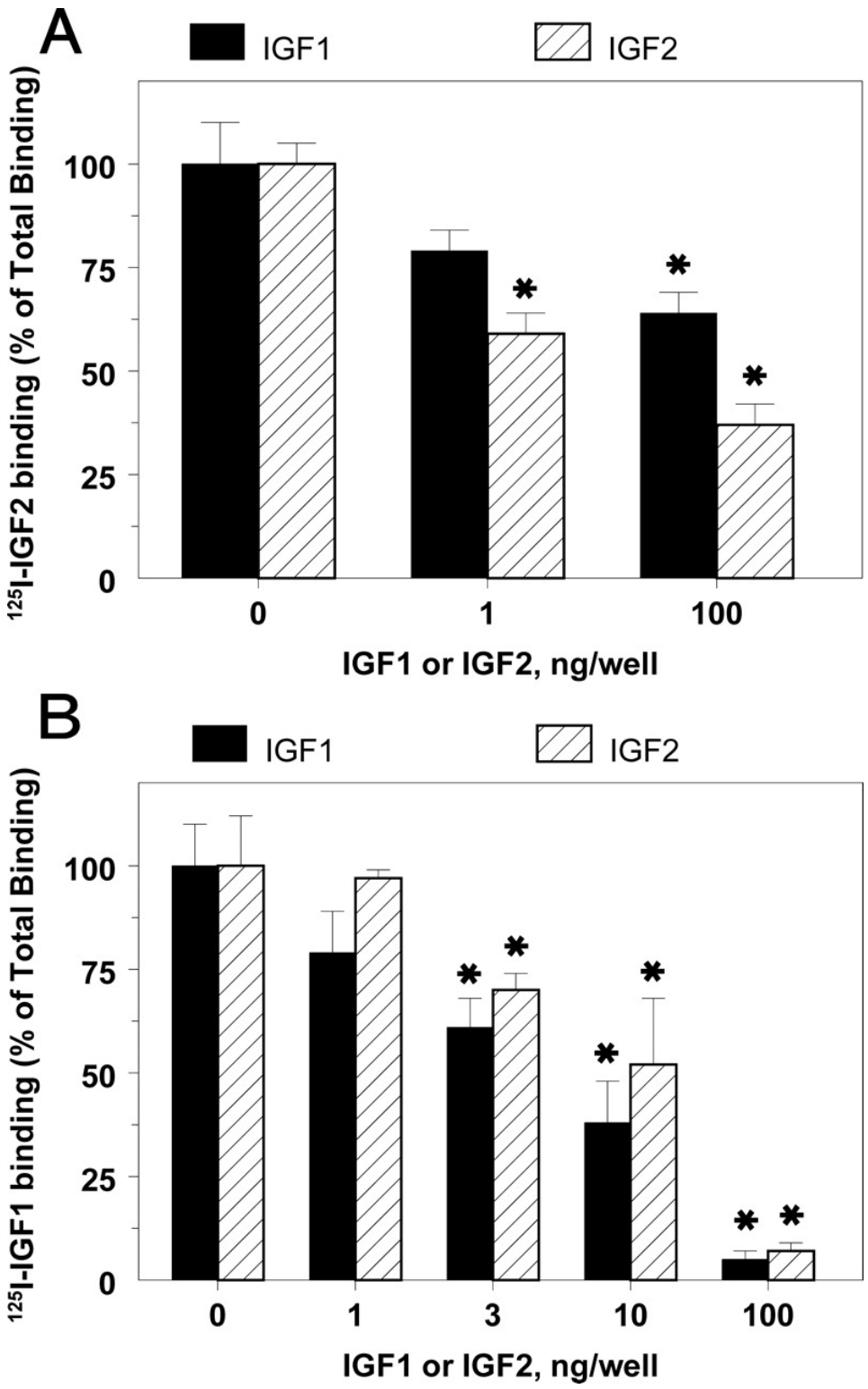

A
IGF1
IGF2
125I-IGF2 binding (% of Total Binding)
100
75
50
25
0
0
1
100
IGF1 or IGF2, ng/well
B
IGF1
IGF2
125I-IGF1 binding (% of Total Binding)
100
75
50
25
0
0
1
3
10
100
IGF1 or IGF2, ng/well

Figure 4.8. Hormonal control of IGF2R in granulosa cells. Panel A: Effect of IGF1, estradiol, FSH and cortisol on specific binding of ^{125}I-IGF2 to granulosa cells (Experiment 8). Granulosa cells were cultured for 48 h in the presence of 10% FCS, and then cells were washed and treated in serum-free medium for an additional 48 h with either estradiol (300 ng/ml), FSH (30 ng/ml) or cortisol (30 ng/ml) in the absence or presence of 30 ng/ml of IGF1 before binding assays were conducted. Binding assays were conducted with 250,000 cpm of ^{125}I-IGF2 in the absence or presence of 200 ng/well of IGF2. Values are means of three separate experiments and are expressed as cpm of specific ^{125}I-IGF2 binding per 100 granulosa cells. *Within a treatment, mean differs ($P < 0.05$) from its respective control without IGF1. Panel B: Effect of IGF1 on FSHR and IGF2R mRNA levels in granulosa cells (Experiment 9). Granulosa cells from small follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated without or with 30 ng/ml of IGF1 for 24 h. Values are means of three separate experiments (± SEM) and normalized to constitutively expressed 18S ribosomal RNA. *Within a panel, mean differs from its respective control.

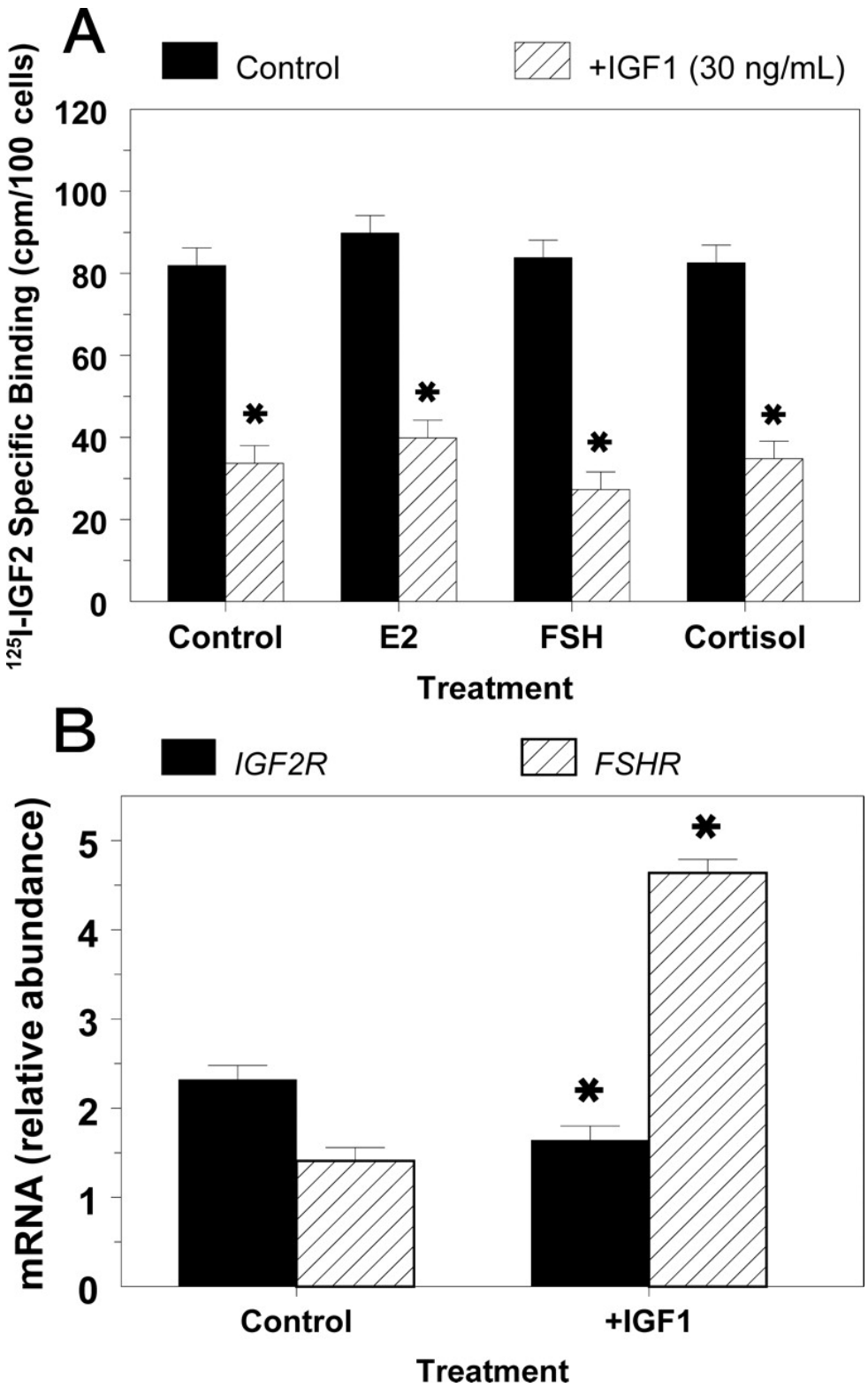

A
Control
+IGF1 (30 ng/mL)
125I-IGF2 Specific Binding (cpm/100 cells)
120
100
80
60
40
20
0
Control
E2
FSH
Cortisol
Treatment
B
IGF2R
FSHR
mRNA (relative abundance)
5
4
3
2
1
0
Control
+IGF1
Treatment

Figure 4.9. Effect of FSH on FSHR mRNA (Panel A) and IGF2R mRNA (Panel B) in granulosa cells (Experiment 10). Granulosa cells from small and large follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated in the presence of 30 ng/ml of IGF2 without or with 30 ng/ml of FSH for 24 h. Cellular RNA was extracted and real-time RT-PCR conducted. Values are means of three separate experiments (± SEM) and normalized to constitutively expressed 18S ribosomal RNA. No significant differences were observed.

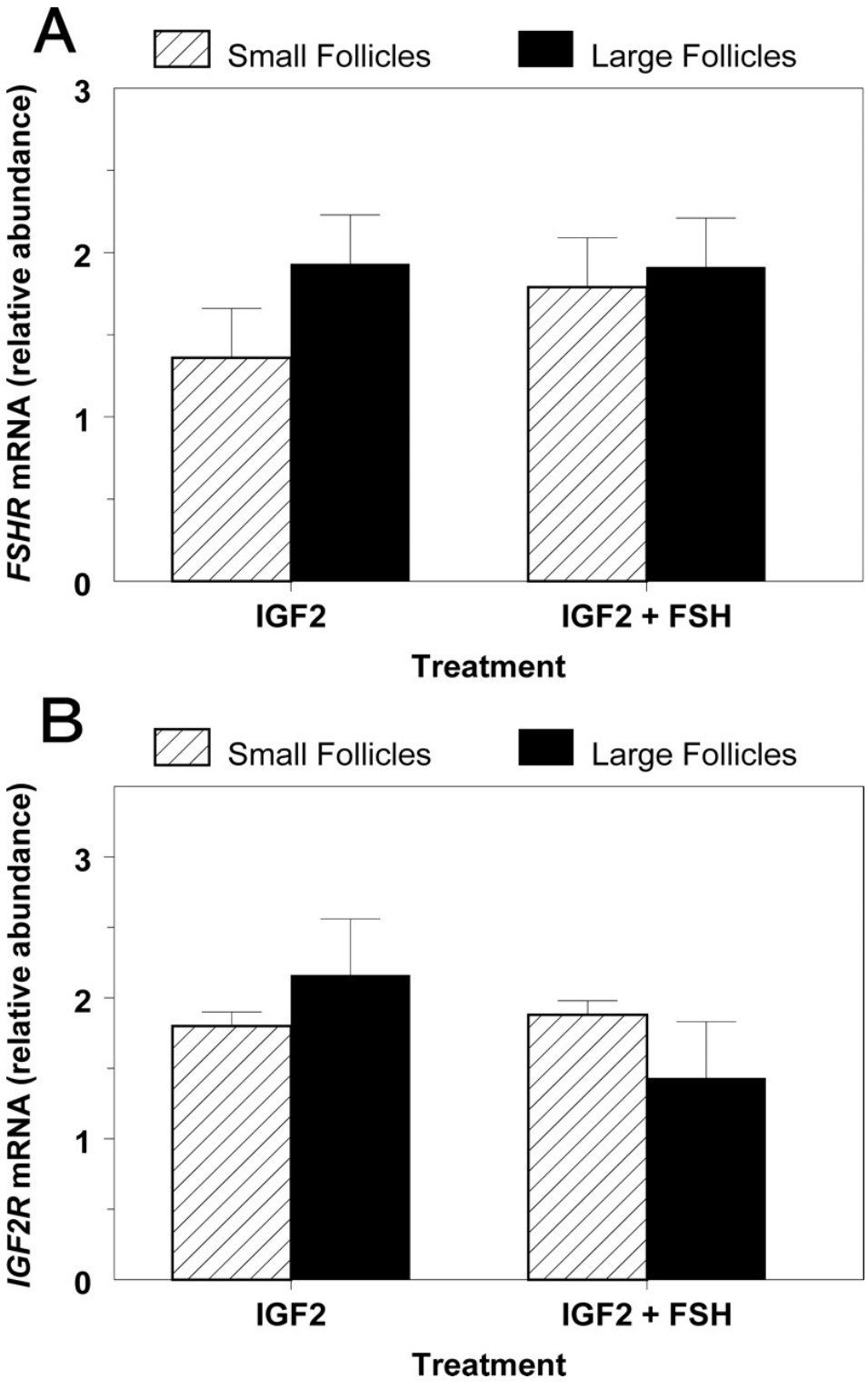
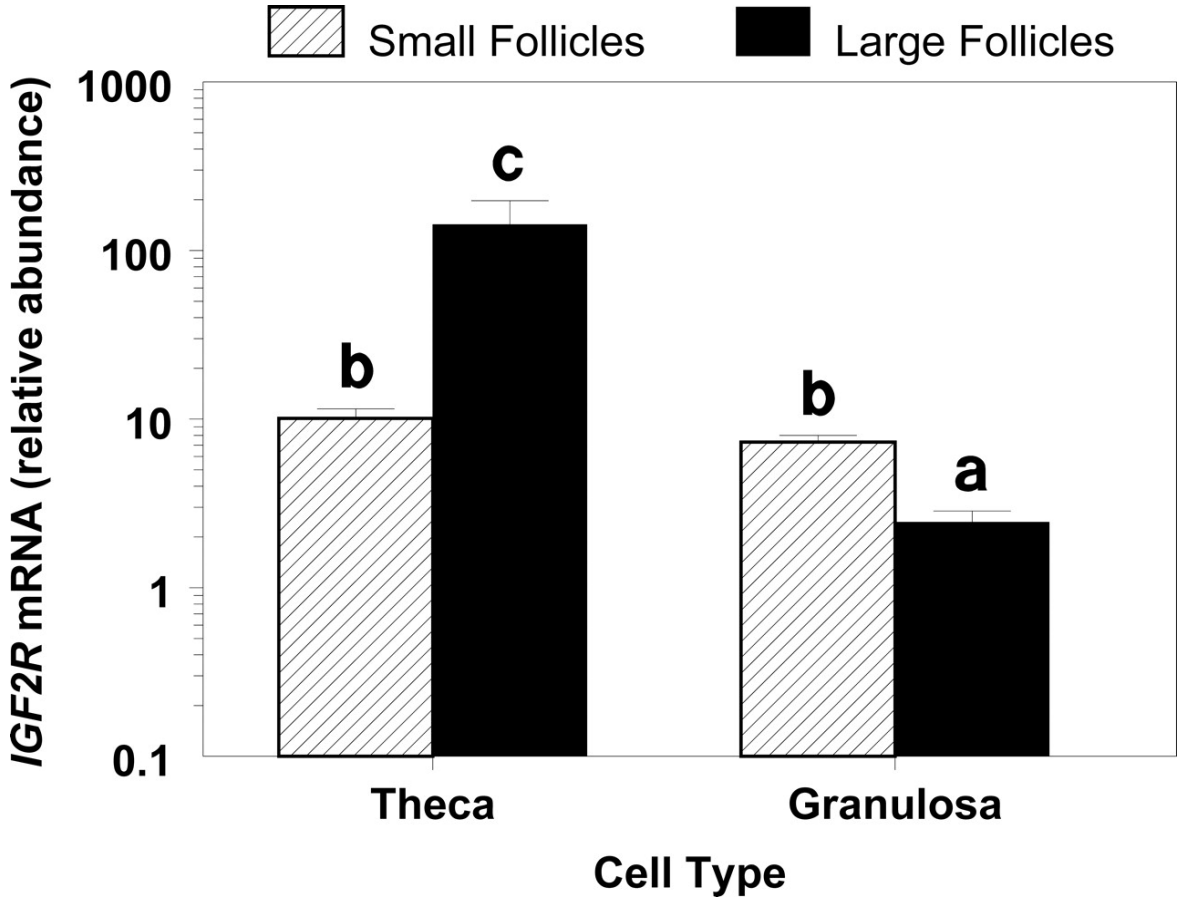

A
Small Follicles
Large Follicles
FSHR mRNA (relative abundance)
3
2
1
0
IGF2
IGF2 + FSH
Treatment
B
Small Follicles
Large Follicles
IGF2R mRNA (relative abundance)
3
2
1
0
IGF2
IGF2 + FSH
Treatment

Figure 4.10. Effect of follicle size and cell type on IGF2R mRNA abundance (Experiment 11). Granulosa and theca cells from small and large follicles were collected, RNA extracted and real-time PCR conducted. Values are means of 6 separate pools of cells and normalized to constitutively expressed 18S ribosomal RNA. a,b,c Means (± SEM) without a common superscript differ ($P < 0.05$). Note log scale on y-axis.


Small Follicles
Large Follicles
IGF2R mRNA (relative abundance)
1000
100
10
1
0.1
c
b
b
a
Theca
Granulosa
Cell Type

TABLES

Table 4.1. Effect of recombinant human IGF2 on granulosa cell numbers in Experiment 1. Granulosa cells from small (1 to 5 mm; Exp. 1A) and large (8-22 mm; Exp. 1B) bovine follicles were cultured as described in Materials and Methods, and treated for 48 h with FSH (0 or 30 ng/ml) and 0 to 100 ng/ml of IGF2

	IGF2 (ng/ml)			
Treatment	0	10	30	100
Experiment 1A				
Control	0.45^{a}	0.52^{ab}	0.56^{b}	0.80^{c}
FSH (30 ng/ml)	0.46^{a}	0.50^{ab}	0.52^{ab}	0.98^{d}
Experiment 1B				
Control	0.99^{a}	1.35^{bc}	1.51^{cd}	1.47^{cd}
FSH (30 ng/ml)	1.15^{ab}	1.57^{cd}	1.74^{d}	1.62^{cd}
SEM				
Control	0.03	0.03	0.03	0.03
FSH (30 ng/ml)	0.11	0.11	0.11	0.11

a-d Within an experiment, means without a common letter differ ($P<0.05$).

Table 4.2. Effect of FSH and recombinant human IGF1 and IGF2 on granulosa cell viability in Experiment 4. Granulosa cells from large bovine follicles were cultured as described in Materials and Methods, and treated for 48 h with no additions (Control), FSH (30 ng/ml), IGF1 (100 ng/ml) or IGF2 (100 ng/ml)

Treatment	**Cell Viability (%)**	**Cell numbers (x105/well)**	**Estradiol (pg/105 cells per 24 h)**	**Progesterone (ng/105 cells per 24 h)**
Control	64.6[a]	0.26[a]	98.1[a]	32.9[a]
FSH (30 ng/ml)	60.7[a]	0.30[a]	165.9[a]	50.0[b]
IGF1 (100 ng/ml	55.7[a]	0.46[b]	1418.3[b]	241.7[d]
IGF2 (100 ng/ml)	69.1[a]	0.42[b]	1912.6[c]	196.1[c]
SEM	5.0	0.02	131.5	15.7

a-d Within a column, means without a common letter differ (P<0.05).

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Amsterdam, A., K. Tajima, V. Frajese, and R. Seger. 2003. Analysis of signal transduction stimulated by gonadotropins in granulosa cells. Mol Cell Endocrinol 202: 77-80.

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CHAPTER V

The hedgehog system in ovarian follicles of cattle selected for twin ovulations and births: evidence of a link between the IGF and hedgehog systems.

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Running Title: Hedgehog system in twinner cattle

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ABSTRACT

The hedgehog system is involved in the regulation of ovarian function in Drosophila but its role in regulating ovarian follicular function in mammals is unclear. Therefore, gene expression of Indian hedgehog ligand (Ihh) and its type 1 receptor, patched 1 (Ptch1), were quantitated in granulosa (GC) and/or theca (TC) cells of cattle. In experiment (exp) 1, cows selected (Twinner) and unselected (Control) for twin births and ovulations were slaughtered between day 3-4 (D3) and 5-6 (D5) of an estrous cycle, and ovaries collected. Individual follicles were snap frozen in liquid nitrogen, and follicular fluid (FF), GC and TC subsequently isolated separately from individual follicles. Follicles were classified as healthy or atretic when estradiol: progesterone ratio was greater than 1 or less than 1, respectively. Additional in vitro experiments (exp 2-5) used GC and TC isolated from abattoir ovaries to evaluate hormonal control of Ihh and Ptch1. In exp 2, GC were treated with 100 ng/ml of IGF1 and/or 30 ng/ml of FSH. In exp 3 and 4, TC were treated with 100 ng/ml of either IGF1 or IGF2 and/or 30 ng/ml of LH. For exp 2-4, total RNA from GC and TC was extracted, and levels of Ihh and Ptch1 mRNA were quantitated using multiplex real-time RT-PCR and expressed as relative mRNA abundance normalized to constitutively expressed 18S ribosomal RNA. In exp 5, serum-starved TC were treated with 10 ng/ml of IGF1 and 0 or 1000 ng/ml of Shh and exposed to ^{3}H-thymidine to measure cell proliferation. In healthy follilces, Ptch1 mRNA in TC were lower ($P < 0.05$) in Twinner versus Control cows, whereas Ihh mRNA abundance in GC did not differ ($P > 0.10$) between Twinner and Control cows. Abundance of Ihh mRNA in GC of healthy follicles was twofold greater ($P < 0.01$) than in atretic follicles and 2.3-fold greater in D3

than D5 follicles, thecal Ptch1 mRNA was greater ($P < 0.05$) at D5 than D3, and Control cows had greater ($P < 0.05$) Ptch1 mRNA than Twinner cows within healthy follicles. Follicular Di Also in exp 1, Di correlated negatively with GC Ihh mRNA ($r = - 0.25$, $P < 0.05$) and with TC Ptch1 mRNA ($r = - 0.24$, $P < 0.01$); GC Ihh mRNA was correlated positively ($r = 0.36$, $P < 0.01$) with GC IGF2R mRNA, and TC Ptch1 mRNA was correlated positively ($r = 0.20$, $P < 0.05$; $r=0.69$, $P < 0.01$) with GC and TC IGF2R mRNA respectively. In exp 2, IGF1 decreased ($P < 0.001$) whereas FSH did not affect ($P > 0.10$) Ihh mRNA in GC. In exp 3 and 4, neither IGF1 nor IGF2 and/or LH treatments for 24 h affected ($P > 0.10$) Ptch1 or IGF2R mRNA in TC, whereas 48 h treatments with IGF1 and IGF2 but not LH decreased Ptch1 mRNA in TC in exp 5. For the first time, an interaction between the ovarian IGF and hedgehog systems has been demonstrated. Increased free IGF1 and IGF2 as follicles grow and develop may suppress Ihh production by GC, and subsequently regulate Ptch1 in TC. Whether the reduction in Ptch1 mRNA in TC is involved in the development of two co-dominant follicles in Twinner cows will require further elucidation.

INTRODUCTION

In growing follicles, communication among oocyte, granulosa and theca cell compartments modulates proliferation and differentiation of these cell layers (Nilsson and Skinner, 2001; 2005). The hedgehog gene family was identified in 1990 as a family of developmentally regulated morphogens controlling basic embryonic developmental processes (Ingham and McMahon, 2001). Three secreted glycoproteins Sonic (Shh),

Indian (Ihh) and Desert (Dhh) hedgehogs have been identified in mammalian cells (Nusslein-Volhard and Wieschaus, 1980; Ingham, 1993; Pangas, 2007), and were localized to the mouse ovarian cell layers including granulosa, theca and the oocyte (Wijgerde et al., 2005; Russell et al., 2007). These ligands work through 2 membrane-bound receptors Patched (Ptch) 1 and 2 (Burke and Basler, 1997; Pangas, 2007). Both receptors bind the membrane-associated signal transducer (Smo) in the absence of ligands and thus suppress its intracellular pathway transcription through the intracellular transcriptional effectors Glioma-associated oncogene homolog (Gli) 1, 2 and 3 (Alcedo and Noll, 1997; Burke and Basler, 1997; Alcedo et al., 2000; Wijgerde et al., 2005; Pangas, 2007). In the ovary, the hedgehog system was first identified to stimulate ovarian somatic stem cell in Drosophila (Zhang and Kalderon, 2000) and to stimulate germ cell proliferation (King et al., 2001) and has recently been identified to stimulate mitogenesis in murine ovarian cells (Russell et al., 2007). In fact, the hedgehog signaling has been involved in regulating cell proliferation and survival, and in determining cell fate, differentiation, and polarity of embryonic cells (Ingham and Kim, 2005; Ingham and Placzek, 2006). Recently, Ihh expression and signaling patterns in the uterus were shown to be modulated by ovarian progesterone in mouse (Takamoto et al., 2002; Lee et al., 2006), and hamster (Khatua et al., 2006) uteri. Whether the hedgehog system is physiologically regulated during ovarian follicular development in mammals is unknown.

The objective of our study was to identify, and characterize the expression of Ihh and Ptch1 in follicles of different sizes at different stages of the estrous cycle in cattle. In addition, we set out to determine whether genetic differences might be governing the expression of these genes in a beef cattle population selected for multiple ovulations and

twin births. In addition, with current research indicating greater levels of serum and follicular fluid IGF1 levels in Twinner cattle (Echternkamp et al., 1990; Echternkamp et al., 2004) and importance of this growth factor as a main regulator of ovarian follicular development and steroidogenesis (Spicer and Echternkamp, 1986; Spicer et al., 1993; Spicer and Echternkamp, 1995; Spicer et al., 2005), cell culture experiments served to investigate the effects of IGF1 and IGF2 on components of the hedgehog system.

MATERIALS AND METHODS

1. Hormones, reagents, instruments and consumables

Hormones and Reagents

Recombinant human IGF1 and IGF2 were obtained from R & D Systems (Minneapolis, MN); LH (15 x NIHFSH-S1 U/mg) was obtained from Scripps Laboratories (San Diego, CA); testosterone was obtained from Steraloids (Wilton, NH); and trypsin, collagenase, DNase, and fetal calf serum (FCS) were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents used for RNA collection and extraction were: RNA*later* reagent and TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 7.0) from Ambion Inc. (Austin, TX); TRIzol from Invitrogen (Carlsbad, CA); Chloroform from Sigma Chemical Co. (St. Louis, MO); Isopropyl alcohol (Pierce Chemical Company (Rockford, IL). RiboGreen® RNA Quantitation Reagent and Kit from Molecular Probes (Eugene, OR) was used to quantitate the RNA. TaqMan® One-Step RT-PCR Master Mix Reagents Kit and TaqMan® Ribosomal RNA Control Reagents from Applied Biosystems

(Foster City, CA) were used for gene expression.

Instruments

Instruments used were: Omni TH tissue homogenizer (Omni International Inc., Marietta, GA) for tissue homogenization; NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and fluorescent plate reader Wallac 1420 (Perkin-Elmer, Boston, MA) for RNA quantitation; and ABI 7700 real-time PCR machine (ABI, Foster City, CA) for quantitative RT-PCR gene expression.

Consumables

Consumables included: for cell culture, plastic 24-well Falcon plates (BD Biosciences, San Jose, CA); for RNA extraction and quantitation, Omni Tip™ disposable generator probes from Omni International Inc. (Marietta, GA), 96-well microplates (Proxiplate™-96F, Packard Bioscience BV, Meridian, CT); for real-time PCR, 96-well MicroAmp™ Optical 96-Well Reaction Plate with Barcode and Optical Adhesive Films (Applied Biosystems, Foster City, CA).

2. Experimental design

Objectives of this study were to: 1) determine expression of the hedgehog system in cattle; 2) identify whether the hedgehog system was genetically regulated in cattle; and

3) study whether an important regulator of ovarian mitogenesis and steroidogenesis such as the IGF system interacts with the hedgehog system. Therefore, Experiment 1 was designed to collect granulosa and theca cells from control cows and cows with ability for double ovulations and twin births (Twinner), whereas Experiments 2 through 6 were designed to determine the effect of IGF1 and/ or IGF2 on the expression of the Indian hedgehog (Ihh) and hedgehog receptor (Patched 1 - Ptch1). All experiments were used to identify any interaction between the 2 hormonal systems.

3. Follicular fluid, fresh granulosa and theca cell collection – Experiment 1

For Experiment 1, 16 MARC I, II and III (control) cows and 16 cows with the propensity for twin births (twinner) were synchronized and ultrasounded as described previously (Chapter 3) to determine functional corpora lutea (CL) and to record the follicular population and confirm ovulation. Cows were slaughtered at follicle recruitment (day 3-4; D3) or deviation (day 5-6; D5) of the estrous cycle. Frozen follicles were processed as described previously (Murdoch et al., 1981) with modifications (Chapter 3). Briefly, individual follicles were snap frozen and stored in liquid nitrogen. Frozen follicles were bisected and one-half of the frozen follicular fluid containing granulosa cells was released into eppendorf tubes. Follicular fluid was then thawed by incubation at 37 °C for 5 min then centrifuged at 1000 x g at 4 °C for 5 min. The follicular fluid supernatant was transferred into clean eppendorf tubes and stored at -20 °C until hormonal assays. The outer follicle wall was slightly thawed in order for the theca cells to be peeled as previously described (Chapter 3). Theca cells were suspended

in 0.5 mL of RNA*later* at 4 °C overnight, then stored at -80 °C until RNA extraction.

Estradiol and progesterone levels in follicular fluid were determined by radioimmunoassay (RIA) as previously described (Spicer and Enright, 1991; Stewart et al., 1996) and as reported in Chapter 3.

4. Granulosa and Theca Cell Culture – Experiment 2 through 8

Ovaries of cattle obtained at slaughter from a nearby abattoir were brought to the laboratory on ice and processed as previously described for obtaining granulosa and theca cells from small (3 to 6 mm - SM-TC) and large (> 8 mm- LG-TC) follicles (Langhout et al., 1991; Spicer and Chamberlain, 1998). For granulosa cell isolation, follicular fluid was aspirated using 20 gauge needles and syringes and centrifuged at 200 x g for 5 min. For theca cell isolation, follicles were cut open after follicular aspiration, washed from granulosa cells with serum-free medium, and then digested for 1 h in 5 mg/ml DNase, 25 mg/ml hyaluronidase, 12.5 mg/ml collagenase and 10 mg/ml protease in serum-free medium mixture, as described previously (Spicer and Chamberlain, 1998, 2000, 2002). These follicle size categories were selected due to their estradiol production potential and their responsiveness to FSH and LH in the presence of IGF1 as described previously (Spicer and Aad, 2007; Spicer et al., 2007). Prior to plating, granulosa and theca cells were resuspended in medium containing 1.25 mg/ml of collagenase and 0.5 mg/ml of DNase. Using trypan blue exclusion method, granulosa cell viability averaged 58 ± 11 % and 79 ± 12 % for small and large follicles respectively, whereas theca cell viability

averaged 77 ± 3 % and 67 ± 6 %, respectively, at time of plating. Approximately 2 x 10^5 viable cells were seeded in each plastic 24-well Falcon plates in 1 ml of medium; medium was a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 containing 0.12 mM gentamycin, 2.0 mM glutamine, and 38.5 mM sodium bicarbonate. Cultures were kept at 38.5°C in a 95% air - 5% CO2 atmosphere, and for all experiments medium was changed every 24 h as described previously (Langhout et al., 1991; Spicer and Chamberlain, 1998; Spicer et al., 2007).

5. Cell culture Experimental design

Granulosa and theca cells were cultured for 48 h in medium containing 10 % FCS for the first 48 h, washed twice with 0.5 ml of serum-free medium, and treated for an additional 24 h with the various hormones as described in the following section. Experiments 2 through 6 were designed to study the effect of IGF1 or IGF2 on the hedgehog ligand (Ihh) or its receptor (Ptch1) mRNA. In exp 2, small granulosa cells were treated with IGF1 (0 or 100 ng/ml) in the presence of 30 ng/ml of FSH. Small and large theca cells were treated with IGF1 (0 or 100 ng/ml), and (or) ovine LH (0 or 30 ng/ml) in exp 3 and 4, respectively. Large theca cells were treated with IGF2 (0 or 100 ng/ml), and (or) ovine LH (0 or 30 ng/mL) in exp 5. In exp 6, both small and large theca cells were treated with IGF1 (0 or 100 ng/ml), IGF2 (0 or 100 ng/ml), LH (0 or 30 ng/ml), or a combination of both. Experiments 7 and 8 were designed to determine whether Shh had any effect on the IGF system, in particular the IGF-type 2 receptor (IGF2R) mRNA. In exp 8, large theca cells were treated for 24 h with IGF1 after which

treatments with Sonic Hedgehog (Shh; 0 or 1000 ng/ml) in the presence of IGF1 were applied for an additional 24 h. At the end of culture, RNA was collected and real-time RT-PCR was used to quantify Indian Hedgehog (Ihh) mRNA (Exp 2), Patched 1 (Ptch1) mRNAs (Exp 3 through 8) and IGF2R mRNA (Exp 5 through 8). All mRNA levels were normalized to constitutively expressed 18S ribosomal RNA as described below.

6. RNA Extraction and RT-PCR

Granulosa and theca cells were lysed in 0.5 ml of TRIzol Reagent (Invitrogen, Carlsbad, CA), RNA extracted, and RNA quantity in Exp 1 was determined using RiboGreen nuclear dye as previously described (Santiago et al., 2005), whereas in all subsequent experiments it was deterermined spectrophotometrically at 260 nm using NanoDrop in all subsequent experiments as previously described (Aad et al., 2006; Spicer and Aad, 2007). Primers and probe for each target gene were designed using Primer Express (Applied Biosystems, Foster City, CA) for TaqMan assay. Exon junctions were determined, when possible, using the spAlign (NCBI, http://www.ncbi.nlm.nih.gov/sutils/splign/) and probes were preferentially designed over exon-exon junctions, and designated by E-E. The target gene primers (forward and reverse) and probe sequences for Indian Hedgehog (Ihh - Accession XM_601000) were CGGCTTCGACTGGGTGTATTAC, AGGGAAGCAGCCACCTGTCT, (E-E) CAAGGCCCACGTGCATTGCTC, respectively; for Patched homolog 1 (Ptch1 - Accession XM_869803) were TGCCCAGGCTACGAGGACTA, CCGGACATTAAAAGGCACATG, and TGACCACGGCCTGTTTGAGGACC,

respectively; for IGF2R (Accession 155 AF342811) were GCAATGCTAAGCTTTCGTATTACG, GGTGTACCACCGGAAGTTGTATG, and ACGCCGGAGTGGGTTTCCCC, respectively (Table 5.1). A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) was also conducted to insure the specificity of the designed primers and probe and to assure that they were not designed from any homologous regions, coding for other genes. Furthermore, the RT-PCR product generated as described in next section was ran on a 3% Agarose I gel (AMRESCO Inc., Solon Industrial, OH) with 0.5 mg/mL ethidium bromide and a lane with PCR markers (Promega, Madison, WI) in a DNA size standard of 50 bp to 1000 bp. The observed imaging verified the length and size of the target gene. The PCR product from each gene was cleaned from the agarose gel using the Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA) following the manufacturer's instructions as described previously (Chapter 3). Briefly, the bands on the agarose gel were excised and excess agarose gel discarded. Bands were then dissolved in agarose dissolving buffer and span in the supplied Zymo Spin I column to discard the flow-through. The columns were washed twice with wash buffer-ethanol mix and the PCR products eluted with water after spinning the column in a 1.5 ml eppendorf. These purified PCR products were quantitated and sequenced to further validate the amplified genes, its sequence and the specificity of the RT-PCR quantification.

7. Multiplex Real-time RT-PCR for mRNA quantification

The differential expression of target gene mRNA in theca cells was quantified using the multiplex one-step real-time RT-PCR reaction for Taqman® Gold RT-PCR Kit (Applied Biosystems, Foster City, CA) as previously described (Voge et al., 2004a; Voge et al., 2004b) and as specified in Chapters 3 and 4. Briefly, based on preliminary optimization results, 100 ng of total RNA was amplified in a total reaction volume of 25 µl consisting of 200 nM forward primer, 200 nM reverse primer and 200 nM fluorescent (FAM/TAMRA) probe for each target gene, optimized levels of 18 S rRNA primers (10 nM for IGF2R mRNA expression, 100 nM for Ihh, Ptch1 and Smo gene expression) and 100 nM of the 18S rRNA VIC-labeled probe, along with 12.5 µl of TaqMan Master Mix without uracil N-glycosylase, and 1 U Multiscribe with RNase inhibitor (Applied Biosystems). Thermal cycling conditions were set to 30 min at 48.8ºC for reverse transcription, 95ºC for 10 min for AmpliTaq Gold Activations, and finished with 50 cycles at 95ºC for 15 sec for denaturing and 60ºC for 1 min for annealing and extension. Extended number of cycles was used as an assurance measure to make sure that no amplification is overlooked, and to further insure the presence of the measured genes in the specific cell-type. Because the total number of samples in Exp 1 was greater than the 96 well-plate capacity, cows were sorted by genotype and cycle. Cows within genotype and cycle were randomly assigned to each plate, with all follicles from one individual cow on the same plate; all individual samples were run in duplicates. The 18S rRNA values were used as internal controls to normalize samples for any variation in amounts of RNA loaded as previously described (Spicer et al., 2006; Spicer et al., 2007). Quantification of gene expression was done by setting an arbitrary threshold (Ct) on the

FAM or VIC curves in the geometric portion of the RT-PCR amplification plot after examining the log view. Relative quantification of target gene mRNAs were expressed using the relative comparative threshold cycle method as previously described (Livak and Schmittgen, 2001; Voge et al., 2004a; Aad et al., 2006). Briefly, the ΔCt was determined by subtracting the 18S Ct value from the target unknown value. For each target gene and within each experiment, the ΔΔCt was determined by subtracting the higher ΔCt (the least expressed unknown) from all other ΔCt values. Fold changes in target gene mRNA expression were calculated as being equal to $2^{-\Delta\Delta Ct}$. To correct for heterogeneity of variance, estradiol production, IGF2R, Ihh, and Ptch1 mRNA were analyzed after transformation natural log (x + 1).

8. Statistical Analyses

Data from experiment 1 were analyzed as a completely randomized design with a 2x2x2 factorial treatment structure with main effects of genotype (control or twinner), day of cycle (recruitment D3, or deviation D5), and follicle estrogenic profile (Healthy E2/P4 ≥ 1, or atretic E2/P4 < 1). Further, a completely randomized design with a 2x2x3 factorial treatment structure was used to characterize the differences among the largest three follicles, where the main effects were genotype (C or T), day of cycle (D3 or D5), and follicle (F1, F2 or F3). In both models, the treatment effects on the dependant variables (e.g., estradiol concentration; Ptch1, IGF2R, etc. mRNA levels) were determined using the MIXED procedure of SAS (SAS, 1996) for Windows (version 8.02, SAS Institute Inc., Cary-NC, USA, 1999-2001), where cow was included as a random

effect. Outliers were detected according to the procedure described by Grubbs (1950) provided by GraphPad Software (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Differences in main effects were considered only when the 2- or 3-way interactions were not significant ($P > 0.05$). Mean differences were determined by Fisher's protected least significant differences test (Ott, 1977), if significant treatment effects in ANOVA were detected. The slice option in the LSMeans statement of SAS was used to separate the main effects mean differences when interactions were significant ($P < 0.05$). Results are presented as the least square means (LSMeans) ± SEM. Main effects means are presented in tables and significant differences are indicated only when there are no interactions. To determine the relationships between diameter follicles and the various herein analyzed parameters (Ihh, Ptch1 mRNA mRNA, etc), data for each of these parameters were regressed against diameter and slopes tested for deviation from "zero" (x axis) or between genotypes using regression analysis procedure of SAS. Regression equations were generated as a result of the regression analysis of SAS. The regression equation slope and intercept deviation from zero were tested by assigning a dummy variable to the genotype levels in the regression procedure of SAS. Simple correlations among variables were calculated using the correlation procedure (Pearson) of SAS. Correlations were considered significant when $p < 0.05$.

Data from cell culture experiments (Exp 2 through 8) were analyzed as a completely randomized design with a 2x2 factorial treatment structure only in Exp 3 through 6 using the GLM procedure of SAS. Experimental data are presented as means ± SEM of measurement from replicated experiments. Each experiment from cell culture was replicated three or more times, and within each experiment, treatments were applied

in triplicate culture wells. Each experiment was conducted on a separate pool of granulosa cells obtained from 8 to 15 cows or heifers. The main effects and their interactions on the variables measured were assessed using the GLM procedure of SAS (SAS, 1996) for Windows (version 8.02, SAS Institute Inc., Cary-NC, USA, 1999-2001).

RESULTS

1. Gene expression in bovine granulosa and theca cells of cows selected for twin ovulations and births (Exp 1)

In granulosa cells, Ihh mRNA did not differ ($P > 0.10$) between Twinner and Control cattle regardless of their status or day of an estrous cycle (Table 5.2). Day of cycle x status interaction affected ($P = 0.02$) Ihh mRNA expression, such that Ihh mRNA abundance was increased from D3 to D5 in healthy follicles, but did not change in atretic follicles (Fig. 5.1).

In theca cells, there was a genotype by status interaction ($P < 0.05$) and a day of cycle effect ($P < 0.05$) on Ptch1 gene expression. Thecal Ptch1 mRNA was 1.9-fold greater in follicles at D5 than D3 of an estrous cycle (Fig. 5.2A). In addition, within control cows, healthy follicles had 5.9-fold greater Ptch1 mRNA than their atretic counterparts. Interestingly, within healthy follicles, thecal Ptch1 mRNA was 3-fold lower ($P < 0.05$) in Twinner than Control cows, and Ptch1 mRNA of twinner cows did not change ($P > 0.32$) between D3 and D5 (Fig. 5.2B).

To determine whether the hedgehog system expression was affected by follicular diameter, regression analysis was performed. Granulosa cell Ihh mRNA (Fig. 5.3) relative to follicular diameter did not differ ($P > 0.10$) in Control cows as compared to Twinner cows, whereas theca cell Ptch1 mRNA (Fig. 5.4) relative to follicular diameter differed ($P = 0.089$) in Control cows as compared to Twinner cows. Theca cell Ptch1 mRNA tended to increase ($P = 0.08$) with increased size within Control cows, but tended to decrease ($P = 0.089$) with increased follicular diameter in Twinner cows.

To determine whether the hedgehog system is involved in the selection of the dominant follicle, the largest three follicles (i.e., F1, F2, F3) within Control and Twinner cows were analyzed. In granulosa cells, there was a genotype by follicle interaction on Ihh mRNA levels (Table 5.3), where Ihh mRNA did not differ within F1, F2 and F3 of Control cows, whereas F2 and F3 of Twinner cows had greater Ihh mRNA as compared to F1 (Fig. 5.5A). Theca Ptch1 mRNA abundance was lower at D5 as compared to D3 of an estrous cycle (Table 5.3). Further, Ptch 1 mRNA abundance tended ($P = 0.09$) to be greater in F1 and F2 of Control as compared to Twinner cows (Fig 5.5B).

2. Pearson Correlation analysis

Correlations are presented in Table 5.4. Granulosa Ihh mRNA was negatively correlated with granulosa IGF2R mRNA ($r = -0.30$, $P < 0.01$), estradiol ($r = -0.26$, $P < 0.01$), follicle diameter ($r = -0.25$, $P < 0.01$) and E_2:P_4 ratio ($r = -0.27$, $P < 0.01$). On the other hand, thecal Ptch1 mRNA was positively correlated with thecal IGF2R mRNA ($r =$

0.69, $P < 0.001$), granulosa Ihh mRNA ($r = 0.20$, $P < 0.05$) and negatively correlated with follicle diameter ($r = -0.24$, $P < 0.01$). Granulosa cells, IGF2R mRNA was negatively correlated with estradiol ($r = -0.29$, $P < 0.01$), follicle diameter ($r = -0.24$, $P < 0.05$) and E_2:P_4 ratio ($r = -0.30$, $P < 0.01$).

3. Gene expression in cultured bovine granulosa and theca cells (Exp 2 - 8)

To determine the ability of IGF to affect the hedgehog system, cultured bovine granulosa or theca cells were treated with IGF1 or IGF2; Ihh mRNA was measured in granulosa and Ptch1 measured in theca. In exp 2, small (Di < 5 mm) follicle granulosa cells were treated with IGF1 in the presence of FSH. IGF1 decreased ($P < 0.05$) levels of Ihh mRNA in granulosa cells (Fig. 5.6). Neither IGF1 nor LH treatments for 24 h affected ($P > 0/10$) Ptch1 mRNA expression in bovine theca cells from small (Di < 6 mm – exp 3) or large (Di > 8 mm - exp 4) follicles (data not shown). In large follicle theca cells, IGF2 tended to decrease the IGF2R mRNA by 1.3-fold, whereas LH did not affect ($P > 0.10$) the Ptch1 gene expression in large follicle theca cells (Fig. 5.7). Longer treatments (for 48 h) with these hormones were tested in both small and large theca (exp 6). Both IGF1 and IGF2 decreased Ptch1 mRNA expression (Fig. 5.8) in small and large theca cells. In addition, 48 h treatment of theca cells with LH decreased Ptch1 mRNA in small but not large theca cells (Fig. 5.8). When combined with IGF1 or IGF2, LH decreased IGF1 and IGF2 inhibition on Ptch1 expression, whereas LH had no effect on Ptch1 mRNA in large theca cells (Fig. 5.8).

Further, we tested the effect of hedgehog ligand on its own receptor Ptch1 (exp 7) and possible effect of hedgehog on IGF2R, given the significant correlations between the mRNAs found in exp 1. Shh (1000 ng/mL) increased ($P < 0.01$) Ptch1 mRNA levels in small and large-follicle theca cells (Fig 9A), whereas 1000 ng/mL of Shh did not affect ($P > 0.10$) IGF2R gene expression in small or large-follicle theca cells (Fig 9B).

DISCUSSION AND CONCLUSION

For the first time in bovine ovaries, we demonstrated the expression of Ihh and Ptch1 in granulosa and theca cells, respectively, using multiplex real-time RT-PCR. Further, we demonstrated that the expression of the hedgehog system changes during follicular growth and that it can be modulated by genetic selection. In addition, for the first time, we demonstrate a link between the IGF and hedgehog system in which IGF1 and IGF2 alters Ihh gene expression.

Components of the hedgehog pathway, Ihh and Ptch1 are highly conserved proteins and were first identified in Drosophila as responsible for the segment polarity (Nusslein-Volhard and Wieschaus, 1980; Tabata et al., 1992), and later detected in the ovary as responsible for stem cell development (Forbes et al., 1996; Song and Xie, 2003). Indeed, excessive Hh signal transduction may lead to division of Drosophila somatic stem cells into two daughter stem cells (Zhang and Kalderon, 2000, 2001). In mice, the ligands Ihh, Dhh and Shh, and their receptor (Ptch1 and Ptch2) mRNA and proteins have been detected in immature and adult ovaries, and murine granulosa cells (Wijgerde et al.,

2005; Russell et al., 2007). In addition, Smo, the seven transmembrane signaling transducer of Hh system, was expressed ubiquitiously in all ovarian tissues, except the oocyte, at all stages of mice follicular development (Wijgerde et al., 2005; Russell et al., 2007). Ptch1 in these tissues (Wijgerde et al., 2005) suppresses Smo transmembrane signaling through Gli, and the binding of Hh to its receptor, Ptch1, derepresses Smo, thus allowing signal transuction. Wijgerde et al. (2005) suggested that Smo gene was not a target for hedgehog signaling, whereas Ptch1 and Gli1 were modulated by Hh (Wijgerde et al., 2005; Russell et al., 2007). The hedgehog system has not been studied in many mammalian species; accordingly our current study is the first to measure Ihh and Ptch1 mRNA in bovine granulosa and theca cells, respectively.

In the present study, granulosa Ihh mRNA did not differ between Control and Twinner cows, but increased during follicle selection between D3 and D5. Also, healthy follicles had greater granulosa Ihh mRNA than atretic follicles on D5 but not D3. In mouse atretic follicles, expression of Ihh and Dhh is rapidly lost (Wijgerde et al., 2005), further supporting the hypothesis that Hh affects cell fate in both mammals and Drosophila (Forbes et al., 1996; Zhang and Kalderon, 2000, 2001). Russell at al. (2007) detected Hh ligands in murine granulosa cells of antral and small follicles in variable amounts, suggesting a control of Ihh during follicular development. The observation that Shh increased murine follicle diameter above controls after 4 days of culture (Russell et al., 2007), together with the current findings that show an increase in granulosa Ihh mRNA at D5 and a greater granulosa Ihh mRNA in healthy versus atretic follicles, indicates that Ihh may control the selection of two rather than one dominant follicle in Twinner cattle. Hh controlled cell fate in ovarian drosophila cells (Forbes et al., 1996;

Zhang and Kalderon, 2000), further supporting a possible involvement of Ihh in follicular growth. In cultured granulosa cells, IGF1 decreased Ihh mRNA indicating that the hedgehog system may be controlled by intrafollicular factors such as IGF1 or IGF2. Consistent with this notion, a positive correlation between abundance of granulosa Ihh and IGF2R mRNA was observed in the present study.

Theca cell Ptch1 mRNA levels increased at deviation (D5), and were lower in atretic than healthy follicles of Control cows. Also, theca cell Ptch1 mRNA levels did not differ in atretic follicles between Control or Twinner cows, whereas Ptch1 mRNA was downregulated in healthy follicles of Twinner cows. In atretic follicles, Hh system affected cell fate in mammals and Drosophila (Forbes et al., 1996; Zhang and Kalderon, 2000, 2001), possibly by modulating the Hh receptor, Ptch. In mouse atretic follicles, expression of Ptch1 was rapidly lost (Wijgerde et al., 2005). In the present study, Ptch1 mRNA decreased in Twinner cows but increased in Control cows with increased follicular diameter. However, theca Ptch1 mRNA was lower in F1 and F2 of Twinner cows as compared to Control cows. Theca cell Ptch1 mRNA decreased between D3 and D5, suggesting that the hedgehog system may be involved in selection/deviation of dominant follicles. Previously, using semi-quantitative immunohistochemistry, the expression of Ptch1 was detected in theca cells and oocytes of antral follicles at different sizes and stages of the murine estrous cycle (Russell et al., 2007). These results indicate a role for Ptch1 in deviation, rather than recruitment of follicles. The differential expression of Ptch1 in Control versus Twinner cows of the present study, indicate a possible genetic difference in the regulation of Ptch1 and thus, a possible involvement of Ptch1 and the hedgehog system in the selection of two, rather than one dominant follicle

in Twinner cows. In the present study, Shh increased Ptch1 mRNA expression, whereas 48 h but not 24 h treatments of theca cells with IGF1, and IGF2 decreased Ptch1 mRNA expression. Thus decreased theca Ptch1 mRNA abundance in Twinner cows may be due to an indirect effect of IGFs through the regulation of Ihh mRNA, or that the exposure of follicles in Twinner cows to greater concentrations of IGF1 and/or IGF2 potentially downregulates Ptch1 mRNA. Follicles collected at day 2 or 8 of an estrous cycle, along with the current findings might help elucidate the control of the hedgehog system by IGF1 and IGF2.

In conclusion, the modulation of both Ihh and Ptch1 mRNA in Twinner versus Control cows indicate a genetic control of the hedgehog system in mammals. In addition, the findings of this study indicate a role for the hedgehog system in deviation, rather than recruitment of the follicles, and the possible involvement of this system in the selection of one, possibly even two dominant follicles. Therefore, we hypothesize that an intrafollicular increase of IGF1 and IGF2 in response to a decrease in IGF2R in Twinner cows leads to the regulation of the hedgehog system via inhibiting the production of granulosa Ihh mRNA. This leads to a decrease in theca Ptch1 mRNA, which induces differentiation of the 2nd dominant follicle in Twinner cattle. These interactions indicate a complex intrafollicular control mechanism of the hedgehog system during follicular growth that requires further investigation.

The extent of the contribution of the hedgehog system to the control of deviation of a dominant follicle should be further investigated using techniques such as RNAi knockout models in our theca cell culture system to decipher the contribution of each of

the herein studied factors. Whether the interaction between the hedgehog system and the IGF system is a key contributor to the selection of two codomitant follicles in Twinner cows requires further investigation.

FIGURES

Figure 5.1: Gene expression for Indian Hedgehog in bovine granulosa cells from cows unselected (Control) or selected (Twinner) for multiple ovulations. Granulosa cells from unselected (Control) or selected (Twinner) cows for multiple ovulations were collected between D3-4 (D3) or D5-6 (D5) of an estrous cycle, follicles classified as Healthy (E2:P4 > 1) or Atretic (E2:P4 < 1), and RNA extracted. Ihh mRNA in granulosa cells quantitated using multiplex real-time RT-PCR and means expressed as relative abundance to 18 S rRNA. $^{a, b}$ Means without a common superscript differ ($P < 0.05$).

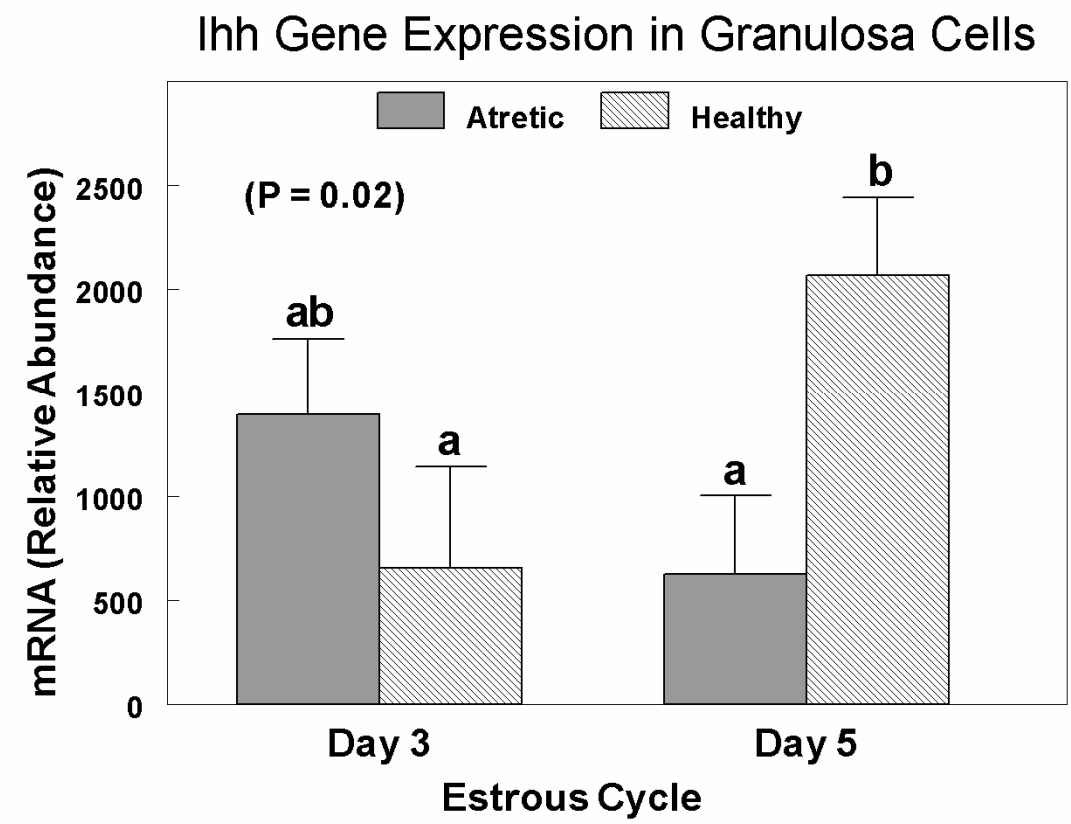

Ihh Gene Expression in Granulosa Cells
Atretic
Healthy
(P = 0.02)
mRNA (Relative Abundance)
2500
2000
1500
1000
500
0
ab
a
a
b
Day 3
Day 5
Estrous Cycle

Figure 5.2: Ptch1 mRNA gene expression in theca cells from cows unselected (Control) or selected (Twinner) for multiple ovulations. Theca cells from unselected (Control) or selected (Twinner) cows for multiple ovulations were collected between D3-4 (D3) or D5-6 (D5) of an estrous cycle, follicles classified as Healthy (E2:P4 > 1) or Atretic (E2:P4 < 1), and RNA extracted. Ptch1 mRNA in theca cells quantitated using multiplex real-time RT-PCR and means expressed as relative abundance to 18 S rRNA. Panel A: Day of cycle main effect on Ptch1 gene expression in theca cells. Panel B: Genotype by follicle status interaction. $^{a, b}$ Within each panel, means without a common superscript differ ($P < 0.05$).

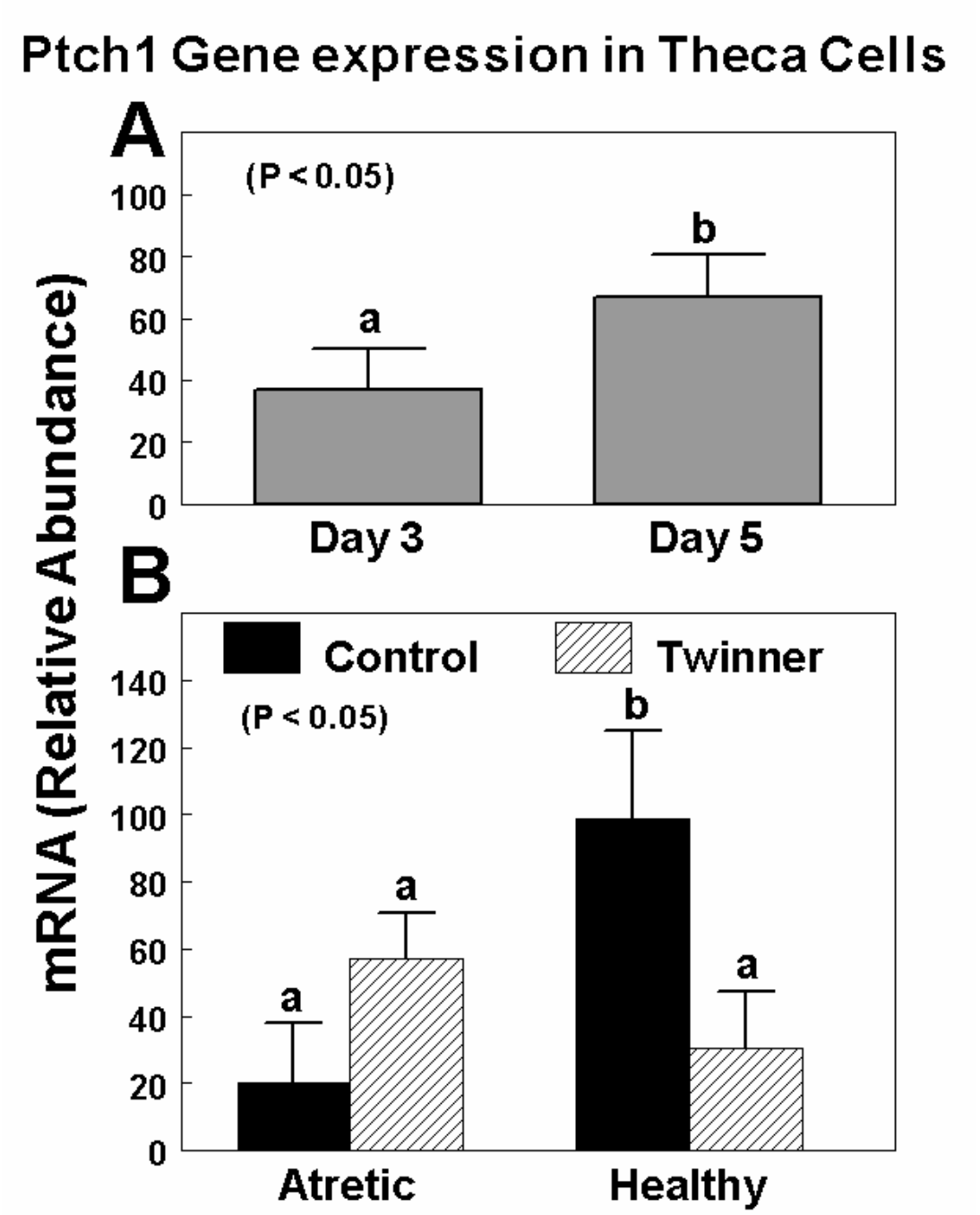

Ptch1 Gene expression in Theca Cells
A
(P < 0.05)
100
80
60
40
20
0
a
b
Day 3
Day 5
B
Control
Twinner
(P < 0.05)
140
120
100
80
60
40
20
0
a
a
b
a
Atretic
Healthy
mRNA (Relative Abundance)

Figure 5.3: Linear regression analysis of Ihh mRNA in granulosa cells of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. Ihh mRNA levels in granulosa cells of follicles from Control cows (—) is represented by the line $y = - 0.09 x + 5.4$; Ihh mRNA levels in granulosa cells of follicles from Twinner cows (- - -) is represented by the line $y = -0.07 x + 5.9$.

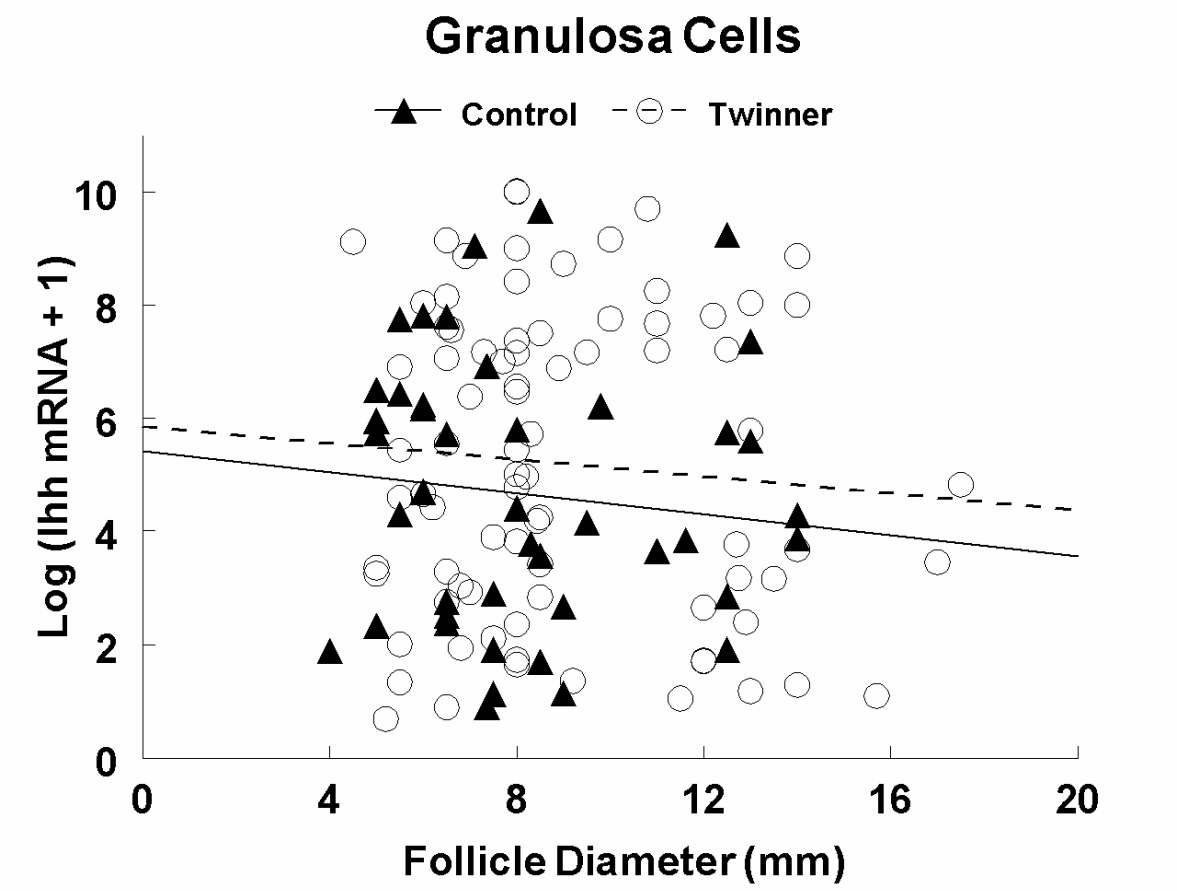

Granulosa Cells
Control
Twinner
Log (Ihh mRNA + 1)
0
2
4
6
8
10
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 5.4: Linear regression analysis of Ptch1 mRNA in theca cells of cattle unselected (Control) or selected (Twinner) for multiple ovulations and twin births. Ptch1 mRNA levels in theca cells of follicles from Control cows (—) is represented by the line $y = 0.04\ x + 2.02$; Ptch1 mRNA levels in theca cells of follicles from Twinner cows (- - -) is represented by the line $y = -0.08\ x + 3.13$.

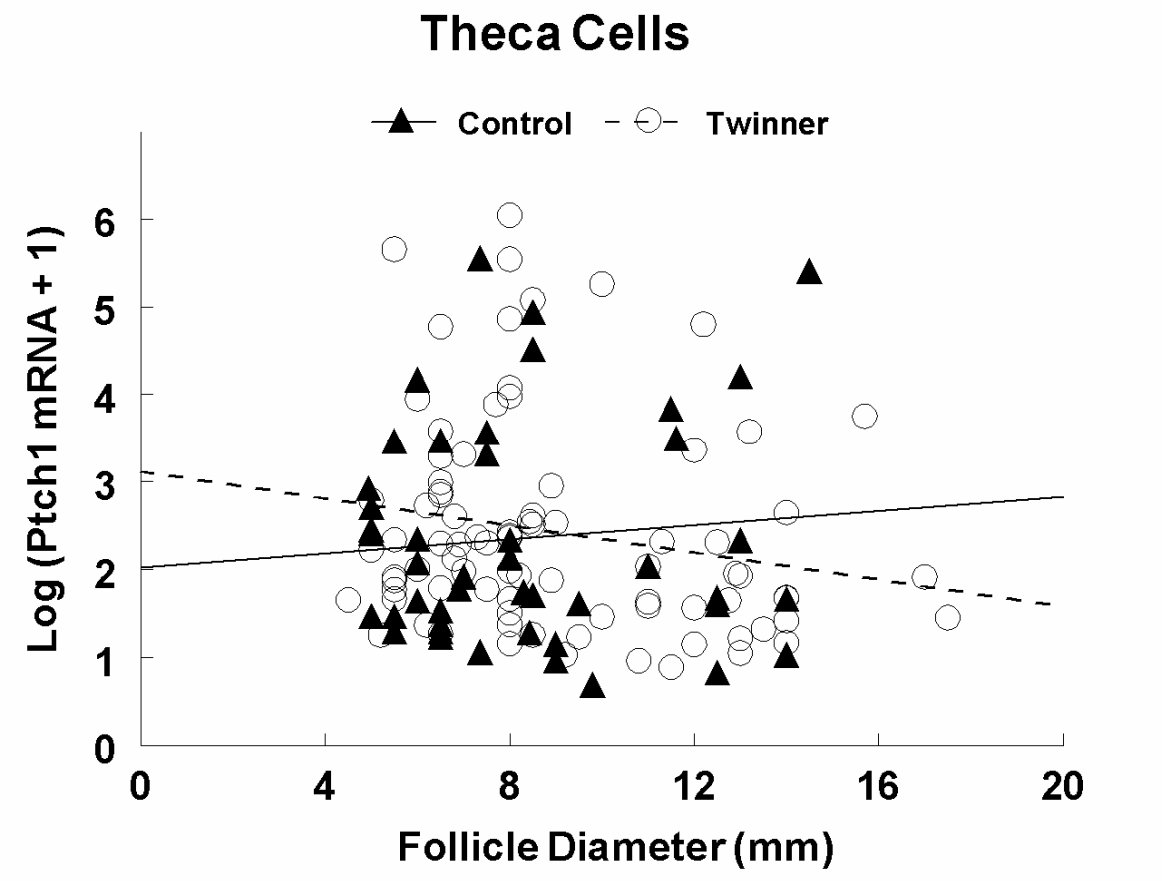

Theca Cells
Control
Twinner
Log (Ptch1 mRNA + 1)
0
1
2
3
4
5
6
0
4
8
12
16
20
Follicle Diameter (mm)

Figure 5.5: Indian Hedgehog and Patched 1 Gene expression in granulosa and theca cells of the largest 3 bovine follicles from cows unselected (Control) or selected (Twinner) for multiple ovulations. Granulosa and theca cells from unselected (Control) or selected (Twinner) cows for multiple ovulations were collected between D3-4 (D3) or D5-6 (D5) of an estrous cycle, follicles classified as Healthy (E2:P4 > 1) or Atretic (E2:P4 < 1), and RNA extracted. Ihh mRNA in granulosa cells and Ptch1 mRNA in theca were quantitated using multiplex real-time RT-PCR and means were expressed as relative abundance to 18 S rRNA. Panel A: Genotype by follicle interaction on Ihh gene expression in granulosa cells. $^{a, b}$ Means without a common superscript differ ($P < 0.05$). Panel B: Genotype by follicle interaction on Ptch1 gene expression in theca cells. *Within each follicle, mean differs from Control ($P < 0.05$).

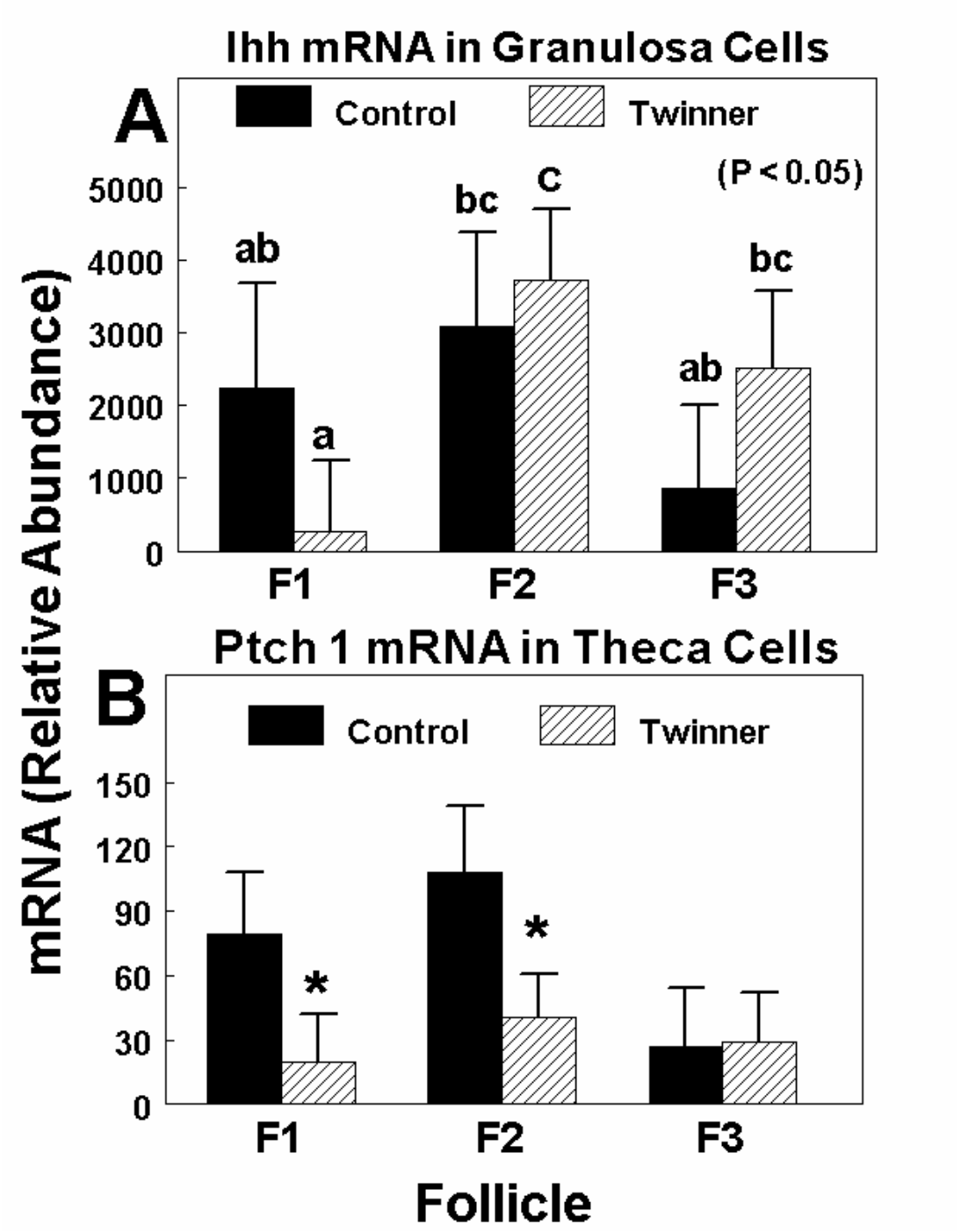

Ihh mRNA in Granulosa Cells
A
Control
Twinner
(P < 0.05)
5000
4000
3000
2000
1000
0
ab
a
bc
c
ab
bc
F1
F2
F3
Ptch 1 mRNA in Theca Cells
B
Control
Twinner
150
120
90
60
30
0
*
*
F1
F2
F3
mRNA (Relative Abundance)
Follicle

Figure 5.6: Indian hedgehog mRNA in cultured small granulosa cells. Granulosa cells from small (2-6 mm) follicles were cultured for 2 days in 10 % FCS, and then cells were washed and treated with IGF1 (0 or 100 ng/ml) in the presence of 30 ng/ml of FSH (Exp 2).

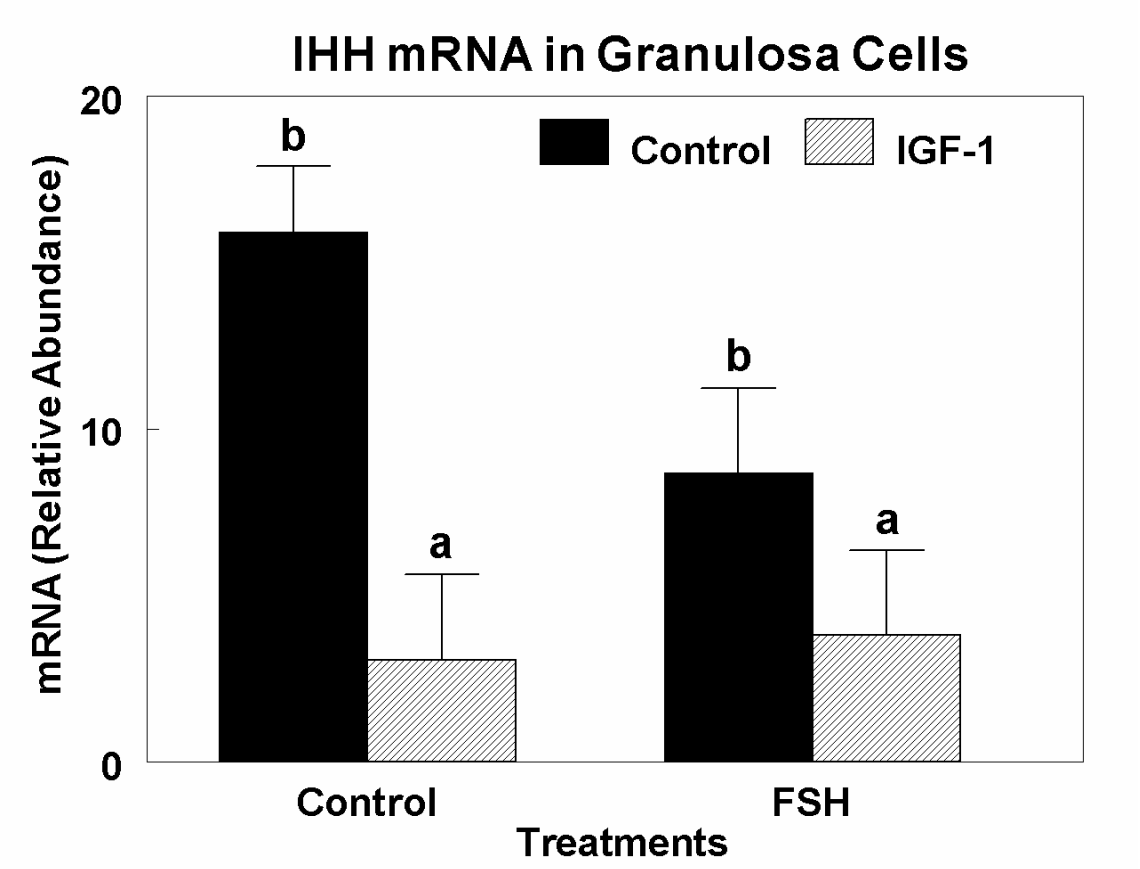

IHH mRNA in Granulosa Cells
20
10
0
mRNA (Relative Abundance)
b
a
b
a
Control
IGF-1
Control
FSH
Treatments

Figure 5.7: Ptch1 gene expression in cultured large theca cells treated with IGF2. Theca cells from large (8-22 mm) follicles were cultured for 2 days in 10 % FCS, and then cells were washed and treated with IGF2 (0 or 100 ng/ml), and (or) ovine LH (0 or 30 ng/mL) (Exp 5). Panel A: Ptch1 mRNA in cultured theca cells. Panel B: IGF2R mRNA in cultured theca cells. [a, b] Within each panel, means without a common superscript differ ($P < 0.05$).

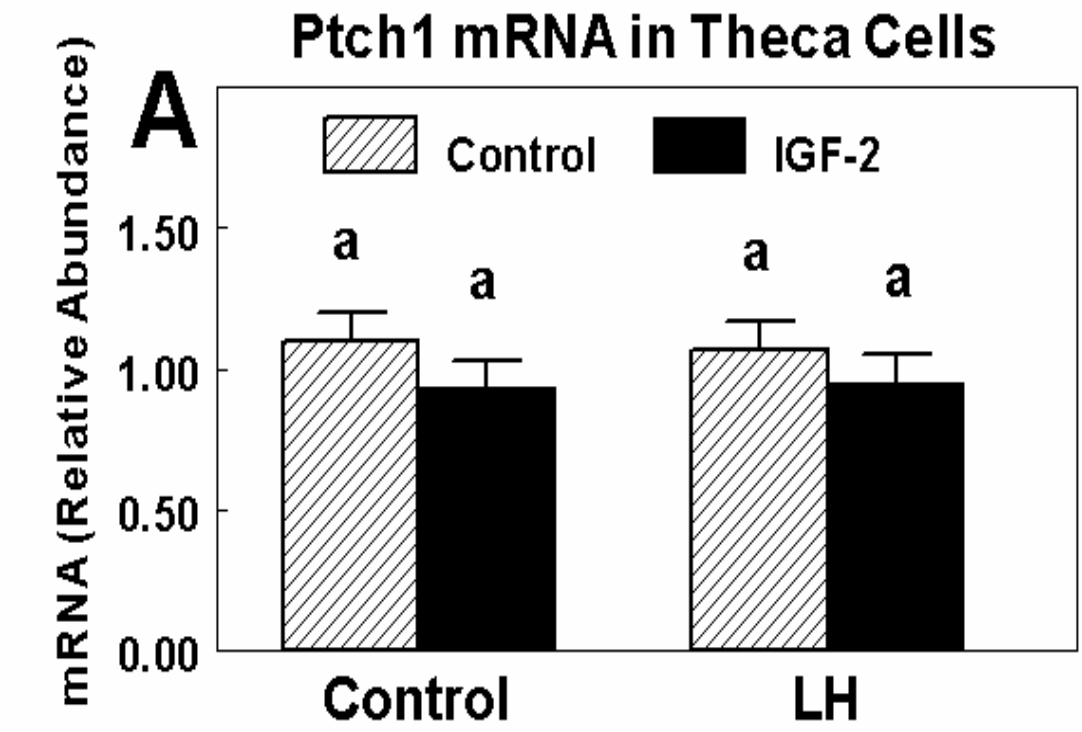

Ptch1 mRNA in Theca Cells
A
Control
IGF-2
mRNA (Relative Abundance)
1.50
1.00
0.50
0.00
a
a
a
a
Control
LH

Figure 5.8: Ptch 1 mRNA gene expression in small and large theca cells treated for 48 h. Theca cells from small (3-6 mm) and large (8-22 mm) follicles were cultured for 2 days in 10 % FCS, and then cells were washed and then treated for 48 h with IGF1 (0 or 100 ng/ml) or IGF2 (0 or 100 ng/ml) and (or) LH (0 or 30 ng/ml) (Exp 6). $^{a, b,c,d,e,f}$ means without a common superscript differ ($P < 0.05$).

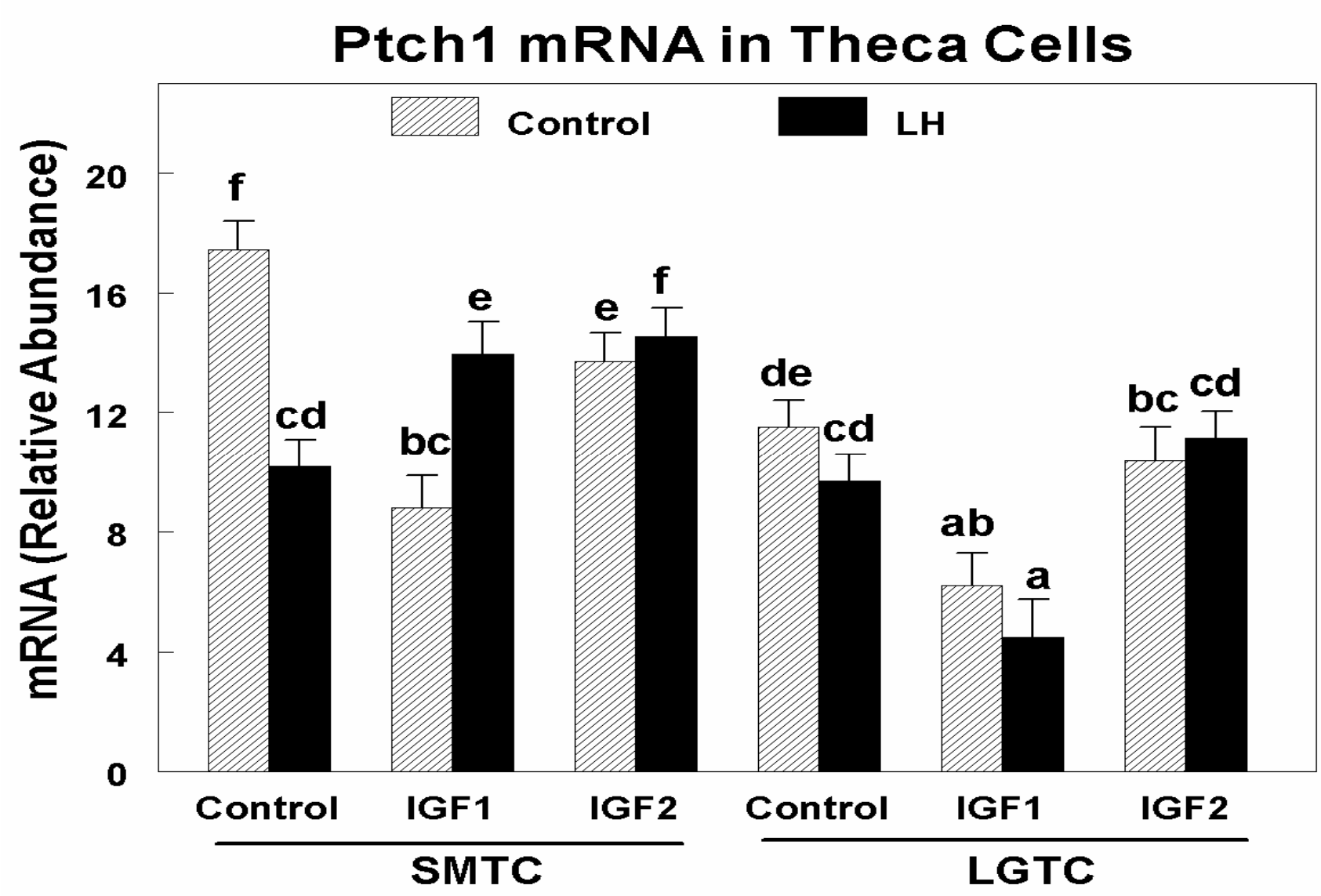
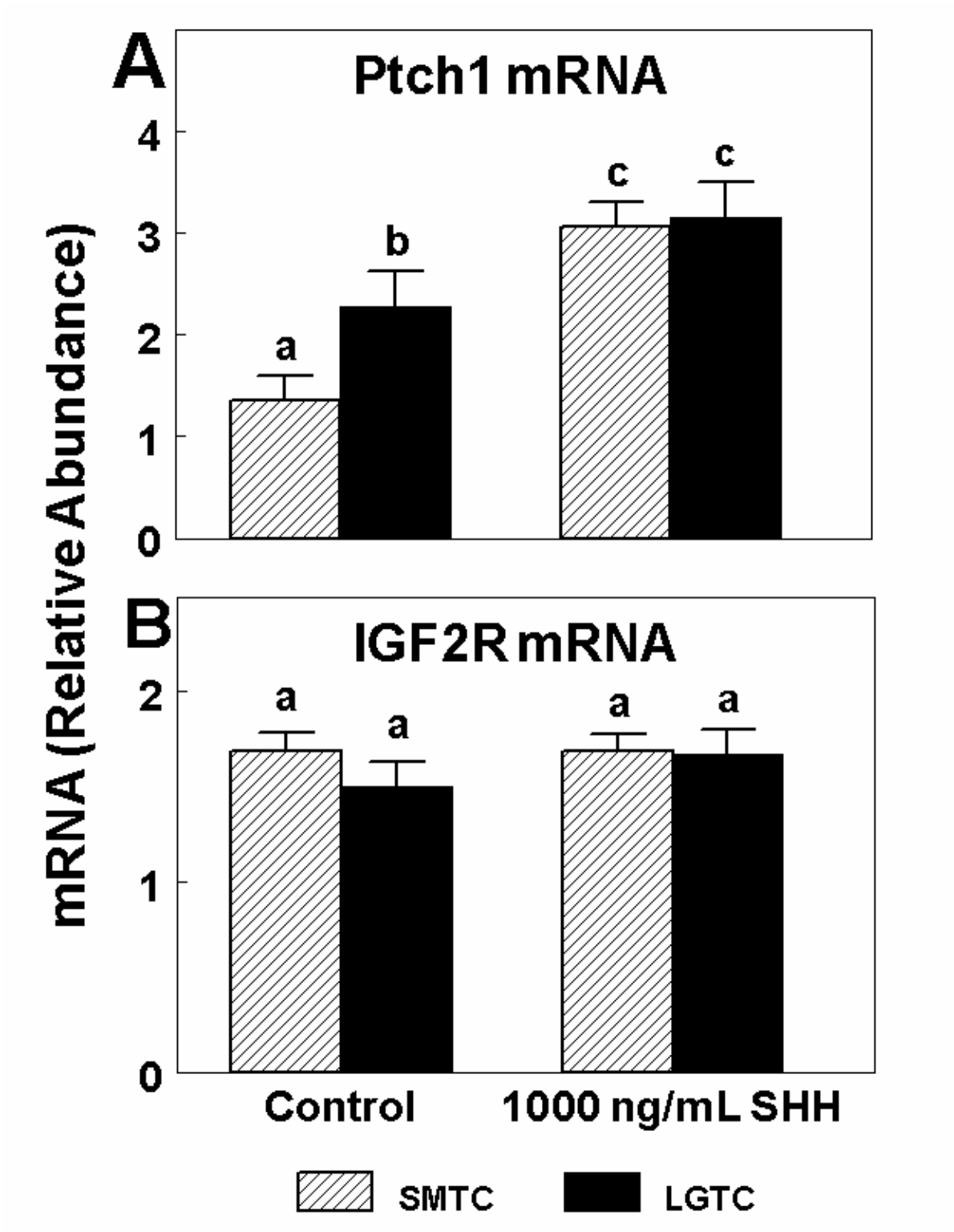

Ptch1 mRNA in Theca Cells
Control
LH
mRNA (Relative Abundance)
0
4
8
12
16
20
f
cd
bc
e
e
f
de
cd
ab
a
bc
cd
Control
IGF1
IGF2
Control
IGF1
IGF2
SMTC
LGTC

Figure 5.9: Ptch1 and IGF2R mRNA treated with 1000 ng/mL Shh in cultured theca cells. Theca cells from large (8-22 mm) follicles were cultured for 2 days in 10 % FCS, and then cells were washed and treated for 24 h with IGF1 after which treatments with Sonic Hedgehog (0 or 1000 ng/ml) in the presence of IGF1 (Exp 8). Panel A: Ptch1 mRNA in cultured theca cells. Panel B: IGF2R mRNA in cultured theca cells. [a, b, c] Within each panel, means without a common superscript differ ($P < 0.05$).


A
Ptch1 mRNA
4
3
2
1
0
a
b
c
c
B
IGF2R mRNA
2
1
0
a
a
a
a
mRNA (Relative Abundance)
Control
1000 ng/mL SHH
SMTC
LGTC

TABLES

Table 5.1: Sequences, accession numbers and melting temperatures of the bovine-specific primers and probes for the selected target genes of interest. Accession numbers are from the national center for biotechnology information (National library of medicine and national institutes of health), available at http://www.ncbi.nlm.nih.gov/.

Gene	Oligo[1]	Sequence	Accession	Anneals between	Tm[1] (°C)
Indian Hedgehog (Ihh)	FWD	CGGCTTCGACTGGGTGTATTAC	**XM_601000**	1009-1030	59
	REV	AGGGAAGCAGCCACCTGTCT		1105-1086	60
	Probe*	CAAGGCCCACGTGCATTGCTCC		1036-1057	69
Patched homolog 1 (Ptch1)	FWD	TGCCCAGGCTACGAGGACTA	**XM_869803**	130-149	59
	REV	CCGGACATTAAAAGGCACATG		204-184	59
	Probe	TGACCACGGCCTGTTTGAGGACC		159-181	68

[1]FWD: forward primer, REV: reverse primer, Tm: melting temperature

* Exon-spanning probe

Table 5.2: The Hedgehog system gene expression in granulosa and theca cells from unselected (Control) or selected (Twinner) cows for multiple ovulations. Ihh and Ptch1 mRNA were measured using multiplex real-time RT-PCR and means expressed as relative abundance to 18 S rRNA.

		Indian hedgehog mRNA		Patched 1 mRNA		
Up to 10 follicles within a cow		**LSMean***	**SEM**	**LSMean***	**SEM**	
Genotype						
Control		991.33	381.12	56.98	17.43	
Twinner		1381.02	259.43	44.32	13.06	
	p-value**	**0.51**		**0.57**		
Day of cycle						
Day 3		1347.15	345.94	66.36	15.59	b
Day 5		1025.20	304.78	34.49	15.21	a
	p-value**	**0.40**		**0.04**		
Status						
Atretic		1011.37	262.32	36.92	11.21	
Healthy		1360.99	379.14	64.38	15.58	
	p-value**	**0.62**		**0.12**		
Genotype x cycle	**p-value****	**0.77**		**0.89**		
Genotype x status	**p-value****	**0.27**		**0.005**		
Cycle x status	**p-value[1]**	**0.02**		**0.62**		
Genotype x cycle x status	**p-value****	**0.19**		**0.89**		

[a, b] Means without a common superscript differ ($P < 0.05$).

* LSMean of the mRNA relative abundance ($2^{-\Delta\Delta Ct}$)

** P-value of ANOVA on Log transformed data.

[1] P-value of ANOVA on mRNA abundance

Table 5.3: The Hedgehog system gene expression in granulosa and theca cells from unselected (Control) or selected (Twinner) cows for multiple ovulations from the largest 3 follicles per cow. Ihh and Ptch1 mRNA were measured using multiplex real-time RT-PCR and means expressed as relative abundance to 18 S rRNA.

PCR and means expressed as relative abundance to 18 S rRNA.

Up to 10 follicles per cow			Granulosa Ihh mRNA		Theca Ptch1 mRNA		
		N	LSMean*	SEM	LSMean*	SEM	
Genotype	**Control**	46	991.33	381.12	56.98	17.43	
	Twinner	85	1381.02	259.43	44.32	13.06	
	P-value**		**0.51**		**0.57**		
Day of cycle	**Day 3**	66	1347.15	345.94	66.36	15.59	b
	Day 5	65	1025.2	304.78	34.49	15.21	a
	P-value**		**0.4**		**0.04**		
Status	**Atretic**	83	1011.37	262.32	36.92	11.21	
	Healthy	48	1360.99	379.14	64.38	15.58	
	P-value**		**0.62**		**0.12**		
Genotype x Cycle	P-value**		**0.77**		**0.89**		
Genotype x Status	P-value**		**0.27**		**0.005**		
Cycle x Status	P-value**		**0.02**		**0.62**		
Genotype x Cycle x Status	P-value**		**0.19**		**0.89**		

[a, b] Within effect, means without a common superscript differ ($P < 0.05$) if interaction is not significant.

* LSMean of the mRNA relative abundance ($2^{-\Delta\Delta Ct}$)

** P-value of ANOVA on Log transformed data.

Table 5.4: Pearson Correlation coefficients among diameter, follicular fluid hormones and follicular cell mRNAs of IGF2R, Ihh and Ptch1.

		Follicular Fluid Hormones			Granulosa Cell mRNA		Theca Cell mRNA	
		E_2	**P_4**	**E_2:P_4**	**IGF2R**	**Ihh**	**IGF2R**	**Ptch1**
	Di	0.78***	0.06	0.75***	-0.24*	-0.25**	-0.12	-0.24**
FFL	E2	-	0.005	0.83***	-0.29**	-0.26**	-0.08	-0.16
	P4	-	-	-0.07	-0.06	-0.13	0.04	-0.13
	E2:P4	-	-	-	-0.30**	-0.27**	-0.14	-0.15
GC	IGF2R	-	-	-	-	0.36**	0.11	0.14
	Ihh	-	-	-	-	-	0.08	0.20*
TC	IGF2R	-	-	-	-		-	0.69***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Abbreviations: Di: diameter; FFL: follicular fluid; GC: granulosa cells; TC: theca cells; E2: estradiol; P4: progesterone; IGF2R: IGF type 2 receptor; Ihh: indian hedgehog; Ptch1: patched 1; and n = 104-106

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CHAPTER VI

SUMMARY: The IGF and Hedgehog system in cattle: insight into follicular development in cattle selected for double ovulations and twin births

Gene expression in granulosa and theca cells in follicles from cattle not selected (Control) or selected for multiple ovulations and twin births (Twinner) using sensitive quantitative multiplex RT-PCR, at two different stages of the follicular cycle were conducted in Chapter 3. E-A follicles had greater E_2 (164.6 ± 13.8 vs. 3.7 ± 11.2 ng/mL) and lower P_4 levels (27.0 ± 18.1 vs 90.8 ± 14.1 ng/mL) than E-I follicles, and expression of granulosa FSHR mRNA was greater ($P < 0.05$) at D3 versus D5 healthy follicles, and in Control versus Twinner cows at D3. Levels of granulosa or thecal LHR mRNA in E-A follicles did not differ ($P > 0.10$) between Control versus Twinner cows. Granulosa IGF2R mRNA was lower ($P > 0.10$) in E-A versus E-I follicles, and lower ($P < 0.05$) in GC at D5 of an estrous cycle. Within the largest three follicles, Di was greater in Twinner versus Control cows and F2 in Twinner cows had the same ($P > 0.10$) Di as F1 in Control cows. Granulosa and thecal LHR mRNA did not differ ($P > 0.10$) between F1 and F2, but granulosa LHR mRNA was lower in F3 of Twinner than Control cows. Granulosa IGF2R mRNA was greater in Control than Twinner cows at D3 and D5 of an estrous cycle; F2 had greater ($P<0.05$) thecal IGF2R mRNA than F1 and F3 of Control and F1, F2 and F3 of Twinner cows. Thus we hypothesize that decreased IGF2R in granulosa and theca cells of Twinner cows increases bioavailable IGF2, which in turn enhances follicular development of two, rather than one follicle.

To further characterize the importance of the increase in bioavailable IGF2 observed to potentially enhance follicular development in Twinner, granulosa cells were collected from small (1–5 mm) and large (8–22 mm) bovine follicles and were treated with IGF2 for 1–2 days in serum-free medium, and steroid production, cell proliferation, specific ^{125}I-IGF2 binding, and gene expression were quantified in Chapter 4. IGF2 increased both estradiol and progesterone production by granulosa cells, and cells from large follicles were more responsive to the effects of IGF2 than those from small follicles. Abundance of aromatase (*CYP19A1*) mRNA was stimulated by IGF2 and IGF1. IGF2 also increased proliferation of granulosa cells by 2- to 3-fold, as determined by increased cell numbers and ^{3}H-thymidine incorporation into DNA. Treatment with IGF1R antibodies reduced the stimulatory effect of IGF2 and IGF1 on estradiol production and cell proliferation. Specific receptors for ^{125}I-IGF2 existed in granulosa cells, and 2-day treatment with estradiol, FSH, or cortisol had no significant effect on specific ^{125}I-IGF2 binding. Also, FSH treatment of small- and large-follicle granulosa cells had no effect on *IGF2R* mRNA levels, whereas IGF1 decreased *IGF2R* mRNA and specific ^{125}I-IGF2 binding. Granulosa cell *IGF2R* mRNA abundance was 3-fold greater in small than in large follicles. These findings support the hypothesis that both IGF2 and its receptor may play a role in granulosa cell function during follicular development.

In Chapter 5, we studied novel genes recently reported to be important in mammalian species in follicular development. Thus, for the first time in bovine ovarian follices, the hedgehog system was investigated in the Twinning model and further in cultured granulosa and theca cells. Abundance of Ihh mRNA in GC of healthy follicles was twofold greater ($P < 0.01$) than in atretic follicles and 2.3-fold greater in D3 than D5

follicles, thecal Ptch1 mRNA was greater ($P < 0.05$) at D5 than D3, and Control cows had greater ($P < 0.05$) Ptch1 mRNA than Twinner cows within healthy follicles. Follicular Di correlated negatively with granulosa Ihh mRNA ($r = - 0.25$, $P < 0.05$) and with TC Ptch1 mRNA ($r = - 0.24$, $P < 0.01$); GC Ihh mRNA was correlated positively ($r = 0.36$, $P < 0.01$) with GC IGF2R mRNA, and TC Ptch1 mRNA was correlated positively with GC ($r = 0.20$, $P < 0.05$) and TC ($r=0.69$, $P < 0.01$) IGF2R mRNA. In cell culture, IGF1 decreased ($P < 0.001$) whereas FSH did not affect ($P > 0.10$) Ihh mRNA in GC. 48 h treatments of theca cells with IGF1 and IGF2 but not LH decreased ($P < 0.01$) Ptch1, but 24 h treatments did not affect Ptch1 or IGF2R mRNA in TC. For the first time, an interaction between the ovarian IGF and hedgehog systems has been demonstrated.

Because IGF2R sequesters free IGF-2 and, thus, reduces its binding to the IGF type I receptor, we hypothesize that reduced thecal IGF2R levels in follicles of Twinner cows may increase the amount of free or bioavailable IGF-2, which may act in an autocrine or paracrine fashion to stimulate development of a second dominant follicle. In particular, increased free IGF1 in developing follicles may decrease synthesis of IGF2R, thereby allowing for more IGF2 to be bioavailable (free) for induction of steroidogenesis and mitogenesis via the IGF1R. Increased free IGF1 and IGF2 as follicles grow and develop may suppress Ihh production by GC, and subsequently regulate Ptch1 in TC. Whether the reduction in Ptch1 mRNA in TC is involved in the development of two co-dominant follicles in Twinner cows will require further elucidation.

CHAPTER VII

CONCLUSION AND IMPLICATIONS

Follicular development in cattle starts with the recruitment of some follicles that will undergo atresia or grow towards dominance. The recruitment of the follicles is preceded by an increase in FSH, an event widely believed to cause the initiation of this process. FSH induces estradiol production by the follicles, and the synthesis of LH receptors in granulosa cells, and both are responsible for increasing follicle sensitivity to gonadotropins and switching follicles from FSH-dependant to LH-dependant (Campbell et al., 1995; Campbell, 2003). The FSH and LH sensitization depends on the presence and expression of gonadotropin receptors and thus increased estradiol production by granulosa cells following uptake of androgens synthesized by theca cells. Thus, a proper communication among these two cell layers is paramount. The hedgehog system in mammalian cells is responsible for cell-cell communication and controls cell fate in the same manner as IGF1, an anti-apoptotic factor. IGF1 and IGF2 play a major role in regulating the growth rate of the follicle and protecting it from atresia. IGF1 and IGF2 stimulates mitogenesis and steroidogenesis in both granulosa (Spicer et al., 1993) and theca cells (Spicer and Aad, 2007) in vitro.

In cattle populations selected for increased ovulation rate and multiple births, plasma and follicular fluid IGF1 levels were greater than contemporary unselected populations, indicating a role of the IGF system in the selection of multiple follicles within a follicular wave (Echternkamp et al., 1990; Echternkamp, 2000; Echternkamp et

al., 2004). In this population, the development of multiple follicles within an estrous cycle provides an excellent model to study ovarian follicular development and to gain insight into intra-follicular factors regulating/controlling follicular selection and dominance.

The purpose of this study was to describe the gonadotropin receptor (LHR and FSHR) expression patterns in Twinner versus Control cattle at different stages of the follicular cycle, and determine the expression and regulation of IGF2R in growing and atretic antral follicles from these two cattle populations. In addition, in an effort to observe the importance of cell communication factors recently identified to play an important role in folliculogenesis and organogenesis in Drosophila and mice, we quantified gene expression of two of the hedgehog system components, Ihh and Ptch1, in ovarian granulosa and theca cells, respectively.

In this study, we found greater FSHR gene expression at recruitment in Control than Twinner cows. Theca LHR gene expression was not different between recruitment and deviation in Control cows, whereas Twinner cows had greater theca LHR gene expression at recruitment than at deviation. Collectively, these results suggest a possibility of precocious follicular deviation in Twinner cows, a model similar to proposed mechanisms of control of twinning in sheep (Souza et al., 1997a; Souza et al., 1997b). IGF2R, a membrane bound binding protein that controls the level of bioavailable IGF2 was downregulated in Twinner cows. The role of IGF2 in the bovine ovary and the hormonal control of IGF2R in vitro were further evaluated. IGF2 increased estradiol and progesterone production by granulosa cells and stimulated

aromatase gene expression in a manner similar to IGF1. In addition, the in vitro downregulation of IGF2R by IGF1 but not FSH. The downregulation of IGF2R in Twinner versus Control cows emphasize the importance of the locally produced IGF2 and its contribution to intrafollicular bioavailable IGF and its role as a key factor controlling selection and maintenance of dominance in cattle, and further emphasizes the involvement of the IGF system in the selection of multiple dominant follicles.

For the first time in the bovine ovary, indian hedgehog (Ihh) and its receptor patched 1 (Ptch1) were expressed in granulosa and theca cells of antral follicles from 4 to 17 mm in diameter, respectively. Both granulosa cell Ihh and theca cell Ptch1 gene expression increased at deviation in estrogen-active follicles, thus indicating a role for the Ihh in controlling cell-fate and deviation of the follicle. The decrease of theca cell Ptch1 gene expression in Twinner cows, combined with the IGF1-induced decrease of Ihh mRNA gene expression in cultured granulosa cells, along with an IGF1 and IGF2 decrease of Ptch1 mRNA in cultured theca cells indicate, for the first time, a relationship between the IGF and hedgehog system in controlling the selection of multiple follicles in cattle. Other candidate genes regulating follicular development and the levels of intrafollicular IGF levels, such as IGFBP-4 and -5, and PAPP-A, in addition to the IGF2R gene might be of interest for future studies.

Insight into the role of the IGF system (namely IGF2 and IGF2R) and its interaction with the hedgehog system (a newly identified player in the control of ovarian follicular development) will pave the way for the discovery of interactions within the

follicle, and the discovery of new factors involved in the selection of the dominant follicle in cattle.

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APPENDIX

Appendix 1: Master mix preparation for real-time RT-PCR optimization. FWD is forward primer, REV is reverse primer. Rx mix is for the reaction mix containing the dNTPs and the AmpliTaq Gold® DNA polymerase. M scribe is for multiscribe containing the reverse transcriptase.

Master Mix		Oligo	nM	Master Mix		Oligo	nM
1	Target Gene	FWD	200	2	Target Gene	FWD	200
		REV	200			REV	200
		Probe	100			Probe	100
	18 S rRNA	FWD	100		18 S rRNA	FWD	20
		REV	100			REV	20
		Probe	100			Probe	100
	Rx mix				Rx mix		
	m scribe				m scribe		
	DEPC water				DEPC water		
3	Target Gene	FWD	200	4	Target Gene	FWD	200
		REV	200			REV	200
		Probe	100			Probe	100
	18 S rRNA	FWD	10		18 S rRNA	FWD	1
		REV	10			REV	1
		Probe	100			Probe	100
	Rx mix				Rx mix		
	m scribe				m scribe		
	DEPC water				DEPC water		
5	Target Gene	FWD	200	6	18 S rRNA	FWD	100
		REV	200			REV	100
		Probe	100			Probe	100
	Rx mix				Rx mix		
	m scribe				m scribe		
	DEPC water				DEPC water		

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: GENE EXPRESSION IN OVARIAN GRANULOSA AND THECA CELLS IN CATTLE SELECTED FOR DOUBLE OVULATIONS AND TWIN BIRTHS

Pages in Study: 241 Candidate for the Degree of Doctor of Philosophy

Major Field: Animal Breeding and Reproduction

Scope and Methods of Study: Components of the IGF system and gonadotropin receptors (FSHR and LHR) might be involved in the control of the development of multiple dominant follicles leading to twinning. Gene expression of FSHR, LHR, IGF2R and the hedgehog system (Ihh and Ptch1) gene expression in granulosa and/or theca cells from cattle not selected (Control) or selected for multiple ovulations (Twinner) were quantitated at two different stages (Day 3, D3; Day 5, D5) of the follicular cycle and in the largest 3 follicles. The effect of IGF2 in small (1–5 mm) and large (8–22 mm) bovine granulosa cell cultures on steroid production, cell proliferation, and gene expression were also investigated.

Findings and Conclusions: Granulosa FSHR mRNA was greater ($P < 0.05$) at D3 versus D5 in healthy estrogen-active (E-A) follicles and in Control versus Twinner cows at D3. Granulosa IGF2R mRNA was lower ($P < 0.05$) in E-A versus atretic estrogen-inactive (E-I) follicles, and lower ($P < 0.05$) at D5. Granulosa IGF2R mRNA was greater ($P < 0.05$) in Control than Twinner cows at D3 and D5; Control F2 had greater ($P<0.05$) thecal IGF2R mRNA than F1, F2 and F3 of Twinner cows. IGF2 increased granulosa estradiol and progesterone production, stimulated aromatase mRNA and increased proliferation of granulosa cells. IGF1R antibodies reduced the stimulatory effect of IGF2 and IGF1 on estradiol production and cell proliferation. Granulosa Ihh mRNA was two-fold greater ($P < 0.05$) in healthy than atretic follicles and in D3 than D5; thecal Ptch1 mRNA was lower ($P < 0.05$) at D3 than D5, and in Twinner versus Control cows. In cultured granulosa cells, IGF1 decreased ($P < 0.001$) Ihh mRNA abundance, and both IGF1 and IGF2 decreased ($P < 0.01$) thecal Ptch1 mRNA abundance. Decreased IGF2R in granulosa and theca cells of Twinner cows likely increases bioavailable IGF2, which in turn enhances follicular development of two, rather than one follicle and further supports the idea that IGF2 and its receptor (IGF2R) may play a role in follicular development. For the first time, we demonstrate an interaction between the ovarian IGF and hedgehog systems.

ADVISER'S APPROVAL: Dr. Leon J. Spicer