RELATIONSHIPS AMONG GRANULOSA CELL INSULIN- LIKE GROWTH FACTOR BINDING PROTEINS AND PREGNANCY-ASSOCIATED PLASMA PROTEIN-A mRNA EXPRESSION AND FOLLICULAR FLUID CONTENTS IN DOMINANT AND LARGE SUBORDINATE FOLLICLES OF PREOVULATORY CATTLE

By

CONSUELO AMOR T. SANTIAGO

Bachelor of Science

University of the Philippines Los Banos

Laguna, Philippines

1998

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillments of
the requirement of
the Degree of
MASTER OF SCIENCE
May, 2004

RELATIONSHIPS AMONG GRANULOSA CELL INSULIN- LIKE GROWTH FACTOR BINDING PROTEINS AND PREGNANCY-ASSOCIATED PLASMA PROTEIN-A mRNA EXPRESSION AND FOLLICULAR FLUID CONTENTS IN DOMINANT AND LARGE SUBORDINATE FOLLICLES OF PREOVULATORY CATTLE

Thesis Approved:

Thesis Adviser

Dean of Graduate College

ACKNOWLEDGEMENT

First and foremost I would like to thank my adviser and committee members. Dr. Leon Spicer, thank you for giving me the opportunity to be part of your research team. I considered my mistakes and success in each experiment as precious tools in my never ending journey towards learning. Thank you for always being patient and understanding. After being under your direction, I hope to do the same to my future advisees. Know that you have not only helped me grow as individual, but have helped my home country as well. Dr. Robert Wettemann, thank you for being my role model in the art and science of teaching. I have enjoyed each and every class that you taught. Dr. Udaya DeSilva, thank you for the "biotech experience" and for giving me the chance to work with Xiaoping and Charaka who have been both very helpful.

I thank God for Victoria Lehloenya for being my constant companion, prayer warrior and older sister. We may never see each other again, but you will never be forgotten. To my fellow graduate students in Animal Science thank you for the company and help that you extended during my stay.

Special thanks to Dr. Adele Tongco whose motherly love became the source of my daily strength, and to Drs. Edralin Lucas, Rose Muralla, Yolly Lim, Tat Morillo for the support, concern, and encouragements. Thank you to my closest friends, Pai Gadiaware and Julian Cacho, for the wonderful and memorable friendship that made my stay worthwhile.

Thank you to my dear Institute of Animal Science (IAS-UPLB) family, for being my inspiration to strive for the very best.

Thank you to the Fulbright-Philippine Agriculture Scholarship Program for giving me the chance to help my country by financing my education here in OSU.

Thank you to my family (everybody under the Santiago household), especially my parents, Benny and Mel, for the love and prayers that you gave. People come and go, but you stood firm in my worst and best moments...

To my three little treasures, Larma Mel Soliven, Lorenzo Gil Soliven, and Jeanne Ashley Santiago, for bringing joy to my life.

To my fiancée, Richard Estrella, whose love never fails.

To God, who made all things possible.

" Walk with faith and you will be truly led to your own true path. "

TABLE OF CONTENTS

CHAPTER **Page**

I. INTRODUCTION.....1

II. REVIEW OF RELATED LITERATURE.....5

Insulin-like Growth Factor-I (IGF-I).....5

Sources of IGF-I in the follicular fluid.....5

Forms of IGF-I: Total vs. Free.....7

IGF-I functions.....9

Role of insulin-like growth factor binding proteins (IGFBPs) in follicle dominance.....13

Localization and gene expression of IGFBPs in the ovary.....16

Changes in IGFBP protein levels in the follicular fluid.....19

Relationship of hormones with ovarian IGFBP.....22

Role of Pregnancy-associated plasma protein-A in follicle dominance.....25

Localization and expression of PAPP-A in follicular cells.....29

Hormonal regulation of PAPP-A mRNA.....30

References.....32

III. RELATIONSHIPS OF GRANULOSA CELL INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND PREGNANCY-ASSOCIATED PLASMA PROTEIN-A mRNA EXPRESSION AND FOLLICULAR FLUID CONTENTS IN DOMINANT AND LARGE SUBORDINATE FOLLICLES OF PREOVULATORY CATTLE

Abstract.....44
Introduction.....45
Materials and Methods.....49
Results.....58
Discussion.....64
Literature Cited.....69

IV. SUMMARY AND CONCLUSIONS.....83

LIST OF TABLES

Table Page

1. Characteristics of some insulin-like growth factor binding proteins.....15
2. Quantitative real-time PCR analysis of IGFBP-2, -3, -4, -5 and PAPP-A mRNA expression in dominant and subordinate follicles collected at 24 or 48 h post PGF2α.....81
3. Pearson correlation coefficients of diameter, follicular fluid hormones, and IGFBPs and PAPP-A.....82

LIST OF FIGURES

Figure Page

1. Parallelism between PAPP-A, IGFBP-5, and 18S rRNA.....75

2. Mean diameters and free IGF-I concentrations of dominant and subordinate follicles averaged across time.....76

3. Estradiol concentrations in dominant and subordinate follicles collected at 24 or 48 h post PGF2α.....77

4. Progesterone and androstenedione concentrations of dominant and subordinate follicles averaged across time.....78

5. Binding activities of IGFBP-2, IGFBP-4 (total) and IGFBP-5 in follicular fluid of dominant and subordinate follicles.....79

6. Relative mRNA abundance of IGFBP-2 (panel A), IGFBP-4 (panel B) and IGFBP-5 (panel C) in granulosa cells of dominant (DF) and subordinate (SF) follicles collected 24 or 48 h after PGF2α.....80

CHAPTER I

INTRODUCTION

Antral follicular growth in farms animals such as cattle, goats, sheep, horses, and buffaloes occurs in a cyclic pattern called follicular waves. For monovular species, cattle have become a good model in studying follicular dynamics. The 2 to 3 follicular waves occur on day 2 and 11, or 2, 9, and 16 of the estrous cycle, respectively, following an increase in serum FSH levels. Usually, the last follicular wave generates the ovulatory follicle unless the dominant follicle of earlier waves is induced to ovulate by PGF2α (Fortune et al., 2001). It is however, the first follicular wave that is popularly used in most studies because it is unaffected by any previous wave making key events such as follicle emergence, selection, and dominance predictable.

Follicle wave emergence is the first day when 4 mm antral follicles are detectable via ultrasonography (Evans, 2003). Studies utilizing the first follicular wave (Mihm et al., 1997; 2000; Austin et al., 2001) showed that FSH concentrations peak to about 30-40 ng/mL, coincident with ovulation on day 1 of the estrous cycle (Ireland and Roche, 2000). This rise in FSH stimulates about 24 follicles to emerge and grow beyond 3 mm in diameter. About 29% of these follicles (7 out of 24) grow $\geq$ 6 mm and enter the selection phase by day 3 of the estrous cycle, while the rest grow to a maximum diameter of 4 to 5 mm (Ginther et al., 1996).

The reduction in the number of follicles growing in the cohort marks the beginning of the selection phase by day 3 of the estrous cycle. The size of the future dominant follicle may not necessarily be greater than its competitors at this point, but diameter deviation becomes obvious by day 4.8 post-estrus (or 2.8 days after follicle wave emergence). In Holstein heifers, diameter deviation, as detected by ultrasound, occurs when the largest follicle has an average diameter of 8.2 mm (Ginther et al., 2003), and is approximately 1.5 mm larger than the next largest follicle. Diameter deviation indicates the end of the selection phase, and onset of dominance. Coincident with the dominant follicle's enlargement is its greater estrogenic capacity than the other large follicles. Intrafollicular estradiol from the largest follicle may therefore drive FSH concentrations to its basal level of about 12 to 19 ng/mL (Mihm et al., 1997; 2000). At low circulating FSH, the dominant follicle continues to grow reaching the preovulatory size of 15 mm (Ireland and Roche, 2000; Mihm et al., 2000; Evans, 2003), while the large subordinate follicles stagnate at 8 mm. Tenure of dominance lasts until day 8 to 10 of the estrous cycle, and the dominant follicle becomes estrogen inactive after day 10. By then, another significant 2-fold increase in FSH (Ireland and Roche, 2000) will initiate the next follicular wave. Regression of the dominant follicle to 4-5 mm diameter, or until it is undetectable may extend to the next follicular wave (Ireland and Roche, 2000).

Although follicle size is a good morphological basis of dominance via ultrasound, there are intrafollicular factors that are responsible for the differential responsiveness of follicles to the same gonadotropin environment. Intrafollicular factors such as ovarian steroids (estrogen, progesterone, androstenedione), and more importantly, insulin-like

growth factor (IGF)-I and its associated IGF binding proteins (IGFBP). Differences in levels of intrafollicular factors of the dominant and subordinate follicles may serve as important biochemical markers of follicle growth.

IGF-I is known to stimulate granulosa cell proliferation and synergize with FSH to enhance its steroidogenic capabilities, such as FSH-induced estrogen production (Spicer and Echternkamp, 1995; Fortune et al., 2001). Estrogenic dominant as compared with estrogen-inactive subordinate follicles may have the same total IGF-I concentrations (Stewart et al., 1996) but reduced low molecular weight IGFBP-2, -4, and –5. Thus, the IGFBP are primarily involved in the regulation of IGF-I bioavailability within the follicle. Indeed, recent studies verify that first wave dominant follicles also have 4 to 8 ng/mL of increased "free" or bioavailable IGF-I (Beg et al, 2001; 2002; Rivera and Fortune, 2003a).

Changes in the concentrations of low molecular weight IGFBP in bovine preovulatory follicles may be regulated by: gene expression in ovarian tissues, proteases such as the pregnancy-associated plasma protein-A (PAPP-A), or a combination of both factors. To date, there are no studies linking the gene expression of the IGFBP and PAPP-A in granulosa cells with the follicular fluid contents of dominant (preovulatory) and the largest subordinate follicle in cattle during luteal regression. Therefore, the objectives of this study were

1) to evaluate if gene expression levels of IGFBP-2,-3,-4, and –5, and PAPP-A differ between the dominant and largest subordinate follicle during luteal regression (24 or 48 h post PGF-2α injection) prior to ovulation, and

2) to determine if these changes in gene expression are associated with changes in follicular fluid concentrations of total and free IGF-I, ovarian steroids (estradiol, progesterone, and androstenedione) or IGFBP protein levels

CHAPTER II

REVIEW OF RELATED LITERATURE

Insulin-like Growth Factor-I (IGF-I)

Insulin-like growth factor (IGF)-I and –II are growth promoting hormones with structural homology with insulin (Rajaram et al., 1997). Both IGFs perform their mitogenic and steoroidogenic functions by binding to IGF type I receptor. The IGF type I receptor preferentially binds IGF-I, but IGF-II also cross-reacts to a much lesser degree (Sara and Hall, 1990). IGF type II receptor on the other hand, is structurally unrelated to the IGF-I receptors, and has very high affinity for IGF-II only. Because of IGF type II receptor's high specificity for IGF-II, it is thought to regulate the level of extracellular IGF-II via degradation (Firth and Baxter, 2002). IGF type II receptor's role in mediating IGF action is less well defined (Baxter, 2000). In humans, levels of IGF-II but not IGF-I are associated with several variables of follicle development (Yoshimura, 2003). Compared to IGF-I, IGF-II is the major peptide in the human ovary abundantly expressed in granulosa cells (Giudice, 1995). Most of the published literature, however, in domestic animals evaluates the role of IGF-I rather than IGF-II.

Sources of IGF-I in the follicular fluid. Insulin-like growth factor (IGF)-I is ubiquitous, acting as an endocrine, paracrine or autocrine hormone. The ovary is considered as one of the top IGF-I producing organs aside from the liver and the uterus

(Yoshimura, 2003). Majority of the IGF-I found in systemic circulation is in a 150-kDa complex; bound to a growth hormone-sensitive transport protein known as the insulin-like growth factor binding protein (IGFBP)-3 (Yoshimura, 2003). It was reported that although IGFBP-3 was identified in the follicular fluid, it was not found in follicular cells (granulosa and theca cell homogenates from medium and small follicles) of beef cattle, and thus may have been sequestered from the blood (Echternkamp et al., 1994). The same 150-kDa complex found in serum was also characterized in ovine follicular fluid (Hodgkinson et al., 1989). However, the degree of seric IGF-I diffusion in the follicular fluid may be influenced by the capillary vasculature surrounding the theca interna or follicular wall of different follicle types. Recent studies showed that an estrogenic preovulatory dominant follicle had richer dilated capillaries and increased blood flow as compared to subordinate or atretic bovine follicles (Acosta et al., 2003; Jiang et al., 2003). Regardless of the amount of IGF-I diffusion, follicular cells are capable of local IGF-I production and secretion. IGF-I transcripts are found in granulosa cells of rats, pigs, cattle (reviewed in Spicer and Echternkamp, 1995), and sheep (Leewenberg et al., 1995). Human theca cells were also seen to express IGF-I transcripts (reviewed in Spicer and Echternkamp, 1995). While it was reported that there were little or no detectable IGF-I mRNA expression in bovine granulosa cells and theca externa of healthy antral follicles using in situ hybrdization (Armstrong et al., 2000), IGF-I mRNA was detected in pools of granulosa and theca cells using northern blotting (Spicer and Echternkamp, 1995). Differences in results may be due to techniques used. More recently, it has been reported that in contrast to what has been found in humans, bovine granulosa cells produce greater amounts of IGF-I than bovine theca cells and this granulosa cell IGF-I

production is regulated by insulin, FSH and LH in vitro, whereas theca cell IGF-I production is not hormonally responsive (Spicer et al., 2002).

In cattle (Spicer and Echternkamp, 1995) and sheep (Monget et al., 1992), IGF-I in the serum is greater than those found in the follicular fluid. In sheep, it was reported that a positive relationship exists between the two IGF-I sources (Monget et al., 1992). Pigs and cattle treated with somatotropin (reviewed in Spicer and Echternkamp, 1995; Mazerbourg, et al., 1995) had increased IGF-I levels in the serum and follicular fluid. In vitro, somatotropin increased levels of IGF-I protein and mRNA in porcine granulosa cells two-fold (Hsu et al., 1987; Samaras et al., 1996) but had no effect on IGF-I production by bovine granulosa cells (Spicer et al., 2002). This implies that both systemic and intrafollicular IGF-I sources are influenced directly or indirectly by somatotropin, and which source is paramount may be species dependent.

In summary, total IGF-I found in the follicular fluid may be due to diffusion of the 150-kDa complexes from the blood and (or) local biosynthesis from follicular cells. However, specific contributions of both sources to the overall IGF-I pool in the follicular fluid remain to be elucidated.

Forms of IGF-I: Total vs Free. Concentration of total IGF-I in the follicular fluid is about 100 ng/mL in bovine (Spicer et al., 2002), and 244 ng/mL in large normal ovine follicles (Monget et al., 1992). IGF-I concentration was found to be greater in follicular fluid of estrogen active (>50 ng/mL estradiol and estrogen:progesterone ratio of >1) follicles with an average diameter of 13.5 mm, than estrogen inactive follicles with an average diameter of 11.9 mm in beef cows (Echternkamp et al., 1994). In another

study (Stewart et al., 1996) during the first follicular wave of dairy cows, the dominant follicle's total IGF-I concentration did not differ from those of the large (≥ 6 mm) and small (<6 mm) subordinate follicles. Total IGF-I concentration was positively correlated with diameter in mares (Bridges et al., 2002) and increases with follicle size in sheep (Spicer et al., 1995) and cattle (reviewed in Spicer and Echternkamp, 1995). It is however the bioavailable IGF-I, i.e. the "free" or unbound form of IGF-I, that is dramatically different between the dominant follicle and its associated subordinate follicles (Mazerbourg et al., 2003), and is therefore considered to be a more useful biochemical marker of dominance than the total IGF-I concentrations in the follicular fluid.

In humans, the concentration of free IGF-I found in the serum is about 1.3 ng/mL (Stoving et. al., 1999) and 14 ng/mL in the follicular fluid of the future dominant follicle (F1) in Holstein heifers (Ginther et al., 2003). Seemingly, there is a biphasic increase in free IGF-I concentrations during selection or by the beginning of diameter deviation, such that it peaks when the F1 reaches 7.6 mm, decreases at 7.8 mm, but starts to increase at 8.2 mm, and peaks again at 8.4 mm (Ginther et al., 2003). Free IGF-I concentrations were significantly (6 to 8 ng/mL) greater between the F1 and its associated F2 (future largest subordinate follicle) when the F1 was 7.6 mm, 8.2 mm and 8.4 mm (Ginther et al., 2003). Estradiol simultaneously increases with free IGF-I in the future dominant follicle during selection. Similarly, Rivera and Fortune (2003a) reported that concentrations of free IGF-I was 1 to 6 ng/mL higher in the F1 compared to F2 during and after time of follicle deviation when the F1 was 8 and 9 mm, respectively. Also, free IGF-I during follicle selection was positively correlated with IGFBP-4 and –5

proteolytic activity (Rivera and Fortune, 2003a). Free IGF-I in F2 continuously decreased up to 1 ng/mL, as F1 of Holstein heifers grew from 7.5 to 10.5 mm (Beg et al., 2001). To date, there are no reports quantifying free IGF-I in follicular fluid of dominant preovulatory follicles in cattle.

In summary, there are no significant differences in total IGF-I concentrations between the dominant (estrogenic) and subordinate follicles, but free IGF-I concentrations are seen to be significantly increased in future dominant follicles. Thus, making free IGF-I concentration a more useful biochemical marker in follicle selection and dominance than total IGF-I concentration.

IGF-I functions. In vivo (e.g., knock-out mice) and in vitro (e.g., cell cultures) experiments were used to determine the significant intraovarian role of IGF-I. In pigs, rats, and cattle, IGF-I is known to stimulate granulosa cell proliferation or mitogenesis and enhance FSH-induced steroidogenesis (Spicer and Echternkamp, 1995). Female IGF-I knock-out mice were infertile, with follicles arrested at the preantral to early antral stage, and had no sign of mature graafian follicles, similar to what have been observed in female FSH knock-out mice (Zhou et al., 1997). Granulosa cell IGF-I gene expression in FSH knock-out mice ovaries was observed to be similar to those of the wild type mice (Zhou et al., 1997). These results imply that IGF-I expression is indispensable both for proliferation of granulosa cell and stimulation of FSH action. FSH is important for follicle development but is not considered as a major regulator of ovarian IGF-I expression. Reduced proliferation and impaired FSH responsiveness of the follicle observed from IGF-I knock-out mice were attributed to the lack of FSH receptor

expression (Kadakia et al., 2001). IGF-I potentiates FSH action by the upregulation of FSH receptor expression, and in turn creates a positive feedback loop, such that increased FSH concentrations augment IGF-I action by increasing IGF-I receptor expression (Zhou et al., 1997; Mazerbourg et al., 2003; Yoshimura, 2003).

Cell culture experiments of ovine granulosa cells showed that IGF-I stimulates proliferation in small (1-2 mm) follicles and steroidogenesis in large (5-8 mm) preovulatory follicles (Monniaux et al., 1994). Similar observations were seen in pigs, wherein IGF-I stimulatory action on steroidogenesis of theca and granulosa cells increased and its mitogenic action decreased as follicular development progressed (Kolodziejczyk et al., 2003). In cattle, FSH and LH enhanced the mitogenic or proliferative effect of IGF-I in granulosa cells from small (less than or equal to 5 mm) follicles, but not in large (less than 10 mm) follicles (Spicer and Echternkamp, 1995). While an inverse relationship may seem to appear between proliferation and differentiation, Monniaux et al. (1994) proposed an "uncoupling" between the proliferation and differentiation. Ovine and bovine granulosa cells from small follicles treated with IGF-I still had the capacity to secrete progesterone (Monniaux et al., 1994) and estradiol (Spicer et al., 1993), respectively, although at lower concentrations than granulosa cells from large follicles. Thus, differentiation by itself cannot explain the granulosa cell's loss of proliferative activity.

IGF-I also regulates theca cell proliferation. IGF-I knock-out mice's ovaries had immature theca cell development, suggesting IGF-I's paracrine role in proliferation (Zhou et al., 1997). Porcine theca cell culture showed that IGF-I stimulated theca cell proliferation in all stages of follicular development, such as those from small, medium

and large follicles (Kolodziejczyk et al., 2003). In bovine theca cell culture, treatment of IGF-I (30 and 100 ng/mL) alone increased theca cell numbers (Stewart et al., 1995).

Differentiated follicular cells acquire the capability to produce steroids. IGF-I is known to enhance gonadotropin-induced granulosa and theca cell steroidogenesis partly via enhancing steroidogenic enzymes involved in the synthesis of progesterone, androstenedione and estradiol (Stewart et al., 1995; deMoura et al., 1997). Steroidogenesis starts with de novo cholesterol synthesis and (or) cholesterol uptake, and ends with important steroid products (i.e., progesterone, androstenedione, and /or estradiol) that serve as markers of the physiological status of the follicle. IGF-I and II have been shown to stimulate de novo cholesterol synthesis in porcine (Veldhuis et al., 1984) and bovine (Spicer et al., 1996) granulosa and theca cells, as well as stimulate granulosa cell uptake and metabolism of cholesterol (Veldhuis et al., 1984). Inside the cell, cholesterol must be translocated from the outer to the inner mitochondrial membrane by a steroid-regulatory protein named, steroidogenic acute regulatory protein (StaR). Cholesterol is then converted to pregnenolone by an enzyme, cytochrome P450 cholesterol side-chain cleavage (P450scc). Pregnenolone is converted to progesterone via the enzyme, 3β-hydoxysteroid dehydrogenase (3β-HSD), or converted to 17α-hydroxypregnenolone by the enzyme cytochrome P450 17α-hydroxylase ($P450_{17\text{-}OH}$). LH binding to its receptor on theca cells stimulates enzyme cytochrome $P450_{c17,20}$-lyase (P450c17) to convert the precursor, 17α-hydroxyprogesterone to androstenedione. In bovine, rat, pig and human theca cells, IGF-I (10 to100 ng/mL) acts synergistically with LH to increase progesterone and androstenedione production (Spicer and Echternkamp, 1995; Stewart et al., 1995; Yoshimura, 2003). Increases in androstenedione production

are partly due to the stimulation of LH receptors by IGF-I, as seen by increased in LH receptor mRNA expression in theca cells (Magoffin and Weitsman, 1994) and specific binding of ^{125}I labeled hCG (Stewart et al., 1995). P450c17 is only expressed in theca cells, so progesterone produced by granulosa cells are not metabolized further, and are only secreted. Theca cells convert androstenedione to testosterone with the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD), and both androgens are secreted and absorbed by adjacent granulosa cells. In the granulosa cells, androstenedione is preferentially metabolized into estrone by enzyme cytochrome P450 aromatase (P450arom), and then estrone is metabolized further into estadiol by 17β-HSD. mRNA for FSH receptor and P450arom were shown to be localized exclusively to granulosa cells, while mRNA for P450c17 and StaR are localized within theca cells (Bao and Garverick, 1998). Eventually, the granulosa cells of the dominant follicle develop LH receptors during the time of selection (Stewart et al., 1996), allowing the cells to synthesize estradiol in response to LH as well as FSH (Fortune et al., 2001).

While StAR mRNA was expressed in bovine theca cells and perhaps luteinized granulosa cells (Bao et al., 1998), it was seen to be upregulated in porcine granulosa follicles treated with IGF-I and FSH. Basal granulosa cell expression of StAR mRNA and protein from small to medium sized porcine follicles was stimulated by IGF-I (100 ng/mL) plus FSH (100 ng/mL) 26-fold and 56- fold, respectively, compared to untreated controls (Balasubramanian et al., 1997). In human granulosa-lutein cells, IGF-I increased StAR mRNA and protein 3- and 4- to 5-fold, respectively (Devoto et al., 1999). IGF-I synergized with FSH to enhance progesterone biosynthesis of rat granulosa cells by stimulating a 2.6 and 1.8-fold increase in P450scc and 3β-HSD enzymes, respectively

(deMoura et al., 1997). In theca interstial cells of rats (stimulated to differentiate in vitro), IGF-I (50 ng/mL) alone, or in the presence of LH, stimulated a dose-related three-fold increase in P450ssc over unstimulated theca cells (Magoffin and Weitsman, 1993a). Also in the theca interstial cells of rats, IGF-I alone (0.1 to 100 ng/mL) did not stimulate P450c17 mRNA, but in the presence of LH (100 ng/mL), stimulated a maximum 3-fold increase (Magoffin and Weitsman, 1993b). In bovine granulosa cells from 2 to 5 mm estrogenic follicles, reduction in IGF-I and insulin concentrations caused a decrease in P450arom mRNA levels (Silva and Price, 2002). Also, estradiol and P450arom mRNA levels were highly correlated (Silva and Price, 2002). At 100 ng/mL, IGF-I alone increased estradiol production by 2-fold in granulosa cells of small and large follicles, while in the presence of FSH (30 ng/mL), IGF-I increased estradiol production by 17-fold in small follicles and 13-fold in large follicles (Spicer et al., 2002); in the presence of FSH the ED_{50} of IGF-I averaged 5 and 6 ng/mL in small and large follicle granulosa cells, respectively. This showed that at nadir FSH concentrations in vivo, the low doses of free IGF-I detected in dominant follicles (i.e., 4 to 8 ng/mL) would likely stimulate aromatase activity (Spicer et al., 2002).

In summary, the intraovarian role of IGF-I includes stimulation of follicular cell proliferation and steroidogenesis (e.g., progesterone, androstenedione, and estrogen biosynthesis).

Role of insulin-like growth factor binding proteins (IGFBPs) in follicle dominance

Both IGF-I and II are bound in plasma and other biological fluids by a family of proteins known as insulin-like growth factor binding proteins (IGFBPs) that regulate the

availability of IGFs to their target cell (Yoshimura, 2003). IGFBPs are cysteine-rich proteins that are relatively conserved among species and have the unique ability to bind to IGFs with high affinity (Hwa et al., 1999). To date, there are about seven IGFBPs reported (Kostecka and Blahovec, 2002), three of which (IGFBP-2,-4, and –5) have been implicated in the regulation of ovarian IGF-I availability (Firth and Baxter, 2002) and are therefore, considered important players in follicular development and atresia. Compared to the IGF type I receptor, IGFBPs have 10- to 100-fold higher affinity for IGFs (Baxter, 2000). Between IGF-I and IGF-II, most of the low molecular weight IGFBPs are more attracted to the former, decreasing IGF-II's potency in activating the IGF type I receptor, as seen in pigs (Grimes and Hammond, 1992). In ovine follicles, IGF-II levels are significantly higher in small than large follicles (Spicer et al., 1995), while the IGF-I:IGF-II ratio increased during follicle growth (Monget et al., 1993). This could be due to again, the strong affinity of IGF-II for ovine follicular fluid IGFBPs (Monget et al., 1993).

These IGFBPs have conserved amino and carboxyl terminal domains, and varying middle domains. Among IGFBP species, homology in the carboxy and amino terminal domains are 58% and 35%, respectively (Hwa et al., 1999). Digested fragments generating C terminal domain or N-terminal domain alone, weakly interact with IGFs (Hwa et al., 1999). Mutagenesis of the conserved regions of both the amino and carboxy disrupts IGF binding, implying a pocket-like structure of the IGFBP enclosing the IGF (Firth and Baxter, 2002). Posttranslational modifications such as glycosylation and phosphorylation occur in the middle domain. Some IGFBP-3, -4 and -5 isoforms are glycosylated. Glycosylation may not affect IGF binding, but may be important in the

IGFBP's resistance to proteolysis (Hwa et al., 1999; Zhou et al., 2003). Unlike the carboxyl and amino terminal domains, the middle domain is susceptible to proteolytic attack of endoproteases (Baxter, 2000). Further studies should be conducted however, to determine the physiological significance of IGFBP glycosylation in ovarian folliculogenesis. Table 1 refers to the distinct characteristics of some IGFBPs (such as molecular weight, IGF affinity and posttranslational modifications).

Table 1. Characteristics of some insulin-like growth factor binding proteins*

IGFBP species	Molecular weight, kDa	IGF affinity	Posttranslational modification
IGFBP-2	32-34	II>I	-
IGFBP-3	43, 40	I=II	glycosylated
IGFBP-4	29 24	I=II	glycosylated non-glycosylated
IGFBP-5	29	II>I	glycosylated

*Information compiled from Kostecka and Blahovec, 1999; Baxter, 2000; and Spicer and Echternkamp,1995

IGFBPs act as carrier proteins that prolong IGF's half-life. IGF-I in ternary (e.g., IGF-I – IGFBP-3 – Acid labile) and binary (e.g., IGF-I – IGFBP-2) complexes have half-lives of 12 to16 h and 30 min respectively, whereas, a free IGF-I has a half-life of 10 min (Kostecka and Blahovec, 1999). Also, IGFBPs regulate IGF-I bioavailability since IGF type I receptor approaches saturation at 5 nM or lower (Baxter, 2000). As long as the IGFs are bound to the IGFBP, it cannot attach to the IGF type I receptor to perform its mitogenic and steroidogenic effects. Thus, while IGF-I is bound to any IGFBP, its half-

life is prolonged but at the same time, its bioactivity inhibited. However, IGF effects can be potentiated (Kostecka and Blahovec, 1999; Baxter, 2000) if the IGFBP is phosphorylated (as in the case of IGFBP-1), bound to the extra cellular matrix (as in the case of IGFBP-5), proteolyzed (as in the case of IGFBP-4 and –5) or expressed differentially (as in the case of gene expression of low molecular weight IGFBPs in follicular cells).

In summary, there is a balancing act between the IGFBP and IGFs, such that IGF bioavailability can be regulated by IGFBP, and IGFBP can also be regulated via proteolysis (or other posttranslational modifications), and gene expression. This balancing act may result to either follicular growth or atresia.

Localization and gene expression of IGFBPs in the ovary. IGFBP-2,-4, and -5 messenger ribonucleic acid (mRNA) were reported to be expressed in follicular cells of several species. In ovine, as confirmed in human, pig and rat, ovarian transcripts of IGFBP-2,-4, and –5 were 1.5-1.8 kb, 2.5-3 kb, and 6 kb, respectively (Besnard et al., 1996a). Expression of IGFBP-2 mRNA was greater in granulosa than theca cells as reported in pigs (Liu et al., 2000), sheep (Besnard et al., 1996a), and cattle (Armstrong et al., 1998; Yuan et al., 1998; Roberts and Echternkamp, 2003). In rats and mice, IGFBP-2 mRNA levels were restricted to the theca-interstial and granulosa cells, respectively (Nakatani et al., 1991; Wandji et al., 1998).

Contrary to IGFBP-2 mRNA, IGFBP-4 mRNA levels were greater in theca than granulosa cells of pigs (Liu et al., 2000) and cattle (Armstrong et al., 1998; Schams et al., 2002). IGFBP-4 mRNA levels were restricted to granulosa cells of atretic follicles in

rats (Nakatani et al., 1991), and to theca cells of healthy antral (small, medium and large) follicles of cattle (Armstrong et al., 1998) and granulosa cells from large healthy follicles of cattle (Roberts and Echternkamp, 2003) in cattle. No difference in IGFBP-4 mRNA expression were seen between theca cells of large and small healthy follicles in ewes (Besnard et al., 1996a) or between theca and granulosa cells of large follicles in cows (Roberts and Echternkamp, 2003).

Levels of IGFBP-5 mRNA on the other hand, was detected in theca cells of healthy follicles in ewes (Besnard et al., 1996a). In mice, IGFBP-5 mRNA was detected only in granulosa cells of primary and secondary follicles and marginally expressed in antral follicles (Wandji et al., 1998).

IGFBP-3 mRNA was detected in granulosa and theca cells of all growing follicles in pigs (Wandji et al., 2000). In cattle using in situ hybridization, IGFBP-3 mRNA was not detected in one study (Yuan et al., 1998) but widely detected in theca of most follicles in another study (Canty et al., 2002; 2003). The presence of IGFBP-3 mRNA in small and large granulosa and theca cells was detected using real time RT-PCR, a very sensitive technique (Voge et al., 2004). IGFBP-3 mRNA was also detected in theca cells of large atretic follicle and vascular endothelial cells in cattle using northern blotting (Roberts and Echternkamp, 2003). In rats, IGFBP-3 mRNA was localized in the corpus luteum but not granulosa or theca cells (Nakatani et al., 1991), and in human follicles, it was lowly detectable in fresh ovarian tissues (i.e., granulosa, theca, and stromal samples; Voutilainen et al., 1996).

Collectively, IGFBP mRNA is expressed in follicular cells, and the prominence of which in a given cell type would depend on the species involved. This implies that local

production and secretion of IGFBP proteins in the follicular fluid is possible and species specific. Changes in mRNA expression in either compartment (such as granulosa or theca cell) or due to size or physiological state (atretic or estrogenic) of the follicle likely affect the amount of IGFBP proteins found in the follicular fluid.

During follicle growth, levels of different IGFBP mRNA expression change dramatically. IGFBP-2 mRNA levels were less in granulosa cells of dominant follicles as compared to its subordinate follicles (Yuan et al., 1998). IGFBP-2 mRNA was also easily detected in granulosa cells of small healthy follicles as compared to large healthy follicles in cattle (Armstrong et al., 1998) and sheep (Besnard et al., 1996a). No change in IGFBP-4 mRNA localized in the theca cells of healthy follicles was observed in cattle (Armstrong et al., 1998) and sheep (Besnard et al., 1996a). In pigs, granulosa and theca cells from large follicles had more IGFBP-2 and -4 mRNA levels detected than in small follicles (Liu et al., 2000). IGFBP-5 mRNA in healthy ovine theca cells slightly decreases during follicular growth (Besnard et al., 1996a). IGFBP-5 mRNA was greatly expressed in granulosa cells of small follicles as compared to large follicles in cows (Roberts and Echternkamp, 2003). In rats, mRNA for IGFBP-2,-3,-4 and –5 were not detected in dominant follicles (Erickson et al., 1992).

In terms of physiological state, atretic follicles have greater IGFBP mRNA levels as compared to healthy follicles. IGFBP-2 mRNA levels were greater in granulosa and theca cells of large atretic antral versus large healthy follicles in ewes (Besnard et al., 1996a) and cows (Roberts and Echternkamp, 2003). IGFBP-5 mRNA levels were most abundant in granulosa cells of small and large atretic follicles in cows (Roberts and Echternkamp, 2003) and rats (Erickson et al., 1992; Wandji et al., 1998) and started to

increase from early to late atresia in ewes (Besnard et al., 1996a). In estrogenic follicles, IGFBP-2 and –5 mRNA were weakly detected in granulosa cells in cows (Schams et al., 2002). IGFBP-4 mRNA was localized in granulosa cells of atretic follicles in rats (Erickson et al., 1992) and levels in theca cells increased during late atresia in sheep (Besnard et al., 1996a). IGFBP-3 mRNA was the same between the granulosa cells of healthy and atretic follicles in pigs (Wandji et al., 2000), and was suggested not to be associated with growth or atresia since generally, its mRNA expression was low and (or) poorly detected (Mazerbourg et al., 2003).

In summary, follicular growth is associated with a decrease in IGFBP-2 and –5 mRNA levels, while increase in IGFBP-2,-4, and –5 mRNA levels is more linked with follicular atresia. Changes in mRNA levels of low molecular weight IGFBPs, if correlated with IGFBP protein levels, can explain differences in the amount of "free" IGF-I between follicle types (such as dominant and subordinate follicles). However, careful interpretation of the literature is needed, for follicles are classified broadly and differently between species. There are no studies yet identifying relative gene expression of these IGFBPs specifically between the dominant and its largest subordinate follicle.

Changes in IGFBP protein levels in the follicular fluid. Binding activity or levels of IGFBP in the follicular fluid varies between follicle size (small, medium or large follicles) or physiological status (healthy and estrogen active versus atretic and estrogen inactive follicles). In the bovine, as early as day 3 of the estrous cycle, where no growth deviation has happened yet, IGFBP-2,-3, and –5 were similar among large

follicles, while IGFBP-4 was lowest in the future dominant follicle (Mihm et al., 2000). At the time of follicle selection, the dominant follicle contains the highest estradiol concentration and lowest amount of IGFBPs (particularly IGFBP-4 and –5) among the large follicles (Austin et al., 2001; Rhodes et al., 2001). During dominance, IGFBP-2,-4, and –5 were higher in large and small subordinate follicles compared to the dominant follicle (Stewart et al., 1995; de la Sota et al., 1996).

Follicle diameter was inversely related to IGFBP-2,-4, and -5 in cows (Austin et al., 2001) and sheep (Monget et al., 1993; Besnard et al., 1996a). Similarly in pigs, small follicles (4 mm) contain greatest IGFBP-2 binding activity, but as follicles progressed into 6 mm, IGFBP-2 decreases (Liu et al., 2000). Total binding activity (summation of binding activities of all IGFBPs) was least in the dominant than the large and small subordinate follicles in bovine (Stewart et al., 1995; de la Sota et al., 1996; Spicer et al., 2001).

Follicular atresia was associated with increased follicular fluid activity of IGFBP-2,-4, –5 (Kojima et al., 2003) and –3 (Manikkam et al., 1997) in bovine follicles. Large bovine estrogen active follicles had undetectable IGFBP-2, -4, and –5 as compared to large estrogen inactive or atretic follicles (Echternkamp et al., 1994; de la Sota et al., 1996). This is also true in ovine follicles, where, IGFBP-2, -4,-5 increased during early and late atresia (Besnard et al., 1996a). In mares, concentrations of IGFBP-5 were greater in large nonvoulatory and small atretic follicles (Bridges et al., 2002). In pigs, IGFBP-2 and –4 increase during atresia, while IGFBP-5 and –3 vary slightly (Besnard et al., 1997).

On the other hand, IGFBP-3 levels were unchanged between the dominant and subordinate follicles in cattle (Echternkamp et al., 1994; de la Sota et al., 1996; Funston et al., 1996; Stewart et al., 1996; Spicer et al., 2001) and small to large follicles (from the follicular phase) in horses (Bridges et al., 2002), but tend to increase in sheep (Monget et al., 1993) and pigs (Liu et al., 2000) during follicular growth.

Recently, Roberts and Echternkamp (2003) reported differences in binding activities of the different IGFBPs in granulosa and theca cell homogenates, which accounted for IGFBPs present in bovine follicular fluid. Follicular growth and increased estradiol concentrations were associated with decreased IGFBP-2 mRNA, and thus was reflected by less IGFBP-2 binding activity detected in granulosa and theca cells of large healthy follicles. While IGFBP-4 mRNA was increased in granulosa cells of large healthy follicles as compared to small and large atretic follicles, IGFBP-4 protein was not detected in the follicular fluid. IGFBP-5 mRNA was greatest in granulosa cells from small and large atretic follicles but was undetectable in other follicle types, and thus may be the reason why binding activity in the follicular fluid was less for large healthy follicles. IGFBP-3 was detected in theca and granulosa cells, but the total amount of IGFBPs accounted for by IGFBP-3 was more in the follicular fluid as what could have been produced by these follicular cells; and thus, again, pointing to the fact that IGFBP-3 may have transudated into the follicle from serum as previously suggested (Echternkamp et al., 1994). In pigs, a strong correlation between plasma and follicular fluid IGFBP-3 gives credence to a peripheral source of IGFBP-3 found in the follicular fluid (Howard and Ford, 1992).

In summary, low molecular weight IGFBP levels are generally low in large or dominant follicles as compared to its subordinate follicles, while IGFBP-3 remain unchanged in some species. Moreover, atretic follicles contain the greatest amount of these IGFBPs as compared to healthy follicles.

Relationship of hormones with ovarian IGFBP. Gonadotropic hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) play important roles not only by directly stimulating steroidogenesis, but perhaps indirectly by regulating intraovarian IGFBP production and (or) degradation. FSH was reported to increase IGFBP-4 mRNA expression in cultured granulosa cells of hypophysectomized rats treated with diethylstilbesterol (Putowski et al., 1995), while FSH reduced IGFBP-2 mRNA in bovine granulosa cells (Armstrong et al., 1998). Another study showed that FSH had no influence on IGFBP-4 and -5 mRNAs of moderately differentiated porcine follicles (Grimes et al., 1994). In bovine granulosa cells from small and large follicles, using real-time RT PCR, FSH had no effect on IGFBP-2, -4 and –5 mRNA (Voge et al., 2004). FSH decreased amount of IGFBP-4 (Piferrer et al., 1997) and –5 (Fielder et al., 1993) proteins in cultured rat granulosa cell. FSH had no effect on bovine (Chamberlain and Spicer, 2001) and ovine (Armstrong et al., 1996) granulosa cell production of IGFBP-5. Also, no changes in IGFBP-3 levels were seen coincident with changes in serum FSH in cattle (Austin et al., 2001). FSH treatment of human (Iwashita et al., 1998) and rat (Fielder et al., 1993) granulosa cells stimulated proteolytic activity degrading IGFBP-4 and –5, respectively. In cattle, FSH treatment stimulated follicular development in cows (Echternkamp et al., 1994) and resulted in the formation of co-

dominant follicles (Rivera and Fortune, 2001) having an IGFBP-4 proteolytic activity similar to a single dominant follicle, implying that FSH may have enhanced proteolytic activity in another large follicle causing co-dominance. Thus, as the dominant follicle grows, FSH may be involved in regulating IGFBP-4 and –5 mRNA expression (depending on the species), and (or) inducing FSH-dependent protease(s) for IGFBP degradation. It is likely that while IGFBP mRNA levels remain unchanged, and IGFBP protein levels decrease, hormonally induced protease activity may be increasing.

PMSG-primed rats had 3.5-fold more IGFBP-4 mRNA 24 h after hCG administration (vs 0 h; Putowski et al., 1997). Four-day treatment of LH also stimulated IGFBP-4 mRNA in bovine theca cells (Armstrong et al., 1998). One-day treatment of LH inhibited IGFBP-2/-5 and –4 production by granulosa cells from large bovine follicles but had no effect on theca cell production of IGFBP-4 protein (Spicer and Chamberlain, 2002) or its mRNA (Voge et al., 2004). In ovine granulosa cells, LH increased IGFBP-4 protein production (Armstrong et al., 1996). One-day treatment of LH had no effect on IGFBP-3 production or mRNA levels in bovine granulosa cells (Spicer and Chamberlain, 2002; Voge et al., 2004) and human theca cells (Voutilainen et al., 1996). In vivo, IGFBP-3 levels remained constant among follicles before and after the ovulatory surge of LH in cattle (Funston et al., 1996). Therefore, the effect of LH on production of a specific ovarian IGFBP may depend on duration of treatment, species and (or) cell type.

Steroids are good biochemical markers of healthy and atretic follicles. Follicular fluid progesterone is the precursor future steroid synthesis (such as androstendione, cortisol, etc.). Both theca and granulosa cells produce large amounts of progesterone

and this increase near the time of ovulation is a result of preovulatory granulosa cell luteinization or inhibition of aromatization (Spicer and Echternkamp, 1985). Increased progesterone was considered to be associated with follicular atresia (Manikkam et al., 1997; Kojima et al., 2003;). Androstenedione on the other hand, serve as a precursor to estradiol production, and increases just before and during the LH surge. Decrease in androstenedione levels was coincident with decreased estradiol concentrations and increased progesterone concentrations in large follicles (Spicer and Echternkamp, 1985). Impaired conversion of androstenedione to estradiol accompanied by progesterone accumulation in the follicular fluid, causes atresia. Estrogen: progesterone ratio (EPR) was correlated with morphological atresia. EPR of greater than 1 characterizes a histologically healthy and estrogenic follicle, while an EPR less than 1 means the follicle is atretic or estrogen-inactive (de la Sota et al., 1996; Manikkam et al., 1997; Mihm et al., 2000). Estradiol and EPR were greater in the future dominant follicle as compared to the future largest or second largest subordinate follicle during day 3 of the estrous cycle, while progesterone and IGFBP-2,-3, and –5 protein levels remain similar (Mihm et al., 2000). In vitro, estradiol inhibited IGFBP-4 and –2/-5 production by granulosa cells of bovine large follicles (Spicer and Chamberlain, 2002), and increased IGFBP-2 production by equine small follicle granulosa cells (Bridges et al., 2002). Estradiol also decreased IGFBP-5 mRNA levels in large granulosa cells, and IGFBP-2, -3, and –4 mRNA in theca cells of bovine follicles (Voge et al., 2004). This is also in agreement with Schams et al. (2002) where IGFBP-2 mRNA in bovine theca cells were seen to decrease as estradiol concentrations increase in follicular fluid. Estradiol had no effect on IGFBP-3 protein levels in granulosa cells of pigs (Mondschein et al., 1990) and both granulosa and theca

cells of cattle (Spicer and Chamberlain, 2002). Overall, the inhibitory effect of estradiol to low molecular weight IGFBPs and perhaps, stimulatory effect to protease activity, may result in increased bioavailable IGF allowing the dominant follicle's continued development and growth.

In summary, follicular fluid contents (such as steroid hormones) of dominant and subordinate follicles differ, together with gonadotropic hormones, can direct the fate of the follicle (to grow or become atretic) by regulating IGFBP.

Role of pregnancy-associated plasma protein-A in follicle dominance

Proteolyzed IGFBP fragments have 50- to 100-fold decreased affinity to IGF-I (Kostecka and Blahovec, 2002). Ligand blotting technique can be used to determine the amount of intact IGFBPs but not proteolyzed IGFBP fragments binding IGFs with low affinity (Hwa et al., 1999). Proteolysis of some, but not all, IGFBPs occur during follicular growth. Follicular fluid of preovulatory follicles failed to degrade IGFBP-2 to a significant extent as seen in cows and heifers (Spicer et. al, 2001; Rivera and Fortune,2003b), mares (Bridges et al., 2002), ewes (Mazerbourg et al., 1999), but not in sows (Besnard et al., 1997). Proteolysis of IGFBP-2 in preovulatory follicles in pigs was estimated at about 80% (Besnard et al., 1997). This would imply that low levels of IGFBP-2 protein in the follicular fluid may not be due to its proteolysis in most but not all species. However, preovulatory follicles of ewes (Mazerbourg et al., 1999), sows (Besnard et al., 1997), heifers (Rivera and Fortune, 2003 a and b) and cows (Spicer et al., 2001) had strong proteolytic activity for IGFBP-4. IGFBP-5 proteolysis was detected in sows (Besnard et al., 1997), mares (Bridges et al., 2002), ewes (Besnard et al., 1996b),

and cattle (Spicer et al., 2001; Rivera and Fortune, 2003a and b). As compared to IGFBP-2, marked proteolysis of IGFBP-4 and –5 was observed, and this may account for the undetectable levels of IGFBP-4 and –5 in the follicular fluid in dominant follicles. IGFBP-3 proteolytic activity on the other hand decreases during follicular growth in ewes (Besnard et al., 1996b) but had no significant change in preovulatory follicles of mares (Bridges et al., 2002), sows (Besnard et al., 1997) and cows (Spicer et al., 2001). This suggests that any free IGF-I the ovary utilizes comes mainly from the regulation of low molecular weight IGFBPs.

IGFBP proteolytic activity detected in follicular fluid may be due not only to one but perhaps by the combined efforts of many proteases. IGFBP proteases were characterized using chemicals (such as EDTA, 1,10 phenantroline, phenylmethanesulfonyl or PMSF, etc) that act as specific protease inhibitors. EDTA (a metalloprotease inhibitor) and 1,10 phenanthroline (a specific chelator of zinc) blocked IGFBP-4 and –5 proteolytic degradation in pigs (Besnard et al., 1997), sheep (Besnard et al., 1996b), cattle (Spicer et al., 2001) and horses (Bridges et al., 2002). PMSF (inhibitor for serine protease) blocked IGFBP-3 and –5 proteolytic activity in small ovine atretic follicles, but had little effect on IGFBP-4 and –5 in preovulatory follicles (Besnard et al., 1996b). It also inhibited IGFBP-5 degradation in mares (Bridges et al., 2002) and IGFBP-2 and –4 in sows (Besnard et al., 1997). Seemingly, protease or proteases involved in IGFBP-4 and –5 bovine and equine follicular fluid proteolysis have characteristics of a serine metalloprotease (Spicer, 2004). Metalloproteases or metalloendopeptidases are mostly zinc-dependent enzymes, which can degrade inner peptide bonds (Gomis-Ruth, 2003) such as IGFBP's central domain. Proteases can also

be classified based on specific inhibitor sensitivities and be termed as serine-, cysteine-, aspartic-, or metallo protease (Fowlkes and Winkler, 2002).

Pregnancy-associated plasma protein-A (PAPP-A) is most likely the major serine protease found in follicular fluid, but others such as human kallikrein-3 and –2 (Rehault et al., 2001; Geisert et al., 2001), matrix metalloproteases (MMP-2 and –9) observed to have the capability to degrade IGFBPs (Besnard et al., 1996b), and still, others that remain to be discovered, should also not be excluded. It is also possible that PAPP-A might be physically associated with an unidentified proteinase, which becomes responsible for IGFBP cleavage, or PAPP-A might function to activate an unknown proteinase by binding to it (Boldt et al., 2001). PAPP-A was shown to interact with serine proteinases, such as human plasmin and bovine trypsin, by having a conformational change causing their entrapment (Zorin, et al., 1995). It was shown that the proteinase bound to PAPP-A was protected from inhibition by large substrate inhibitors such as α1-proteinase inhibitor and soybean trypsin inhibitor, but was still accessible to inactivation by aprotinin and PMSF (Zorin et al., 1995). Thus, even though it traps proteinases, it does not necessarily block the proteinase's active site. Alternatively, endogenous inhibitors for PAPP-A, although not yet identified, may be regulating PAPP-A proteolytic activity, as does tissue inhibitor of metalloprotease-1 (TIMP) for MMPs (Riley et al., 2001; Fowlkes and Winkler, 2002).

PAPP-A is a large glycoprotein (i.e., 200 kDa) that contains an elongated zinc-binding motif and a conserved methionine 1,4 B-turn, that are known to be conserved within the metzincin super family of zinc peptidases (Giudice, 2001; Fowlkes and Winkler, 2002; Gomis-Ruth, 2003). PAPP-A was first purified from pregnancy sera

(Lawrence et al., 1999). In pregnancy serum, it is bound to eosinophil major basic protein (pro-MBP), and had no instance of degrading IGFBP-4 (Soe et al., 2002). While this is the case, PAPP-A in other systems remains to be an active homodimer capable of proteolytic activities (Gomis-Ruth, 2003). Its main site of synthesis during pregnancy is the placenta, but it is present and secreted in several reproductive and nonreproductive tissues like the ovary, endometrium, seminal fluid, fallopian-tube mucosa, cervical mucosa, testis, breasts, kidneys, colon, marrow stromal cells, osteoblasts, fibroblasts, and vascular smooth muscle cells (Fiavola et al., 2002; Soe et al., 2002; Gomis-Ruth, 2003). It is a serine protease like plasmin and kallikrein (Spicer, 2004). PAPP-A was first identified as the IGFBP-4 protease in human fibroblasts and osteoblast cells (Lawrence et al., 1999). PAPP-A was later identified as the IGFBP-4 protease in human (Giudice, 2001), equine, bovine, and porcine follicular fluid (Mazerbourg et al., 2001). In cattle, using immunoprecipitation of follicular fluid from preovulatory follicles with anti-PAPP-A antibodies abrogated both IGFBP-4 and IGFBP-5 proteolysis (Rivera and Fortune, 2003b), also suggesting that PAPP-A may be involved in IGFBP-5 degradation. Also, recently, PAPP-A was hypothesized to degrade IGFBP-2 at a much slower rate as compared to IGFBP-4 and –5 degradation (Monget et al., 2003).

It is also possible that changes in IGFBP levels regulate each other's proteolysis. In ovine follicles, addition of IGFBP-3 and –5 led to the inhibition of IGFBP-4 proteolytic degradation (Mazerbourg et al., 1999). The heparin-binding domain in the C-terminal region of IGFBP-3 and –5 inhibits IGFBP-4 proteolysis by directly interacting with its proteases (Mazerbourg et al., 1999). Follicular fluid IGFBP-2 in heifers, sows, and mares, inhibits IGFBP-4 degradation by sequestering IGF-I; while, follicular fluid

IGFBP-5 in heifers and ewes inhibits IGFBP-4 degradation by the direct interaction of its heparin-binding domain with the protease (Mazerbourg et al., 2000).

In summary, low amounts of IGFBP present in the follicular fluid of large preovulatory follicles may be due to low levels of mRNA levels (as in the case of IGFBP-2 protein) or proteolysis (as in the case of IGFBP-4 and –5). IGFBP-4 and –5 protease(s) have serine metalloprotease characteristics, and the foremost protease may be PAPP-A. The less efficient IGFBP-2 degradation by PAPP-A may not be the major cause of low follicular fluid IGFBP-2 protein levels. Because levels of IGFBP-3 remain unchanged in bovine and equine follicular fluid, IGFBP-3 proteolysis does not play a major role in the release of IGF-I.

Localization and expression of PAPP-A in follicular cells. PAPP-A mRNA is detectable in granulosa cells from cattle and pigs (Mazerbourg et al., 2001), humans (Hourvitz et al., 2000), and rats (Hourvitz et al., 2002). PAPP-A mRNA expression was highest in granulosa cells from fully differentiated large follicles in cattle and pigs (Mazerbourg et al., 2001). Intense in situ hybridization signal of PAPP-A mRNA was seen in dominant estrogenic preovulatory follicles, also in granulosa-luteal cells, in humans (Hourvitz et al., 2000; Giudice, 2001). It is also considered as marker of follicle selection, because selected follicles with a diameter of 9 mm in humans express PAPP-A mRNA (Conover et al., 2001). In contrary, PAPP-A mRNA was lowly detected in small healthy and atretic antral follicles (regardless of size) in humans (Hourvitz et al., 2000). Even though PAPP-A mRNA was expressed in healthy antral follicles, there was no protein staining detected in human antral follicles, only in

granulosa and theca cells of primordial (intermediate and mature follicles) and small and large luteal cells (Vlasak et al., 2003). Seemingly, PAPP-A mRNA expression and protein secretion of granulosa cells may not always occur at parallel times or more sensitive techniques (i.e., real-time PCR) are needed to detect changes in PAPP-A mRNA in antral follicles.

In summary, PAPP-A mRNA is localized in granulosa cells of estrogenic dominant preovulatory follicles, and luteal cells, implying that PAPP-A mRNA is a marker of follicle selection and lutenization. However, PAPP-A mRNA levels in dominant and subordinate follicles have not been compared.

Hormonal regulation of PAPP-A mRNA. As previously cited, IGFBP-4 proteolysis commence as early as follicle selection, where FSH are at nadir concentrations, implying that the future dominant follicle acquires an FSH-inducible IGFBP-4 and –5 protease in bovine (Rivera and Fortune, 2003a). In rat granulosa cells, FSH induced the production of an IGFBP-5 protease (Fielder et al., 1993; Resnick et al., 1998). Thus, transcription of PAPP-A may change during the preovulatory, ovulatory and postovulatory phases of the ovarian cycle (Hourvitz et al., 2002). In immature rats, PMSG treatment increased whole ovarian PAPP-A mRNA at 24 h and 36 h after treatment, but levels fell to non-detectable levels in dominant preovulatory follicles 48 h after treatment (Hourvitz et al., 2002). However, whole ovarian PAPP-A mRNA expression increased again after hCG treatment and was sustained at high levels throughout ovulation and luteinization (Hourvitz et al., 2002). This may imply that PAPP-A mRNA is not continually expressed in granulosa cells and therefore acutely

regulated (Spicer, 2004). Whether the recently identified IGFBP-5 specific papalysin, PAPP-A2 (Overgaard et al., 2001) is regulated concomitantly with PAPP-A remains to be determined.

PAPP-A protease activity on IGFBP-4 degradation is IGF dependent (Lawrence et al., 1999), while on IGFBP-5 is IGF independent (Laursen et al., 2001). IGF-I may cause conformation changes on IGFBP-4, making it more susceptible to proteolysis (Mazerbourg et al., 2001). However, in early and late atretic ovine follicles with reduced proteolytic activity, IGF-I did not enhance IGFBP-4 degradation (Mazerbourg et al., 1999). In humans, PAPP-A activity is IGF-II dependent (Conover et al., 2001). Also, estradiol and androstenedione were positively correlated with IGFBP-4 and –5 proteolytic activity, which may imply that these steroids may regulate these IGFBPs via induction of IGFBP protease (Spicer, 2004). Future research should focus on developing bovine (and other farm animal species) specific reagents in order to measure changes in PAPP-A and –A2 concentrations in follicular fluid.

In summary, PAPP-A mRNA may be acutely regulated by gonadotropins, while its protease activity is IGF dependent. Steroids such as estradiol and androstenedione may also play a part in IGFBP protease induction.

REFERENCES

Acosta, T.J., K.G. Hayashi, M. Ohtani, and A. Miyamoto. 2003. Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. Reproduction 125: 759-767.

Armstrong, D.G., C.O. Hogg, B.K. Campbell, and R. Webb. 1996. Insulin-like growth factor (IGF)-binding protein production by primary cultures of ovine granulosa and theca cells: the effects of IGF-I, gonadotropin, and follicle size. Biol Reprod 55: 1163-1171.

Armstrong, D.G., G. Baxter, C.G. Gutierrez, C.O. Hogg, A.L. Glazyrin, B.K. Campbell, T.A. Bramley, and R. Webb. 1998. Insulin-like growth factor binding protein-2 and –4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. Endocrinology 139: 2146-2154.

Armstrong, D.G., C.G. Gutierrez, G. Baxter, A.L. Glazyrin, G.E. Mann, K.J. Wood, C.O. Hogg, R. Webb. 2000. Expression of mRNA encoding IGF-I and type I IGF receptor in bovine ovarian follicles. J Endocrinol 120: 429-438.

Austin, E.J., M. Mihm, A.C.O.Evans, P.G. Knight, J.L.H. Ireland, J.J. Ireland, and J.F. Roche. 2001. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. Biol Reprod 64: 839-848.

Balasubramanian K., H.A. Lavoie, J.C. Garmey, D.M. Stocco, and J.D. Veldhuis. 1997. Regulation of porcine granulosa cell steroidogenic acute regulatory protein (StAR) by insulin-like growth factor I: synergism with follicle-stimulating hormone or protein kinase A agonist. Endocrinology 138: 433-439.

Bao, B., M.D. Calder, S.Xie, M.F. Smith, B.E. Salfen, R.S. Youngquist, H.A. Garverick. 1998. Expression of steroidogenic acute regulatory protein messenger ribonucleic acid is limited to theca of healthy bovine follicles collected during recruitment, selection, and dominance of follicles of the first follicular wave. Biol Reprod 59: 953-959.

Bao, B. and H.A. Garverick. 1998. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. J Anim Sci 76: 1903-1921

Baxter, R.C. 2000. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am J Physiol Endocrinol Metab 278: E967-976.

Beg, M.A., D.R. Bergfelt, K. Kot, M.C. Wiltbank, and O.J. Ginther. 2001. Follicular fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. Biol Reprod 64: 432-441.

Besnard, N., C. Pisselet, D. Monniaux, A. Locatelli, F. Benne. F. Gasser, F. Hatey, and P. Monget. 1996. Expression of messenger ribonucleic acid of insulin-like growth factor binding proteins-2,-4, and –5 in ovine ovary: localization and changes during growth and atresia of antral follicles. Biol Reprod 55: 1356-1367.

Besnard, N., C. Pisselet, J. Zapf, W. Hornebeck, D. Monniaux, and P. Monget. 1996b. Proteolytic activity is involved in changes in intrafollicular insulin-like growth factor binding protein levels during growth and atresia of ovine ovarian follicles. Endocrinology 137: 1599-1607.

Besnard, N., C. Pisselet, D. Monniaux, and P. Monget. 1997. Proteolytic activity degrading insulin-like growth factor-binding protein-2,-3,-4 and –5 in healthy growing and atretic follicles in the pig ovary. Biol Reprod 56: 1050-1058.

Boldt, H.B., M.T. Overgaard, L.S. Laursen, K. Weyer, L. Sottrup-Jensen and C. Oxvig. 2001. Mutational analysis of the proteolytic domain of pregnancy-associated plasma protein-A (PAPP-A): classification as a metzincin. Biochem J 358: 359-367.

Bridges, T.S., T.R. Davidson, C.S. Chamberlain, R.D. Geisert, and L.J. Spicer. 2002. Changes in follicular fluid steroids, insulin-like growth factors (IGF) and IGF-binding protein concentration, and proteolytic activity during equine follicular development. 2002. J Anim Sci 80: 179-190.

Canty, M.J, M.P. Boland, and M.A. Crowe. 2002. Insulin-like growth factor binding protein-2, -3, and –4 messenger ribonucleic acid expression in bovine ovarian follicles. Biol Reprod 66: 73 Suppl. 1.

Canty, M.J., M.P. Boland, and M.A. Crowe. 2003. Insulin-like growth factor binding preotin-3 localization in bovine ovarian follicles. Reprod Domest Anim 38: 333.

Chamberlain C.S. and L.J. Spicer. 2001. Hormonal control of ovarian cell production of insulin-like growth factor binding proteins. Mol Cell Endocrinol 182: 69-81.

Conover, C.A., G.F. Faessen, K.E. Ilg, Y.A. Chandrasekher, M. Christiansen, M.T. Overgaard, C. Oxvig, L.C. Giudice. 2001. Pregnancy-associated plasma protein-A is the insulin-like growth factor binding protein-4 protease secreted by human ovarian granulosa cells and is a marker of dominant follicle selection and the corpus luteum. Endocrinology 142: 2155-2158.

de la Sota, R.L., Simmen, F.A., Diaz, T., Tatcher, W.W. 1996. Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. Biol Reprod 55: 803-812.

deMoura, M.D., D. Choi, E.Y. Adashi, and D.W. Payne. 1997. Insulin-like growth factor-I-mediated amplification by cultured rat granulosa cells: enhancement of steroidogenic enzyme activity and expression. Biol Reprod 56: 946:953.

Devoto, L., L.K. Chirstenson, J.M. McAllister, A. Makrigiannakis, and J.F. Strauss III. 1999. Insulin and insulin-like growth factor-I and –II modulate human granulosa-lutein cell steroidogenesis: enhancement of steroidogenic acute regulatory protein (StAR) expression. Mol Hum Reprod 15: 1003-1010.

Echternkamp, SE, H.J. Howard, A.J. Roberts, J. Grizzle, and T. Wise. 1994. Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. Biol Reprod 51: 971-981.

Erickson, G.F., A. Nakatani, N. Ling, S. Shimasaki. 1992. Cyclic changes in insulin-like growth factor-binding protein-4 messenger ribonucleic acid in the rat ovary. Endocrinology 130: 625-636.

Evans, A.C.O. 2003. Characteristics of ovarian follicle development in domestic Animals. Reprod Dom Anim 28: 240-246.

Fiavola, M. and Malbohan, I.M. 2002. Pregnancy-associated plasma protein-A (PAPP-A): theoretical and clinical aspects. Bratisl Lek Listy 103: 194-205.

Fielder, P.J., H. Pham, E.Y. Adashi, and R.G. Rosenfeld. 1993. Insulin-like growth factors (IGFs) block FSH-induced proteolysis of IGF-binding protein-5 (BP-5) in cultured rat granulosa cells. Endocrinology 133: 415-418.

Firth S.M. and R.C. Baxter. 2002. Cellular actions of insulin-like growth factor binding proteins. Endoc Rev 23: 824-954.

Fortune, J.E., G.M. Rivera, A.C.O Evans, and A.M. Turzillo. 2001. Differentiation of dominant versus subordinate follicles in cattle. Biol Reprod 65: 648-654.

Fowlkes, J.L., and M.K. Winkler. 2002. Exploring the interface between metalloprotease activity and growth factor and cytokine bioavailability. Cyt Growth Fac Revs 13: 277-287.

Funston, R.N., G.E. Seidel Jr., J. Kilndt, and A.J Roberts. 1996. Insulin-like growth factor binding proteins in bovine serum and follicular fluid before and after the preovulatory surge of luteinizing hormone. Biol Reprod 55: 1390-1396

Geisert, R.D., C.S. Chamberlain, K.A. Vonnahme, J.R. Malayer, and L.J. Spicer. 2001. Possible role of kallikrein in proteolysis of insulin-like growth factor binding proteins during the oestrous cycle and early pregnancy in pigs. Reproduction 121: 719-728.

Gomis-Ruth X. F. 2003. Structural aspects of the metzincin clan of metalloendopeptidases. Molecular Biotechnology 24: 157-202.

Ginther, O.J., M.C. Wiltbank, P.M. Fricke, J. R. Gibbons, and K. Kot. 1996. Selection of the dominant follicle in cattle. Biol Reprod 55: 1187-1194.

Ginther, O.J., M.A. Beg, K. Kot, C. Meira, and D.R. Bergfelt. 2003. Associated and independent comparisons between the two largest follicles preceding follicle deviation in cattle. Biol Reprod 68: 524-529.

Grimes, R.W., J.A. Barber, S. Shimasaki, N. Ling, and J.M.Hammond. 1994. Porcine ovarian granulosa cells secrete insulin-like growth factor-binding protein-4 and –5 and express their messenger ribonucleic acids: regulation of follicle-stimulating hormone and insulin-like growth factor-I. Biol Reprod 50: 695-701.

Grimes R.W. and J.M Hammond. 1992. Insulin and insulin-like growth factors (IGFs) stimulate production of IGF-Binding proteins by ovarian granulosa cells. Endocrinology 131: 553-558.

Giudice, L.C. 2001. Insulin-like growth factor family in graafian follicle development and function. J Soc Gynecol Investig 8: S26-S29.

Giudice, L.C. 1995. The insulin-like growth factor system in normal and abnormal huan ovarian follicle development. Am J Med 98: 48S-54S.

Hodgkinson, S.C., L. Moore, J.R. Napier, S.R. Davis, J.J. Bass, P.D. Gluckman. 1989. Characterization of insulin-like growth factor binding proteins in ovine tissue fluids. J Endocrinol 120: 429-38.

Howard , H.J. and J.J. Ford. 1992. Relationships among concentrations of steroids, inhibin, insulin-like growth factor-I (IGF-I), and IGF-binding proteins during follicular development in weaned sows. Biol Reprod 47: 193-201.

Hourvitz, A., A.E. Widger, F. T. Filno, R.J Chang, E.Y. Adashi, G.F. Erickson. 2000. Pregnancy-associated plasma protein-A gene expression in human ovaries is restricted to healthy follicles and corpora lutea. J Clin Endocrinol Metab 85: 4916-4919.

Hourvitz, A., A. Kuwahara, J.D. Hennebold, A.B. Tavares, H. Negeshi, T.H. Lee, G.F. Erickson, and E.Y. Adashi. 2002. The regulated expression of the pregnancy-associated plasma protein-A in the rodent ovary: a proposed role in the

development of the dominant follicle and the corpora lutea. Endocrinology 143: 1833-1844.

Hsu, C.J. and J.M. Hammond. 1987. Concomitant effect of growth hormone on secretion of insulin-like growth factor I and progesterone by cultured porcine granulosa cells. Endocrinology 121:1343-1348.

Hwa, V., Y. Oh, and R.G. Rosenfeld. 1999. The insulin-like growth factor binding protein (IGFBP) super family. Endoc Rev 20: 761-787.

Ireland J.J. and J.F. Roche. 1983. Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. Endocrinology 112: 150-156.

Iwashita, M., Y. Kudo, and Y. Takeda. 1998. Effect of follicle stimulating hormone and insulin-like growth factors on proteolysis of insulin-like growth factor binding protein-4 in human granulosa cells. Molec Hum Reprod 4: 401-405.

Jiang, J.Y., G. Macchiarelli, B.K. Tsang, and E.Sato. 2003. Capillary angiogenesis and degeneration in bovine ovarian antral follicles. Reproduction 125: 211-223.

Kadakia, R., J.A. Azzaztoa, C. Bondy, and J. Zhou. 2001. Granulosa cell proliferation is impaired in the IGF-I null ovary. Growth Horm IGF Res 11: 220-224.

Kojima, F.N., E.G.M. Bergfeld, M.E. Wehrman, A.S. Cupp, K.E. Fike, D.V. Mariscal-Aguayo, T. Sanchez-Torres, M. Garcia-Winder, D.T. Clopton, A.J. Roberts and J.E. Kinder. 2003. Frequency of luteinizing hormone pulses in cattle influences duration of persistence of dominant ovarian follicles, follicular fluid concentrations of steroids, and activity of insulin-like growth factor binding proteins. Anim Reprod Sci 77: 187-271.

Kolodziejczyk, J., A. Gertler, H. Leibovich, J. Rzasa, E.L. Gregoraszczuk. 2003. Synergistic action of growth hormone and insulin-like growth factor-I (IGF-I) on proliferation and estradiol secretion in porcine granulosa and theca cells cultured alone of in coculture. Theriogenology 60: 559-570.

Kostecka, Z. and J. Blahovec. 1999. Insulin-like growth factor binding proteins and their functions (minireview). Endoc Rev 33: 90-94.

Kostecka, Z. and J. Blahovec. 2002. Animal insulin-like growth factor binding proteins and their biological functions. Vet. Med. – Czech 47: 75-84.

Lawrence, J.B., C.Oxvig, M.T. Overgaard, L. Sottrup-Jensen, G.J.Gleich, L.G. Hays, J.R. Yates III, and C.A. Conover. 1999. The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A. Proc Natl Acad Sci 96: 3146-3153.

Laursen, L.S., M.T. Overgaard, R.Soe, H.B. Boldt, L. Sottrup-Jensen, L.C. Giudice, C.A. Conover, and C.Oxvig. 2001. Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A. FEBS Letters 504: 36-40.

Liu, J., A.T. Koenigsfeld, T.C. Cantley, C.K. Boyd, Y. Kobayashi, and M.C. Lucy. 2000. Growth and the initiation of steroidogenesis in porcine follicles are associated with unique patterns of gene expression for individual components of the ovarian insulin-like growth factor system. Biol Reprod 63: 942-952.

Magoffin D.A. and S.R. Weitsman. 1993a. Effect of insulin-like growth factor-I on cholesterol side-chain cleavage cytocrome-P450 messenger-ribonucleic acid expression in ovarian theca-interstitial cells stimulated to differentiate in-vitro. Mol Cell Endocrinol 96: 45-51.

Magoffin D.A. and S.R. Weitsman. 1993b. Differentiation of ovarian theca-interstial cells in vitro: regulation of 17α-hydoxylase messenger ribonucleic acid expression by luteinizing hormone and insulin-like growth factor-I. Endocrinology 132: 1945-1951.

Magoffin D.A. and S.R. Weitsman. 1994. Insulin-like growth factor-I regulation of luteinizing hormone (LH) receptor messenger-ribonucleic-acid expression and LH-stimulated signal-transduction in rat ovarian theca-interstitial cells. Biol Reprod 51: 766-775.

Manikkam, M. and R. Rajamahendran. 1997. Progesterone-induced atresia of the proestrus dominant follicle in the bovine ovary: changes in diameter, insulin-like growth factor system, aromatase activity, steroid hormones, and apoptotic index. Biol Reprod 57:580-587.

Mazerbourg, S., J.Zapf, D.R. Brigstock, C. Lalou, M. Binoux, and P. Monget. 1999. Insulin-like growth factor binding protein-4 proteolytic degradation in ovine preovulatory follicles: studies of underlying mechanisms. Endocrinology 140: 4175-4184.

Mazerbourg, S., J. Zapf, R.S. Bar, D.R. Brigstock, and P. Monget. 2000. Insulin-like growth factor (IGF)-binding protein-4 proteolytic degradation in bovine, equine and porcine preovulatory follicles: regulation of IGFs and heparin-binding domain containing peptides. Biol Reprod 63: 390-400.

Mazerbourg, S., M.T. Overgaard, C. Oxvig, M. Christiansen, C.A. Conover, I. Laurendeau, M. Vidaud, G. Tosser-Klopp, J. Zapf, and P. Monget. 2001. Pregnancy-associated plasma protein-A (PAPP-A) in ovine, bovine, porcine, and equine ovarian follicles: involvement in IGF binding protein-4 proteolytic

degradation and mRNA expression during follicular development. Endocrinology 142: 5243-5253.

Mazerbourg, S, C.A. Bondy, J. Zhou, and P. Monget. 2003. The insulin-like growth factor system: a key determinant role in the growth and selection of ovarian follicles? A comparative species study. Reprod Dom Anim 38: 247-258.

Mihm, M., T.E.M.Good, J.L.H. Ireland, J.J. Ireland, P.G. Knight, and J.F. Roche. 1997. Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers. Biol Reprod 57: 1328-1337.

Mihm, M., E.J. Austin, T.E.M Good, J.L.H. Ireland, P.G. Knight, J.F. Roche, and J.J. Ireland. 2000. Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers. Biol Reprod 63:811-819.

Mihm, M. and E.J. Austin. 2002. The final stages of dominant follicle selection in cattle. Domest Anim Endocrinol 23:155-166.

Mondschein, J.S., S.A. Smith, and J.M. Hammond. 1990. Production of insulin-like growth factor binding proteins (IGFBPs) by porcine granulosa cells: identification of IGFBP-2 and –3 and regulation by hormones and growth factors. Endocrinology 127: 2298-2306.

Monget, P., D. Monniaux, C. Pisselet, and P. Durand. 1992. Changes in insulin-like growth factor (IGF-I), IGF-II and their binding proteins during growth and atresia of ovine ovarian follicles. Endocrinology 132:1438-1445.

Monget, P., D. Monniaux, C. Pisselet, and P. Durand. 1993. Changes in insulin-like growth factor-I (IGF-I), IGF-II, and their binding proteins during growth and atresia of ovine ovarian follicles. Endocrinology 132(4):1438-1446.

Monget, P., S. Fabre, P. Mulsant, F. Lecerf, J.M. Elsen, S. Mazerbourg, C. Pisselet, D. Monniaux. 2002. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. Domest Anim Endocrinol 23:139-154.

Monget, P., S. Mazerbourg, T. Delpeuch, M.-C. Maurel, S. Maniere, J. Zapf, G. Lalmanach, C. Oxvig, and M.T. Overgaard. 2003. Pregnancy-associated plasma protein-A is involved in insulin-like growth factor binding protein-2 (IGFBP-2) proteolytic degradation in bovine and porcine preovulatory follicle: identification of cleavage site and characterization of IGFBP-2 degradation. Biol Reprod 68: 77-86.

Monniaux, D., C. Pisselet, J. Fontaine. 1994. Uncoupling between proliferation and differentiation of ovine granulosa cells in vitro. J Endocrinol 142:497-510.

Nakatani, A., S. Shimasaki, G. F. Erickson, and N. Ling. 1991. Tissue-specific expression of four insulin-like growth factor-binding proteins (1,2,3, and 4) in the rat ovary. Endocrinology 129: 1521-1529.

Overgaard, M.T., H.B. Boldt, L.S. Laursen, L. Sottrup-Jensen, C.A. Conover, C. Oxvig. 2001. Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase. J Biol Chem 276: 21849-21853.

Piferrer, F., D. Li, S. Shimasaki, G.F. Erickson. 1997. Transforming growth factor-α stimulates insulin-like growth factor binding protein-4 (IGFBP-4) expression and blocks follicle-stimulating hormone regulation of IGFBP-4 production in rat granulosa cells. Mol Cell Endocrinol 133:9-17.

Putowski, L., R.M. Rohan, D.S. Choi, W.J. Scherzer, E. Ricciarelli, J. Mordacq, K.E. Mayo, and E.Y. Adashi. 1997. Rat ovarian insulin-like growth factor binding protein-4: a hormone-dependent granulosa cell-derived antigonadotropin. J Soc Gynecol Invest 4: 144-151.

Rajaram, S., D.J. Baylink, and S. Mohan. 1997. Insulin-like growth factor-binding Proteins in serum and other biological fluids: regulation and functions. Endoc Rev 18: 801-831.

Rehault, S., P. Monget, S. Mazerbourg, R. Tremblay, N. Gutman, F. Gauthier, and T. Moreau. 2001. Insulin-like growth factor binding proteins (IGFBPs) as potential physiological substrates for human kallikreins hk2 and hk3. Eur J Biochem 268: 2960-2968.

Resnick, C.E., P.J. Fielder, R.G. Rosenfeld, and E. Y. Adashi. 1998. Characterization and hormonal regulation of a rat-ovarian insulin-like growth factor binding protein-5 endopeptidase: an FSH-inducible granulosa cell derived metalloprotease. Endocrinology 139: 1249-1257.

Riley, S.C., A.H. Gibson, R. Leask, D.J.W. Mauchline, H.G. Pedersen, and E.D. Watson. 2001. Secretion of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases into follicular fluid during follicle development in equine ovaries. Reproduction 121: 553-560.

Rivera, G.M., and J.E. Fortune. 2001. Development of codominant follicles in cattle is associated with a follicle-stimulating hormone-dependent insulin-like growth factor binding protein-4 protease. Biol Reprod 65: 112-118.

Rivera, G.M. and J.E. Fortune. 2003a. Proteolysis of insulin-like growth factor binding proteins-4 and –5 in bovine follicular fluid: implications for ovarian follicular selection and dominance. Endocrinology 144: 2977-2987.

Rivera, G.M. and J.E. Fortune. 2003b. Selection of the dominant follicle and insulin-like growth factor (IGF)-binding proteins: evidence that pregnancy-associated plasma protein-A contributes to proteolysis of IGF-binding protein-5 in bovine follicular fluid. Endocrinology 144: 437-446.

Roberts, A.J. and S.E. Echternkamp. 2003. Insulin-like growth factor binding proteins in granulosa and thecal cells from bovine ovarian follicles at different stages of development. J Anim Sci 81:2826-2839.

Samaras, S.E., S.F. Canning, J.A. Barber, F.A. Simmen, and J.M. Hammond. 1996. Regulation of insulin-like growth factor biosynthesis in porcine granulosa cells. Endocrinology 137:4657-4664.

Sara, V.R., and K. Hall. 1990. Insulin-like growth factors and their binding proteins. Phys Rev 79: 591-614.

Schams, D., B. Berisha, M. Kosmann, and W.M. Amserlgruber. 2002. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. Domest Anim Endocrinol 22: 51-72.

Schneider, M.R. and E. Wolf, A. Hoeflich, and H. Lahm. 2002. Insulin-like growth factor binding protein-5: flexible player in the IGF system and effector on its own. J Endocrinol 172: 423-440.

Silva J.M. and C.A. Price. 2002. Insulin and IGF-I are necessary for FSH-induced cytochrome P450 aromatase but not cytochrome P450 side-chain cleavage gene expression in oestrogenic bovine granulosa cells in vitro. J Endocrinol 174: 499-507.

Soe, R., M.T. Overgaard, A.R. Thomsen, L.S. Laursen, I.M. Olsen, L. Sottrup-Jensen, J. Haaning, L.C. Giudice, C.A. Conover, and C. Oxvig. 2002. Expression of recombinant murine pregnancy-associated plasma protein-A (PAPP-A) and a novel variant (PAPP-Ai) with differential proteolytic activity. Eur J Biochem 269: 2247-2256.

Spicer, L.J. and S.E. Echternkamp. 1985. Ovarian follicular growth, function, and turnover in cattle: a review. J Anim Sci 62: 428-451.

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

Spicer, L.J., S.E. Echternkamp, E.A. Wong, D.T. Hamilton, and R.K. Vernon. 1995. Serum hormones, follicular fluid steroids, insulin-like growth factors and their

binding proteins, and ovarian IGF mRNA in sheep with different ovulation rates. J Anim Sci 73:1152-1163.

Spicer, L.J. and S.E. Echternkamp. 1995. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Domest Anim Endocrinol 12: 223-245.

Spicer L.J., S.E. Echternkamp, E.A. Wong, D.T. Hamilton, and R.K. Vernon. 1995. Serum hormones, follicular fluid steroids, insulin-like growth factors and their binding proteins, and ovarian IGF mRNA in sheep with different ovulation rates. J Anim Sci 73:1152-1163.

Spicer, L.J., T.D. Hamilton, and B.E. Keefer. 1996. Insulin-like growth factor I enhancement of steroidogenesis by bovine granulosa cells and theca cells: dependence on de novo cholesterol synthesis. J Endocrinol 151: 365-373.

Spicer, L.J., C.S. Chamberlain, and G.L. Morgan. 2001. Proteolysis of insulin-like growth factor binding proteins during preovulatory follicular development in cattle. Domest Anim Endocrinol 21: 1-15.

Spicer, L.J., C.S. Chamberlain, and S. M. Maciel. 2002. Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. Domest Anim Endocrinol 22: 237-254.

Spicer, L.J., and C.S. Chamberlain. 2002. Estradiol and luteinizing hormone regulation of insulin-like growth factor binding protein production by bovine granulosa and theca cells. Endocrine 17: 161-168.

Spicer, L.J. 2004. Proteolytic degradation of insulin-like growth factor binding proteins by ovarian follicles: a control mechanism for selection of dominant follicles. Biol Reprod (In press).

Stewart, R.E., L.J. Spicer, T.D. Hamilton, and B.E. Keefer. 1995. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine theca cells: involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. J Anim Sci 73: 3719-3731.

Stewart, R.E., L.J. Spicer, T.D. Hamilton, B.E. Keeger, L.J. Dawson, G.L. Morgan, and S.E. Echternkamp, 1996. Levels of insulin-like growth factor binding proteins, LH, and IGF-I receptor and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrous cycles. Endocrinology 137: 2842-2849.

Stoving, R.K., A. Flyvbjerg, J. Frystyk, S. Fisker, J. Hangaard, M. Hansen-Nord, and C. Hangen. 1999. Low serum levels of free and total IGF-I in patients with anorexia

nervosa are not associated with increased IGF-binding protein 3 proteolysis. J Clin Endocrinol Metab 84: 1346-1350.

Veldhuis J.D., Gwynne J.T., Strauss J.F. III, Demers L.M., and Klase P.A. 1984. Role of estradiol as a biological amplifier of gonadotropin action in the ovary: in vitro studies using swine granulosa cells and homologous lipoproteins. Endorcinology 114: 3069-3076.

Vlasak, A.R., G.J. Gleich, P. Bischof, and N. Chegini. 2003. Localization and cellular distribution of PAPP-A and major basic protein in human ovary and corpora lutea throughout the menstrual cycle. Reprod Endocrinol 79: 1833-1844.

Voge, J.L., C.A.T. Santiago, P.Y. Aad, D.W. Goad, J.R. Malayer, and L.J. Spicer. 2004. Quantification of insulin-like growth factor binding protein mRNA using real time PCR in bovine granulosa and theca cells: effects of estradiol, insulin and gonadotropins. Domest Anim Endoc 26: 241-258.

Voutilainen, R., S. Franks, H.D. Mason, and H. Martikainen. 1996. Expression of insulin-like growth factor (IGF), IGF-binding protein, and IGF receptor messenger ribonucleic acids in normal and polycystic ovaries. J Clin Endocrinol Metab 81: 1003-1008.

Wandji, S.A., T.L.Wood, J. Crawford, S.W. Levison, J.M. Hammond. 1998. Expression of mouse ovarian insulin-like growth factor system components during follicular development and atresia. Endocrinology 139: 5205-5214.

Wandji, S.A., J.E. Gadsby, F.A. Simmen, J. A. Barber, and J.M. Hammond. 2000. Porcine ovarian cells express messenger ribonucleic acids for the acid-labile subunit and insulin-like growth factor binding protein-3 during follicular and luteal phases of the estrous cycle. Endocrinology 14: 2638-2647.

Yoshimura, Y. 2003. Insulin-like growth factors and their binding proteins: potential potential relevance to reproduction physiology. Reprod Med Biol 2: 1-24.

Zhou, J. and Bondy. 1993. Anatomy of the human ovarian insulin-like growth factor system. Biol Reprod 48: 467-482.

Zhou, J., T.R.Kumar, M.M. Matzuk, and C. Bondy. 1997. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. Mol Endocrinol 11: 1924-1933.

Zhou, R., D. Diehl, A. Hoeflich, H. Lahm, and E. Wolf. 2003. IGF-binding protein A: biochemical characteristics and functional consequences. J Endocrinol 178: 177-193.

Zorin, N.A., S.G. Zhabrin, N.N. Semenkov. 1995. Interaction of human pregnancy-associated plasma protein-A with serine proteinases. Clinica Chimica Acta 239: 47-55.

CHAPTER III

RELATIONSHIPS AMONG GRANULOSA CELL INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND PREGNANCY-ASSOCIATED PLASMA PROTEIN-A mRNA EXPRESSION AND FOLLICULAR FLUID CONTENTS IN DOMINANT AND LARGE SUBORDINATE FOLLICLES OF PREOVULATORY CATTLE

ABSTRACT

The objectives of this study were to evaluate if 1) expression levels for insulin-like growth factor binding protein (IGFBP) and pregnancy-associated plasma protein-A mRNAs differ between the dominant and largest subordinate follicles during luteal regression, and 2) these differences are associated with differences in follicular fluid (FFL) concentrations of steroids (estradiol, androstenedione, and progesterone), total and free IGF-I, or IGFBPs. To accomplish these goals, estrous cycles of non-lactating Holstein dairy cows (n = 15) were synchronized with two injections of prostaglandin (PGF2α) 11 d apart. Follicular growth was monitored daily via transrectal ultrasonography five days prior to the second injection of PGF2α. Granulosa cells and FFL were collected either 24 h or 48 h after the second injection of PGF2α from dominant and subordinate follicles. Diameter of the dominant follicles was greater ($P<0.0001$) than that of the large subordinate follicles. FFL from dominant follicles had

lower concentrations of progesterone ($P<0.08$) and higher concentrations of estradiol ($P< 0.05$), androstenedione ($P<0.0001$), estradiol:progesterone ratio ($P<0.0001$), free IGF-I ($P<0.0001$) and calculated percentage free IGF-I ($P<0.01$) than large subordinate follicles. Total IGF-I was affected by follicle type by time. IGFBP-2,-4, and –5 levels in the FFL were greater ($P<0.005$) in subordinate than in dominant follicles. IGFBP-3,-4 and PAPP-A mRNA expression did not differ ($P>0.05$) between dominant or subordinate follicles. IGFBP-2 mRNA levels of subordinate follicles was 4-fold greater than the dominant follicles ($P<0.06$). IGFBP-5 mRNA levels at 24 h and 48 h, were several fold greater ($P<0.05$) in subordinate than dominant follicles. Only IGFBP-2 and –5 mRNA levels were positively correlated with their respective proteins in the FFL. We conclude that decreased levels of IGFBP-2 and –5 mRNA in granulosa cells may contribute to the decrease in FFL IGFBP-2 and –5 proteins of preovulatory dominant follicles, and that changes in granulosa cell IGFBP-3,-4, and PAPP-A mRNA levels do not occur during final preovulatory follicular development in cattle.

INTRODUCTION

Cattle have two to three follicular waves per estrous cycle. Each 7- to 10-day follicular wave brings about one dominant follicle that becomes larger than the rest of the cohort subordinate follicles. The dominant follicles from the first and second wave (of a three-wave cycle) are non-ovulatory, and become atretic, unless luteolysis is induced via treatment with PGF2α (Fortune et al., 2001). The first follicular wave is the most convenient to study for predicting and monitoring the process of follicle growth because

it is unaffected by any previous wave (Mihm et al., 2000; Fortune et al., 2001). Studies concerning follicle wave emergence to selection are conducted on days 1 to 3 (Mihm et al., 2000; Austin et al., 2001) and follicle dominance on days 4 to10 of the estrous cycle (Stewart et al., 1996).

As the follicular wave progresses, follicles respond differently to the decreasing FSH and increasing LH concentrations. During functional dominance, the dominant follicle continues to increase in size, as its cohort subordinates lag behind. Although increased diameter is a good visual characteristic to determine the dominant follicle via ultrasonography, it is more of a result rather than the cause of dominance, because intrafollicular factors are initiated even before significant difference in follicle diameter is detected (Mihm et al., 2000; Austin, 2001; Spicer, 2004). These factors include increased levels of estrogen, proteases, and free IGF-I as well as decreased levels of low molecular weight IGF binding proteins (IGFBP)-4 and –5.

Many studies indicate that after follicular wave emergence, the largest follicle (which becomes the dominant follicle) secretes the most estradiol (Stewart et al., 1996; Mihm et al., 2000; Rivera and Fortune, 2003a). Recently, Ginther et al. (2003) reported that concentration of estradiol and free IGF-I simultaneously became greater in the follicular fluid of the largest follicle (F1) as compared to the next largest associated follicle (F2) by the beginning of diameter deviation. This presumably increases circulating estradiol levels, which cause a negative feedback to FSH, hindering the growth of other follicles. However, levels of free IGF-I in bovine preovulatory dominant and subordinate follicles have not yet been reported.

The source of intrafollicular IGF-I is likely from systemic blood as well as from local production by ovarian granulosa, theca, and luteal cells, allowing for endocrine and intraovarian autocrine and paracrine action (Spicer and Echternkamp, 1995). In most species studied, IGF-I stimulates both proliferation and differentiation of granulosa cells (Spicer an Echternkamp, 1995; Mazerbourg et al., 2003), and has the potential to prevent ovarian follicular cell apoptosis (Chun et al., 1994). Increased free IGF-I could either directly enhance estradiol production, indirectly enhance FSH-induced estrogen production and (or) indirectly enhance LH-induced androgen production by the theca cells (Stewart et al., 1995; Spicer et al., 2002). Presumably, continued IGF-I bioavailability is needed by the largest follicle during its development to dominance (Beg et al., 2002; Ginther et al., 2003), however, its use by the aforementioned ovarian cell types is affected by IGFBP (Spicer and Echternkamp, 1995; Mihm et al., 2000; Austin et al., 2001).

IGFBP activity in the follicular fluid of cows (Spicer and Echternkamp, 1995) and mares (Bridges et al., 2002; Mazerbourg et al., 2003;) decreases as follicles develop and become estrogen active, but increases in atretic follicles. Low molecular weight binding proteins $\leq$ 35 kDa, such as IGFBP-2,-4, and -5, regulate the availability of IGF-I to granulosa and theca cells (Spicer et al., 1997; Spicer and Chamberlain, 1999). Decreased amounts of IGFBP-4 and –5 in the follicular fluid of dominant as compared to subordinate follicles occurred before morphological selection, while decreased IGFBP-2 occurred after deviation (Mihm et al., 2000; Rivera and Fortune, 2003a). This characteristic difference between follicle types (dominant and subordinates) is maintained until the growing or preovulatory dominant follicle ovulates or undergoes atresia. The

changes in concentrations of low molecular weight IGFBPs in bovine follicles may be regulated by its own gene expression localized in different ovarian tissues, post translational degradation by hormonally-induced proteases, or both (Spicer et al., 2001; Mihm et al., 2002; Monget et al., 2002). However, IGFBP gene expression in dominant and subordinate follicles has not been evaluated during luteal regression. During follicular growth, IGFBP-2 mRNA levels were less in granulosa cells of large follicles as compared with the smaller follicles in cattle (Yuan et al., 1998), and pigs (Liu et al., 2000). IGFBP-4 mRNA increased in the theca interna of large (8 mm) follicles in pigs (Liu et al., 2000), while changes were not detected in theca cells of heifers (Armstrong et al., 1998). In rats, IGFBP-2,-3,-4 and –5 mRNA expressions were not detected in dominant follicles (Erickson et al., 1992).

Pregnancy-associated plasma protein-A (PAPP-A), known best for its IGFBP-4 protease activity, is found in porcine, bovine, ovine and equine follicular fluid (Mazerbourg et al., 2001; Hourvitz et al., 2002). PAPP-A mRNA expression was localized in granulosa cells of healthy follicles in humans (Conover et al., 2001; Hourvitz et al., 2000), rodents, and domestic animals (Mazerbourg et al., 2001). PAPP-A may also be a protease for IGFBP-5 (Rivera and Fortune, 2003a) and perhaps IGFBP–2 (Monget et al., 2003). PAPP-A may be hormonally regulated since recently, Hourvitz et al. (2002) showed significant changes in PAPP-A mRNA levels in murine ovaries after PMSG and hCG treatment in vivo. However, changes in granulosa cell PAPP-A mRNA during preovulatory follicular development has not been evaluated in any species.

The objectives of this study were to evaluate if amounts of IGFBP and PAPP-A mRNAs differ between the dominant and largest subordinate follicles during luteal

regression (different time points post PGF-2α injection) prior to ovulation, and to determine if these changes are associated with changes in follicular fluid concentrations of total and free IGF-I, steroids (estradiol, progesterone, and androstenedione) or IGFBPs.

MATERIALS AND METHODS

ANIMALS

Estrous cycles of 15 non-lactating Holstein cows were synchronized with two injections of 25 mg IM PGF2α (Lutalyse; Pharmacia and Upjohn, Kalamazoo, MI) 11 days apart during two replicates (replicate 1, n=10; replicate 2, n=5). Follicular growth was monitored daily via transrectal ultrasonography with an Aloka 500V ultrasound scanner attached to a 7.5 mHz probe five days before the second PGF2α injection to identify the dominant follicle and subordinate follicles (Spicer et al., 2001). About 10 mL of blood were collected via the tail vein and plasma were harvested for progesterone determinations.

COLLECTION OF FOLLICULAR FLUID AND GRANULOSA CELLS

Follicular fluid from dominant and large subordinate follicles was collected via transvaginal aspiration 24 (n= 8) or 48 (n=7) h after the second injection of PGF2α. Cows had epidural injection with 2% Lidocaine (5 mL; Butter Co., Columbus, OH), between coccygeal 1 (C1) and 2 (C2) vertebra located by raising the tail in a "pump handle" way. This anesthetic is used to prevent straining during collection. A 55 cm 17-gauge aspiration needle was inserted through a steel needle guide attached to the

ultrasound probe and inserted in the vagina. The dominant and large subordinate follicles were identified and punctured, and the granulosa cell and follicular fluid mixture were aspirated with 10 cc syringes and deposited into 1.5 mL sterile cryotubes. Granulosa cells were obtained by immediate centrifugation (200 x g for 5 min) of the follicular fluid, placed in 0.5 mL of TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD), and frozen in liquid N_2. Granulosa cell and follicular fluid samples were stored in the cryotubes at −80°C until RNA extraction and RIAs, respectively, were conducted. Times after PGF2α injection were selected based on previous studies (Spicer et al., 2001) and because serum LH concentration increases during this time, prior to the anticipated LH surge (Spicer and Roche, 1981; Ireland and Roche, 1982). The dominant follicles were aspirated on average (± SE) on day 8 ± 2.9 post ovulation and had an average (±SE) growth rate of 1.1+0.2 mm during the 5-day ultrasound period.

RADIOIMMUNOASSAYS (RIAs)

Progesterone (P4) concentrations in plasma were determined with a solid-phase RIA kit (Coat-a-Count, Diagnostic Products, Los Angeles, CA) as previously described (Stewart et al., 1996) to determine if cows were exhibiting normal luteal function; the intraassay CV was 10.9%, and the assay sensitivity, defined as 91% of total binding was 0.005 ng/mL. Follicular fluid concentrations of estradiol (E2) were determined by RIA (Spicer and Enright, 1991); the intraassay coefficient of variation was 8.7% and the assay sensitivity, defined as 95% of total binding, was 2.6 pg/mL. Follicular fluid concentrations of progesterone were determined by RIA (Spicer and Enright, 1991); the intrassay coefficient of variation was 14.7% and assay sensitivity, defined as 90% of total

binding, was 8.0 ng/mL. Follicular fluid concentrations of IGF-I were determined by RIA after acid-ethanol extraction (16 h at 4°C; Echternkamp et al., 1990); the intraassay coefficient of variation was 2.5%, and assay sensitivity, defined as 95% of total binding, was 4.5 ng/mL. Follicular fluid concentrations of androstenedione were determined using a solid-phase RIA kit (ICN Biomedicals, Costa Mesa, CA; Stewart et al., 1996); the intraassay coefficient of variation was 4.4%, and assay sensitivity, defined as 90% binding at 12 ng/mL. Follicular fluid concentrations of free IGF-I were determined using Active® Free IGF-I immunoradiometric assay (IRMA; DSL-9400, DSL, Webster, TX). Increasing volumes of pooled bovine follicular fluid (2 μL, 5μL, 10 μL, 20 μL, 40 μL) in 75 μL of PBS (0.25% BSA, pH 7.5) resulted in parallelism to the free IGF-I standard curve. Briefly, in this experiment, 10 μL of follicular fluid sample in 90 μL of PBS was deposited at the bottom of the coated tubes and incubated for 2 h at 4 °C. Tubes were washed twice with 2 mL deionized water, and incubated in a shaking platform (180 rpm) with 200 μL second I^{125} antibody at 25°C for 2 h. Tubes were washed thrice with 3 mL of deionized water and Free IGF-I was quantitated using a gamma counter. The intraassay coefficient of variation was 2.3%, and assay sensitivity, defined as 0.25% binding, was 0.5 ng/mL. Percentage of Free IGF-I was calculated as (free IGF-I / total IGF-I) x 100.

LIGAND BLOTTING

Amounts of IGFBP in follicular fluid were assessed by one-dimensional SDS-PAGE as previously described (Stewart et al., 1996; Spicer et al., 2001). Briefly, 4 μL of

follicular fluid was mixed with 21 μL of Laemmli sample buffer (Bio-Rad, Hercules, CA). Samples were then heat treated (3 min at 100°C) to denature the proteins and centrifuged at approximately 4600 x g for 3 min. Samples were then added to a 12% polyacrylamide gel and electrophoresed overnight at constant current (27 amperes) and varying voltage (overnight at 36 volts and the following morning at 82 volts). Following separation, gels were electrophoretically transferred to nitrocellulose paper (Midwest Scientific, St. Louis, MO) for 2.5 to 3.0 h, and ligand blotted overnight with ^{125}I-IGF-I and ^{125}I-IGF-II (1:1) on a rocking platform at 4°C. After washings and exposure to X-ray film for 48 h at -80°C, band intensity was densitometrically analyzed with Molecular Analyst (Bio-Rad). All values were expressed as arbitrary densitometric units (ADU/4 μL). The inter-gel coefficient of variation, computed by taking the average and standard deviation of IGFBP-3 and –2 levels from pooled follicular fluid (run on each gel and serving as the control) was 27.7%.

mRNA ANALYSES

mRNA extraction. Total cellular RNA was isolated from aspirated granulosa cells by lysis in 0.50 mL TRIzol reagent (Life Technologies, Inc., Gaithersurg, MD) in 1.5 mL eppendorf tubes. Lysed cells were then incubated in TRIzol for 5 min at 22 °C. Next, 0.10 mL of chloroform (Sigma Chemical Co., St. Louis, MO) was added and each sample vortexed for 15 sec. Following 3 min incubation at 22°C, samples were centrifuged (3500 x g for 30 min at 4°C). The upper aqueous phase containing the RNA was transferred to a new eppendorf tube and RNA was precipitated using 0.25 mL of

isopropyl alcohol (Pierce Chemical Co., Rockford, IL). Samples were gently mixed by hand, and then incubated at 22°C for 10 min, followed by centrifugation at 3500 x g for 10 min at 4°C. The supernatant was then removed and the RNA pellet washed with 0.50 mL of 75% ethanol and then centrifuged, as before, for 5 min. Ethanol supernatant was removed and RNA pellet allowed to dry for 4 min. The RNA was then dissolved in 0.03 mL of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 7.4).

Quantification of total RNA. Ribogreen ® RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, OR) were used to quantify total RNA isolated from the granulosa cell samples following manufacturer's specifications with modifications. An aliquot of the isolated RNA from the samples was placed in 1 reaction of Tris-EDTA buffer (1xTE) with a 1 sample:50 TE dilution. The Ribogreen RNA quantitation reagent was diluted 200-fold for the high range assay. The ribosomal RNA standard (100 µg/mL) was diluted 50-fold in 1xTE to make a 2 µg/mL working solution. This working solution was further diluted with appropriate amounts of 1xTE for the standard curve with final RNA concentrations of 1000, 750, 500, 250, 100, 75, 25, 10, and 0 ng/ µL. 50 µL of RNA sample and standard was pipetted into black 100 µL 96-well microplates (Proxiplate™-96F, P/N 6006270, Packard Bioscience BV, Meridian, CT), followed by 50 µL of the aqueous working solution of Ribogreen RNA quantitation reagent. After 5 min incubation at 25 °C, fluorescence was read using Wallac 1420 (Perkin Elmer, Boston, MA). The excitation maximum for Ribogreen reagent bound to RNA is 500 nm, while its emission maximum is 525 nm. The fluorescence value of the reagent blank (0 ng/mL

RNA) was subtracted from each of the standards' and samples' fluorescence to adjust for the background. Fluorescence of the standard RNA was plotted with its corresponding concentrations. Given the adjusted fluorescence value of the samples, RNA concentrations were determined. The intra-assay coefficient of variation was 17%.

RT-PCR Primer and Probe Design. Primers and probes for quantitative RT-PCR were made using Primer Express software (Foster City, CA) with the following manufacturer's restrictions: The temperature melting (Tm) for primers is 50 °C to 60°C with the probe's Tm to be at least 10°C higher. The minimum GC base pair content for the primers and probes should be 20% to 80% avoiding runs of an identical nucleotide. The minimum and maximum length of the strands should be 9 and 40 nucleotides, respectively.

The available bovine sequences for IGFBP-2,-3 ,-4,-5 and PAPP-A found in GENBANK were analyzed by the Primer Express program to determine optimum primer and probe locations. IGFBP-2 (954 bp, Accession AF074854) forward and reverse primers were constructed from bp 466 to 486 with a sequence of GACGGGAACGTGAACTTGATG and from bp 518 to 536 with a sequence of TCCTTCATGCCGGACTTGA, respectively. The probe for IGFBP-2, AGGTGGAGGTGGTGCCGGTCG, was found to anneal from 489 to 509 of the IGFBP-2 sequence. Forward and reverse primers for IGFBP-3 (1568 bp, Accession M76478) were designed from bp 1137 to 1163 with a sequence of AAAGAGATGTTTGAAATGCCTAGTTTT, and from bp 1200 to 1224 with a sequence of TCAAACTCGGTTTCACTGACTACTG for each. Its probe has a sequence of

TTCCACATGGTGAACCTGGCATCTTTC and anneals from 1165 to 1191 of the IGFBP-3 sequence. Forward and reverse primers for IGFBP-4 (2028 bp, Accession S52770) were GAGGAAAGAATGTATGTGCCTGATG and GACCACAAACGGAGGAGGAA, respectively. The forward primer anneals from 1733 to 1757, while the reverse primer anneals from 1808 to1827 of the IGFBP-4 sequence. Taqman probe, CATGCTGGGAGGTGAGGGACTTATCTGG, anneals from 1772 to 1799 of the IGFBP-4 sequence. Forward and reverse primers for IGFBP-5 (335 bp, Accession S52657), GTTTGCCTGAACGAAAAGAGCTA and CGAGTAGGTCTCCTCTGCCATCT, respectively, anneal from 193 to 215 and 275 to 295 of the IGFBP-5 sequence. Taqman probe, AGCCAAGATCGAAAGAGACTCCCGTGAG, anneals from 225 to 252 of the IGFBP-5 sequence. For PAPP-A (851 bp, Accession AF421141), the forward and reverse primers used were CAGATGTTGAGCAGCCCTGTAA and GGGTTGACGGCTGAATTGG, respectively. The forward primer anneals from 557 to 578 of the sequence while the probe, CCAGCGTCCGCACCTGGAGC, anneals from 581 to 600 of the PAPP-A sequence. PCR products of IGFBP-2,-3,-4,-5, and PAPP-A were 70, 87, 94 and 63 base pairs, respectively. The specificity of the desired products was documented using high-resolution gel electrophoresis to verify that the transcripts were of the exact molecular size predicted and further confirmed by sequence analysis.

Quantitative RT-PCR. Real-time quantitative RT-PCR was used to determine differences in mRNA expression levels for IGFBP-2, -3, -4, -5 and PAPP-A between dominant and subordinate follicles collected at either 24 or 48 h after second

administration of PGF2α. Expression levels were quantitated using TaqMan® One Step PCR Mastermix (P/N 4309169, Applied Biosystems, Foster City, CA). The probe anneals between the forward and reverse primer sites. The Taqman probes for each IGFBP contain a 5' reporter dye (TET) and 3' quencher dye (TAMRA). Taqman probe for PAPP-A contains the same quencher dye at the 3' end, but has FAM as its 5' reporter dye. The quencher dye suppresses the reporter's fluorescence until cleavage by the AmpliTaq Gold DNA polymerase. An increase on the reporter's fluorescence corresponds to an equal amount of PCR product. A total reaction volume of 25 μL consisted of: 1μL of 200 nM forward primer (IGFBP-2,-3,-4,-5, and PAPP-A), 1 μL of 200 nM reverse primer (IGFBP –2, -3, -4, -5, and PAPP-A), 0.5 μL of 100 nM fluorescent probe for IGFBP-3 and -4 or 1 μL of 200 nM fluorescent probe for IGFBP-2, -5, and PAPP-A, 12.5 μL of Taqman Master mix without uracil-N- glycosylase (UNG), 0.625 μL of Multiscribe and RNase inhibitor mix (P/N 4309169, Applied Biosystems, Foster City, CA), and 4 μL containing a total of 100 ng RNA, brought to volume with RNase free water.

One step RT-PCR amplification was performed in the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: 30 min at 48°C for reverse transcription, 15 sec for 95°C for denaturing and 1 min at 60°C for annealing and extension. Denaturation, annealing and extension steps repeated for 40 cycles. Running time was approximately 2.5 h. Ribosomal 18S RNA control kit (P/N 4308329, PE Biosystems, Foster City, CA) was used as a housekeeping gene to normalize samples for any variation in RNA loading. A

total reaction volume of 25 μL consisted of: 0.25 μL of 10μM of forward primer, 0.25 μL of 10μM of reverse primer, 0.0625 μL of 40μM probe, 12.5 μL of Taqman Master mix without UNG, 0.625 μL of Multiscribe and RNase inhibitor mix (P/N 4309169, Applied Biosystems, Foster City, CA), and 100 pg of sample RNA in 4 μL brought to volume with RNase free water.

To verify the sensitivity of 18S rRNA housekeeping gene to detect fold changes, varying amounts of RNA were loaded in a parallel fashion with the target probe (IGFBP-2,-3,-4, -5 or PAPP-A). Decreasing amounts of total RNA were analyzed for 18S rRNA (500, 100, 50, 10, 5 or 1 pg) or target mRNA (500, 100, 50, 10, 5 or 1 ng) with results for IGFBP-5 and PAPP-A illustrated in Fig. 1.

Quantification of gene expression was made by setting an arbitrary threshold on the TET or FAM curves in the geometric portion of the RT-PCR amplification plot. Relative quantification of IGFBP-2,-3,-4, -5 and PAPP-A mRNA expression was done using the comparative threshold cycle (Ct) method (Technical Bulletin No. 15, PE Biosystems User Manual). The ΔCt was determined by subtracting the 18S Ct from the target unknown's Ct value (IGFBP-2,-3,-4,-5, or PAPP-A). For each IGFBP and PAPP-A mRNA, the ΔΔCt was determined by subtracting the highest ΔCt from all other ΔCt values. Fold changes in mRNA expression of the target genes were calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Individual sample fold expression was then divided by the lowest treatment mean. Ct values amplifying 2 cycles before or after the no template control (NTC) were discarded. Outliers were detected and determined as described by Ott (1977). The intra-assay (within plate) coefficients of variation based on Ct values

There was significant ($P<0.05$) follicle type by time effect on estradiol concentration. Subordinate follicles are significantly different from each other ($P<0.05$). At 24 h and 48 h, dominant follicles had greater estradiol levels than subordinate follicles ($P<0.0001$). Estradiol levels of dominant follicles collected at 24 and 48 h averaged 518.5 ± 67.5 ng/mL and 459.0 ± 72.2 ng/mL, respectively, while estradiol concentrations in subordinate follicles collected at 24 and 48 h averaged 5.1 ± 84.3 ng/mL and 34.8 ± 73.9 ng/mL, respectively (Fig. 3).

Follicular fluid progesterone concentrations tended to differ ($P<0.08$) between follicle types, but unaffected by time or its interaction. Progesterone concentrations of dominant and subordinate follicles averaged across time were 206.3 ± 62.1 and 413.0 ± 75.0 ng/mL, respectively (Fig. 4).

Follicular fluid androstenedione concentrations were significantly ($P<0.0001$) affected by follicle type but not time or follicle type by time interaction. Androstenedione concentrations of dominant follicles were 23-fold greater as compared to the subordinate follicles ($P<0.0001$). Androstenedione concentrations of dominant and subordinate follicles averaged across time were 393.9 ± 149.4 and 17.7 ± 151.8 ng/mL, respectively (Fig. 4).

Estrogen:progesterone ratio was significantly ($P<0.0001$) affected by follicle type but not by time or follicle type by time interaction. Estrogen:progesterone ratios averaged across time for dominant and subordinate follicles were 2.7 ± 0.4 and 0.2 ± 0.4, respectively.

Total IGF-I concentration was affected ($P<0.05$) by follicle type by time interaction. Total IGF-I concentrations decreased ($P<0.05$) between 24 h and 48 h in

and gene fold expression for the various target genes averaged 0.4 to 1.5% and 13 to 31%, respectively from sample duplicates on each plate.

STATISTICAL ANALYSES AND EXPERIMENTAL DESIGN

Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). Data exhibiting heterogeneous variances (Follicular fluid E2, P4, A4, IGF-I, IGFBP and mRNA data) were analyzed after transformation to log (x+1). Least-square means $\pm$ SE of the non-transformed data were reported. Fixed effects consist of time of collection (24 or 48 h) and follicle type (dominant or subordinate). The random effects were replicate (1 or 2) and cow nested in time, and their interactions were analyzed. If main effects were significant, differences in treatment means were evaluated using LSMEANS with the PDIFF option. Alternatively, LSMEANS with the SLICE option was used to determine differences in treatment means if any interaction was significant. Pearson correlation coefficients were also calculated to determine any significant relationships among the variables measured.

RESULTS

FOLLICLE DIAMETER, STEROIDS, AND IGF-I

Follicle type but not time affected ($P<0.0001$) follicle diameter. Average diameters of dominant follicles collected at 24 and 48 h were greater ($P<0.0001$) than the average of the subordinates. Mean diameters of dominant and subordinate follicles across time were 18.3 $\pm$ 1.5 mm and 8.2 $\pm$ 1.5 mm, respectively (Fig. 2).

subordinate but not dominant follicles and were less in dominant than subordinate follicles at 24 h ($P<0.05$) but greater in dominant than subordinate follicles at 48 h ($P<0.05$). Total IGF-I in dominant follicles collected at 24 and 48 h were 76.2 $\pm$ 29.3 and 90.3 $\pm$ 29.4 ng/mL, respectively, while IGF-I in subordinate follicles at 24 and 48 h were 97.7 $\pm$ 29.6 and 67.6 $\pm$ 29.8 ng/mL, respectively.

Free IGF-I concentrations were significantly ($P<0.0001$) different between follicle types. There was no significant time effect or follicle type by time interaction. Concentrations of free IGF-I in dominant follicles collected at 24 h or 48 h were greater ($P<0.001$) than the subordinates. Concentrations of free IGF-I in dominant and subordinate follicles averaged across time were 18.6 $\pm$ 2.2 and 2.3 $\pm$ 2.7 ng/mL, respectively (Fig. 2).

Calculated percentage free IGF-I was significantly ($P<0.001$) different between follicle types but there was no significant time effect or follicle type by time interaction. Dominant follicles had higher calculated % free IGF-I than the large subordinate follicles at 24 h ($P<0.05$) and 48 h ($P<0.01$). Dominant and subordinate follicles had 22.2 $\pm$ 2.8% and 4.3 $\pm$ 4.2% free IGF-I, respectively.

IGFBP via LIGAND BLOTTING

Concentrations of IGFBP-2 in follicular fluid were significantly ($P<0.05$) affected by follicle type, but not by time or follicle type by time interaction. Averaged across time, IGFBP-2 levels in dominant follicles were 3-fold lower than in subordinate follicles (Fig. 5).

Main effects of time and follicle type were not significant but there was significant ($P<0.05$) follicle type by time interaction effect on IGFBP-3. Levels of IGFBP-3 in follicular fluid increased ($P<0.05$) in dominant (but not subordinate) follicles between 24 h (46.8 ± 10.6) and 48 h (61.7 ± 11.3); IGFBP-3 in subordinate follicle averaged 68.8 ± 10.5 and 50.5 ± 11.4 at 24 h and 48 h, respectively.

The N-glycosylated form of IGFBP-4 was significantly ($P<0.005$) affected by follicle type, but not by time or its interaction with follicle type. Averaged across time, levels of N-glycosylated form of IGFBP-4 in subordinate follicles were 3-fold greater as compared to the dominant follicles. At 24 h, IGFBP-4 levels in dominant and subordinate follicles differed significantly ($P<0.05$) and tended to differ ($P=0.07$) at 48 h. On the other hand, levels of N-nonglycosylated form of IGFBP-4 were not significantly affected by follicle type, time, or its interaction. Total IGFBP-4 (sum of glycosylated and non-glycosylated forms) was significantly affected ($P<0.05$) by follicle types but not by time or its interaction with follicle type (Fig. 5).

Concentration of IGFBP-5 in follicular fluid was significantly affected ($P<0.001$) by follicle type, but not by time or its interaction with follicle type. IGFBP-5 levels in dominant and subordinate follicles differed at 24 h ($P<0.01$) and 48 h ($P<0.05$). Averaged across time, IGFBP-5 levels in subordinate follicles were 4-fold greater as compared to the dominant follicles (Fig. 5).

Total IGFBP concentrations in follicular fluid as determined by the sum of the activity of all four IGFBP, were significantly ($P<0.05$) affected by follicle type by time interaction. Total IGFBP activity in dominant (67.0 ± 16.6 ADU /4 μL) and subordinate (62.7 ± 16.8 ADU/ 4 μL) follicles did not differ ($P>0.10$) at 48 h. At 24 h, total IGFBP

activity differed ($P<0.05$) between dominant (57.7 ± 15.6 ADU / 4 μL) and subordinate (107.2 ± 15.5 ADU / 4 μL) follicles.

IGFBP and PAPP-A FOLD GENE EXPRESSION

IGFBP-3, -4, and PAPP-A mRNA levels in granulosa cells were not significantly ($P>0.50$) affected by follicle type, time or its interaction. In contrast, IGFBP-2 mRNA levels tended to be affected ($P<0.06$) by follicle type by time interaction. At 24 h, the largest subordinate had 26-fold more IGFBP-2 mRNA levels compared to the dominant follicle. At 48 h, IGFBP-2 mRNA levels did not differ between dominant and subordinate follicles. IGFBP-2 mRNA between follicle types differ ($P<0.06$). There were 4-fold greater IGFBP-2 mRNA levels in the subordinate than in dominant follicle (Fig. 6).

IGFBP-5 mRNA levels in granulosa cells were significantly affected by follicle type by time interaction ($P<0.05$). IGFBP-5 mRNA decreased ($P<0.05$) 11-fold between 24 h and 48 h in subordinate follicles but did not significantly change between 24 h and 48 h in dominant follicles. At both 24 h and 48 h, IGFBP-5 mRNA levels in granulosa cells of follicles were several fold greater ($P<0.005$) in subordinate than dominant follicles (Fig. 6).

IGFBP-3 fold gene expression averaged across time was 4.7 ± 1.5 and 3.5 ± 1.5 for the dominant and subordinate follicles, respectively. IGFBP-4 fold gene expression averaged across time was 2.8 ± 1.9 and 3.9 ± 1.9 for the dominant and subordinate follicles, respectively. PAPP-A mRNA fold gene expression averaged across time was

2.5 $\pm$ 0.9 and 1.3 $\pm$ 0.9 for the dominant and subordinate follicles, respectively. Ct and ΔCt values for IGFBP-2, -3, -4, -5 and PAPP-A analyses are presented on Table 2.

PEARSON CORRELATION ANALYSIS

Free IGF-I was positively correlated with estradiol (r=0.73, P<0.001), androstenedione (r=0.56, P<0.05), follicle diameter (r=.67, P<0.05) and negatively correlated with IGFBP-2 (r=-0.53, P<0.05), -4 (r=-0.51, P<0.05), and -5 (r=-0.52, P<0.05) proteins, and IGFBP-5 mRNA levels (r=-0.67, P<0.05). Diameter was positively correlated with estradiol (r=0.75, P<0.001) and androstenedione (r=0.54, P<0.05). Estradiol was negatively correlated with progesterone (r =-0.53, P<0.05), IGFBP-2 protein (r = -0.55, P<0.05), IGFBP-4 protein (r = -0.46, P<0.05), IGFBP-5 protein (r = -0.56, P<0.05), and IGFBP-5 mRNA (r =-0.73, P<0.05), but positively correlated with androstenedione (r = 0.75, P<0.001). Progesterone was positively correlated with IGFBP-5 mRNA (r = 0.50, P<0.05), IGFBP-2 mRNA (r=0.61, P<0.05) and with IGFBP-4 protein (r = 0.57, P<0.05). Androstenedione was negatively correlated with IGFBP-5 mRNA level (r =-.52, P<0.1) and positively correlated (r = 0.47, P<0.05) with total IGF-I.

IGFBP-2 protein was positively correlated with IGFBP-4 (r = 0.57, P<0.001), -5 (r =0.91, P<0.001), and the total IGFBPs (r =0.68, P<0.001) and IGFBP-5 mRNA levels (r = 0.75, P<0.001). IGFBP-2 protein tended to positively correlate with its own mRNA fold expression (r =0.41, P<0.10). IGFBP-5 protein was positively correlated with its own mRNA fold expression (r = 0.81, P<0.001). IGFBP-3 protein was positively correlated with the total IGFBPs (r = 0.91, P<0.001).

IGFBP-2 mRNA levels were positively correlated IGFBP–5 mRNA levels (r = 0.51, P<0.05). IGFBP-4 mRNA levels were also positively correlated with IGFBP-5 mRNA levels (r=0.51, P<0.05; Table 3).

DISCUSSION

Overall, the present study showed that dominant preovulatory follicles had: 1) greater concentrations of free IGF-I, estradiol, and androstenedione than subordinate follicles; 2) less binding activities of IGFBP-2, -4, and –5 than subordinate follicles; 3) less IGFBP-2 and –5 gene expression than in subordinate follicles, and 4) similar IGFBP-3,-4, and PAPP-A mRNA levels to those of subordinate follicles.

The present study pioneered the quantification of free IGF-I in large subordinate and dominant preovulatory follicles in cattle. Others (Beg et al., 2001; Beg et al., 2002; Rivera and Fortune, 2003a; Ginther et al., 2003) have reported 4 to 8 ng/mL increase in free IGF-I when bovine follicles reach 8 mm at the time of deviation. The present study showed 16 ng/mL difference in free IGF-I between preovulatory dominant and subordinate follicles and that free IGF-I was positively correlated with follicular fluid estradiol and androstenedione concentrations. Collectively, these results suggest a continuous increase in free IGF-I may occur during the dominant follicle's tenure, and thus elevated free IGF-I may be a necessary intrafollicular event throughout follicular selection, dominance and preovulatory development.

Concentrations of free IGF-I was negatively correlated with amounts of low molecular weight IGFBP-2,-4, and –5 in the present study. Calculated difference of free

IGF-I (ΔFree) tended to correlate ($r=0.54$, $P>0.10$) with calculated difference of total binding protein activity (ΔTBP) between dominant and the largest subordinate follicles. This positive correlation implies that in the dominant follicle, the lesser the binding proteins, the more bioavailable IGF-I are released to perform its functions. Because IGF-I is needed to activate IGFBP-4 dependent proteolytic activity of PAPP-A (Mazerbourg et al., 2001; Lawrence et al., 1999), perhaps increased free IGF-I also enhances proteolysis of IGFBP-4. In support of this latter suggestion, IGFBP-4 protein but not its mRNA was correlated most significantly with free IGF-I levels.

Calculated percentage free IGF-I in follicular fluid of preovulatory dominant follicles was 5.5-fold greater than in large subordinate follicles. The present study is the first to report the percentage free IGF-I in dominant preovulatory and large subordinate follicles. Calculated percentage free IGF-I in the blood of humans is less than 1% of the total IGF-I (Martin and Baxter, 1986). IGFBP increase the metabolic half-life of IGF-I and-II (Kostecka and Blahovec, 2002). Intrafollicular degradation of these IGFBPs likely amplifies the percentage of free IGF-I in follicular fluid to assure its biological effect on granulosa cells of the follicle. However, it is still not clear how much IGF-I from the circulation contributes to the total IGF-I found in the follicle.

Total IGF-I on the other hand, increased in preovulatory dominant and decreased in subordinate follicles, between 24 and 48 h. Previously, total IGF-I concentrations did not significantly change between day 5 and 10 of the estrous cycle of the first follicular wave or differ between dominant and subordinate follicles in cattle (Stewart et al., 1996). In the present study, it may be possible that diffusion of IGF-I from the blood to the follicle varies between follicle types during preovulatory follicular development when

LH concentrations are increasing. The latter statement is supported by observations in cows where the highest blood flow velocity in preovulatory follicles occurs during increasing plasma LH concentrations, whereas, atretic follicles lack detectable blood flow (Acosta et al., 2003). In sheep (Brown and Driancourt, 1989) it was reported that reduced capillary blood flow rates occur as follicular atresia progresses. Also, in cows (Jiang et al., 2003) and pigs (Jiang et al., 2002), dramatic capillary growth occurs during preovulatory follicular growth. Vascular corrosion casts from bovine ovaries containing estrogenic and non-estrogenic follicles were observed using scanning electron microscopy (SEM), and results showed that dominant follicles had a rich outer layer of normal and dilated capillaries with different angionenic structures (budding, splitting, sprouting from former blood vessels), while atretic follicles (having low estrogen:progesterone ratios) were seen to have degenerative plexuses in the theca interna (Jiang et al., 2003). The increase and decrease of total IGF-I concentrations in the dominant and subordinate follicles, respectively, with time follows the same pattern (although not significant) as that of IGFBP-3 levels in the study, further supporting the notion that the changes in IGFBP-3 and total IGF-I in follicular fluid are due to changes in blood flow and (or) follicle permeability to blood-born factors as previously suggested (Echternkamp et al., 1994). This latter statement is further supported by the fact that granulosa cell IGFBP-3 mRNA did not differ among follicle types and time, or correlate with its protein levels.

As previously reported for cattle (de la Sota et al., 1996; Stewart et al., 1996; Mihm et al., 2000; Austin et al., 2001; Fortune et al., 2001; Spicer et al., 2001; Rivera and Fortune, 2003a), dominant follicles had lower binding activity of IGFBP-2, -4, and –

5 than in subordinate follicles. These changes in binding activity or levels of follicular fluid IGFBPs may be due to decreased mRNA levels, increased proteolytic activity, or both. The present study is the first to report, using quantitative real time RT-PCR, that IGFBP-2 mRNA levels in granulosa cells differ between dominant and subordinate follicles. A significant positive relationship tended to exist between IGFBP-2 protein and its mRNA. A previous study in cattle using in situ hybridization (Yuan et al., 1998) reported that in early (9 mm) and mid dominant (16 mm) follicles, granulosa cell IGFBP-2 mRNA was nearly undetectable as compared to subordinate follicles (5.3-5.6 mm). Because IGFBP-2 proteolysis in preovulatory dominant follicles is significantly less than in subordinate follicles (Spicer et al., 2001) and proteolysis of IGFBP-2 was much less sensitive than IGFBP-4 to PAPP-A (Monget et al., 2003), regulated changes in IGFBP-2 mRNA expression may be the major cause of decreased IGFBP-2 protein levels in dominant follicles of cattle.

In the present study, while follicular levels of IGFBP-4 mRNA did not differ, IGFBP-4 protein were 3-fold less in preovulatory dominant than subordinate follicles. Less IGFBP-4 protein levels in dominant than subordinate follicles agrees with previous reports in cattle (Stewart et al., 1996; Mihm et al., 2000; Austin et al., 2001; Spicer et al., 2001). IGFBP-4 mRNA was detected in bovine (Roberts and Echternkamp, 2003) and ovine (Besnard, et al., 1996) granulosa cells of healthy follicles. No significant change in IGFBP-4 mRNA was seen in granulosa cells of follicles having 0.5 to >180 ng/mL estradiol (Schams et al., 2002). Proteolysis of IGFBP-4 is more evident in the dominant (largest) rather than in subordinate follicles (Spicer et al., 2001; Rivera et al., 2001). Proteolysis of IGFBP-4 by follicular fluid has also been reported for pigs (Besnard et al.,

1997), sheep (Besnard et al., 1996), and horses (Bridges et al., 2002). Because no significant correlation existed between the IGFBP-4 protein and its mRNA expression, and levels of IGFBP-4 mRNA did not differ follicle types, it is likely that decreased levels of IGFBP-4 protein in the follicular fluid may be attributed more to increased proteolysis rather than due to regulated IGFBP-4 mRNA expression in granulosa cells.

Averaged across time, follicular fluid IGFBP-5 mRNA and protein levels in subordinate follicles were 13-fold and 4-fold greater, respectively, than in dominant follicles. Greater IGFBP-5 protein levels in subordinate than dominant follicles agrees with previous reports in cattle (Stewart et al., 1996; Rivera et al., 2001; Spicer et al., 2001). The few studies conducted in other species indicate that IGFBP-5 mRNA is absent in dominant follicles in rats (Erickson et al., 1992) and is dramatically increased in granulosa cells of atretic follicles in sheep (Besnard et al., 1996). For the first time in cattle, IGFBP-5 protein was shown to be positively correlated with its mRNA fold expression, which supports the idea that increased levels of IGFBP-5 in the follicular fluid of subordinate follicles is due to the upregulation of its mRNA. However, greater IGFBP-5 proteolytic activity exists in ovulatory and nonovulatory dominant as compared to subordinate follicles in cattle (Spicer et al., 2001; Rivera and Fortune, 2003b) and horses (Bridges et al., 2002). These latter observations indicate that the lower levels of IGFBP-5 in dominant follicles may be due to both increased proteolysis (Spicer et al., 2001; Rivera and Fortune, 2003b) as well as decreased gene expression (present study). Levels of granulosa cell PAPP-A mRNA were not significantly different between dominant and subordinate follicles, a finding not previously reported in any species. In PMSG-induced immature mice (Hourvitz et al., 2002), a time-dependent 4.8-fold

increase in whole ovarian PAPP-A mRNA levels occurred between 0 and 24 h post PMSG injection; PAPP-A mRNA levels became undetectable by 48 h post PMSG. Thus, it is also possible that PAPP-A mRNA is acutely expressed, and therefore changes have occurred prior to sample collection in the present study. Alternatively, changes in PAPP-A activity (e.g., increased in IGFBP-4 protease) may occur without changes in PAPP-A mRNA levels. In support of the latter suggestion, Mazerbourg et al., (2001) showed that PAPP-A mRNA expression is detectable in granulosa cells of both healthy and atretic follicles, although no proteolytic activity degrading IGFBP-4 was observed in the latter follicle type. The significant negative correlation between estradiol and IGFBP-4 protein (but not mRNA), and positive correlation between progesterone and IGFBP-4 protein (but not mRNA) suggest these steroids may regulate proteolytic activity of PAPP-A. Alternatively, because PAPP-A mRNA fold expression also showed no correlation with the IGFBPs, steroids or IGF-I in the present study, suggests that PAPP-A may not be the only protease involved in IGFBP degradation. Future research will be required to determine if proteolytic activity of PAPP-A is under hormonal regulation, and to determine if proteases like kallikreins (Geisert et al., 2001), known to cleave IGFBP regulate ovarian IGFBP levels.

LITERATURE CITED

Acosta, T.J., K.G. Hayashi, M. Ohtani, and A. Miyamoto. 2003. Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. Reproduction 125: 759-767.

Austin, E.J., M. Mihm, A.C.O. Evans, P.G. Knight, J.L.H. Ireland, J.J. Ireland and

J.F. Roche. 2001. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. Biol Reprod 64: 839:848.

Beg, M.A., D.R. Bergfelt, K. Kot, M.C. Wiltbank, and O.J. Ginther. 2001. Follicular fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. Biol Reprod 64: 432-441.

Beg, M.A., D.R. Bergfelt, K.Kot, O.J. Ginther. 2002. Follicle selection in cattle: dynamics of follicular fluid factors during development of follicle dominance. Biol Reprod 66: 120-6.

Besnard N., C. Pisselet, D. Monniaux, A. Locatelli, F. Benne, F. Gasser, F. Hatey, and P. Monget. 1996. Expression of messenger ribonucleic acids of insulin-like growth factor bindin protein –2,-4, -5 in the ovine ovary: localization and changes during growth and atresia of antral follicles. Biol Reprod 55: 1356-1367.

Besnard, N., C. Pisselet, D. Monniaux, P. Monget. 1997. Proteolytic activity degrading insulin-like growth factor binding protein-2,-3,-4, and –5 in healthy growing and atretic follicles in the pig ovary. Biol Reprod 56: 1050-1058.

Bridges, T.S., T.R. Davidson, C.S. Chamberlain, R.D. Geisert, and L.J. Spicer. 2002. Changes in follicular fluid steroids, insulin-like growth factors (IGF) and IGF-binding proteins concentration, and proteolytic activity during equine follicular development. J Anim Sci 80: 179-190.

Brown, B.W., and M.A. Driancourt. 1989. Blood flow in the ovaries and ovarian follicles of Romanov and Prealpes-de-Sud ewes. J. Reprod Fert 85: 317-323.

Chun, S.Y., Billig, H., Tilly J.L., Furuta, I., Tsafriri A., Hsueh A.J. 1994. Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor I. Endocrinology 135: 1845-53.

Conover, C.A., G.F. Faessen, K. Ilg, Y.A. Chandrasekher, M. Christiansen, M.T. Overgaard, C. Oxvig, L.C. Giudice. 2001. Pregnancy-associated plasma protein-A is the insulin-like growth factor binding protein-4 secreted by human ovarian granulosa cells and is a marker of dominant follicle selection and the corpus luteum. Endocrinology 142: 2155-2158.

de la Sota, R.L., F.A. Simmen, T. Diaz, and W.W. Tatcher. 1996. Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. Biol Reprod 55: 803-812.

Echternkamp, S.E., L.J. Spicer, K.E. Gregory, S.F. Canning, J.M. Hammond. 1990. Concentrations of insulin-like growth factor-I in blood and ovarian follicular fluid of cattle selected for twins. Biol Reprod 43: 8-14.

Echternkamp, SE, H.J. Howard, A.J. Roberts, J. Grizzle, and T. Wise. 1994. Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. *Biol Reprod* 51: 971-981.

Erickson, G.F., A. Nakatani, N. Ling, S. Shimasaki. 1992. Cyclic changes in insulin-like growth factor-binding protein-4 messenger ribonucleic acid in the rat ovary. Endocrinology 130:625-636.

Fortune, J.E., G.M. Rivera, A.C.O. Evans, and A.M.Turzillo. 2001. Differentiation of dominant versus subordinate follicles in cattle. Biol Reprod 65:648-654.

Geisert, R.D., C.S. Chamberlain, K.A. Vonnahme, J.R. Malayer, and L.J. Spicer. 2001. Possible role of kallikrein in proteolysis of insulin-like growth factor binding proteins during oestrus cycle and early pregnancy in pigs. Reproduction 121: 719-728.

Ginther, O.J., M.A. Beg, K. Kot, C. Meira, and D.R. Bergfelt. 2003. Associated and independent comparisons between the two largest follicles preceding follicle deviation in cattle. Biol Reprod 68: 524-529.

Hourvitz A., A. Kuwahara, J.D. Hennebold, A.B.Tavares, H. Negishi, T.H.Lee, G.F. Erickson, and E.Y. Adashi. 2002. The regulated expression of the pregnancy associated plasma protein-A in the rodent ovary: a proposed role in the development of dominant follicles and of corpora lutea. Endocrinology 143: 1833-44.

Ireland, J.J., and J.F. Roche. 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. Endocrinology 111: 2077-86.

Jiang, J.Y., G. Macchiarelli, K. Miyabayashi, and E. Sato. 2002. Follicular microvasculature in the porcine ovary. Cell Tissue Res 310: 93-101.

Jiang, J.Y., G. Macchiarelli, B.K. Tsang, and E.Sato. 2003. Capillary angiogenesis and degeneration in bovine ovarian antral follicles. Reproduction 125: 211-223.

Kostecka, Z., and J. Blahovec. 2002. Animal insulin-like growth factor binding proteins and their biological functions. Vet. Med. – Czech 47(2-3): 75-84.

Lawrence J.B., C. Oxvig, M.T. Overgaard, L.S-Jensen, G.J. Gleich, L.G. Hays, J.R. Yates III, and C.A. Conover. 1999. The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A. Proc. Natl. Acad. Sci USA 96: 3149-3153.

Liu, J. A.T. Koenigsfeld, T.C. Cantley, C.K. Boyd, Y. Kobayashi, and M.C. Lucy.

2000. Growth and the initiation of steroidogenesis in porcine follicles are associated with unique patterns of gene expression for individual components of the ovarian insulin-like growth factor system. Biol Reprod 63: 942-952.

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402-408.

Martin, J.L. and R.C. Baxter. 1986. Insulin-like growth factor-binding protein from human plasma. Purification and characterization. J Biol Chem 261:8754.

Mazerbourg, S., J.Zapf, R.S. Bar, D.R. Brigstock, C. Lalou, M. Binoux, and P. Monget. 1999. Insulin-like growth factor binding protein-4 proteolytic degradation in ovine preovulatory follicles: studies underlying mechanisms. Endocrinology 140: 4175-4184.

Mazerbourg, S, M.T. Overgaard, C.Oxvig, M. Christiansen, C.A. Conover, M. Christiansen, C.A. Conover, I. Laurendeau, M. Vidaud, G.Tosser-Klopp, J. Zapf, and P. Monget. 2001. Pregnancy-associated plasma protein-A (PAPP-A) in ovine, bovine, porcine, and equine ovarian follicles: involvement in IGF binding protein-4 proteolytic degradation and mRNA expression during follicular development. Endocrinology 142: 5243-5253.

Mazerbourg, S., C.A. Bondy, J. Zhou, and P. Monget. 2003. The IGF system: a key determinant role in the growth and selection of ovarian follicles? A comparative study. Reprod Dom Anim 38: 247-258.

Mihm, M. , E.J. Austin, T.E.M. Good, J.L.H. Ireland, P.G. Knight, J.F. Roche, and J.J. Ireland. 2000. Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers. Biol Reprod 63: 811-819.

Mihm, M. , M.A. Crowe, P.G. Knight, and E.J. Austin. 2002. Follicle wave growth in cattle. Reprod Dom Anim 37: 191-200.

Monget, P., S. Fabre, P.Mulsant, F. Lecerf, J.M. Elsen, S. Mazerbourg, C. Pisselet, D. Monniaux. 2002. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. Domest Anim Endocrinol 23: 139-154.

Monget P., S. Mazerbourg, T. Delpuech, M.C. Maurel, S. Maniere, J. Zapf, G. Lalmanach, C. Oxvig, and M.T. Overgaard. 2003. Pregnancy associated plasma protein is involved in insulin-like growth factor binding protein-2 (IGFBP-2) proteolytic degradation in bovine and porcine preovulatory follicles: identification of cleavage site and characterization of IGFBP-2 degradation. Biol Reprod 68: 77-86.

Ott, L. 1977. An introduction to statistical methods and data analysis. North Scituate, MA, Duxbury Press, p384

Rivera, G.M., Y.A. Chandrasekher, A.C.O.Evans, L.C. Giudice, and J.E. Fortune. 2001. A potential role for insulin-like growth factor binding protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle. Biol Reprod 65: 102-111.

Rivera, G.M. and J.E. Fortune. 2003a. Proteolysis of insulin-like growth factor binding protein -4 and -5 in bovine follicular fluid: implications for ovarian follicular selection and dominance. Endocrinology 14: 2977-2987

Rivera, G.M. and J.E. Fortune. 2003b. Selection of dominant follicle and insulin-like growth factor (IGF)-binding proteins: evidence that pregnancy-associated plasma protein A contributes to proteolysis of IGF-binding protein 5 in bovine follicular fluid. Endocrinology 144: 437-446.

Spicer, L.J, J.J. Ireland, and J.F. Roche. 1981. Changes in serum LH, progesterone, and specific binding of ^{125}I-hCG to luteal cells during regression and development of bovine corpora lutea. Biol Reprod 25: 832-41.

Spicer L.J., and W.J. Enright. 1991. Concentrations of insulin-like growth factor I and Steroids in follicular fluid of preovulatory bovine ovarian follicles: effect of daily injections of a growth hormone-releasing factor analog and(or) thyrotropin-releasing hormone. J Anim Sci 69: 1133-9.

Spicer, L.J. and S.E. Echternkamp. 1995. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Domest Anim Endocrinol 12: 233-245.

Spicer, L.J., R.E. Stewart, P. Alvarez, C.C. Francisco, B.E. Keefer. 1997. Insulin-like growth factor-binding protein-2 and –3: their biological effects in bovine thecal cells. Biol Reprod 56: 1458-65.

Spicer, L.J. and C.S. Chamberlain. 1999. Insulin-like growth factor binding protein-3: its biological effect on bovine granulosa cells. Domest Anim Endocrinol 16:19-29.

Spicer, L.J., C.S. Chamberlain, G.L. Morgan. 2001. Proteolysis of insulin-like growth factor binding proteins during preovulatory follicular development in cattle. Domest Anim Endocrinol 21: 1-15

Spicer, L.J. 2004. Proteolytic degradation of insulin-like growth factor binding proteins by ovarian follicles: a control mechanism for selection of dominant follicles. Biol Reprod (In press).

Stewart, R.E., L.J. Spicer, T.D. Hamilton, and B.E. Keefer. 1995. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone induced steroidogenesis of bovine thecal cells: involvement of glucose

and receptors for insulin-like growth factor I and luteinizing hormone. J Anim Sci 73: 3719-3731.

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

Wandji, S.A., T.L. Wood, J. Crawford, S.W. Levison, and J.M. Hammond. 1998. Expression of mouse ovarian insulin growth factor system components during follicular development and atresia. Endocrinology 139: 5205-5214.

Yuan, W., B. Bao, H.A. Garverick, R.S. Youngquist, and M.C. Lucy. 1998. Follicular dominance in cattle is associated with divergent patterns of ovarian gene expression for insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein-2 in dominant and subordinate follicles. Domest Anim Endocrinol 15: 55-63.

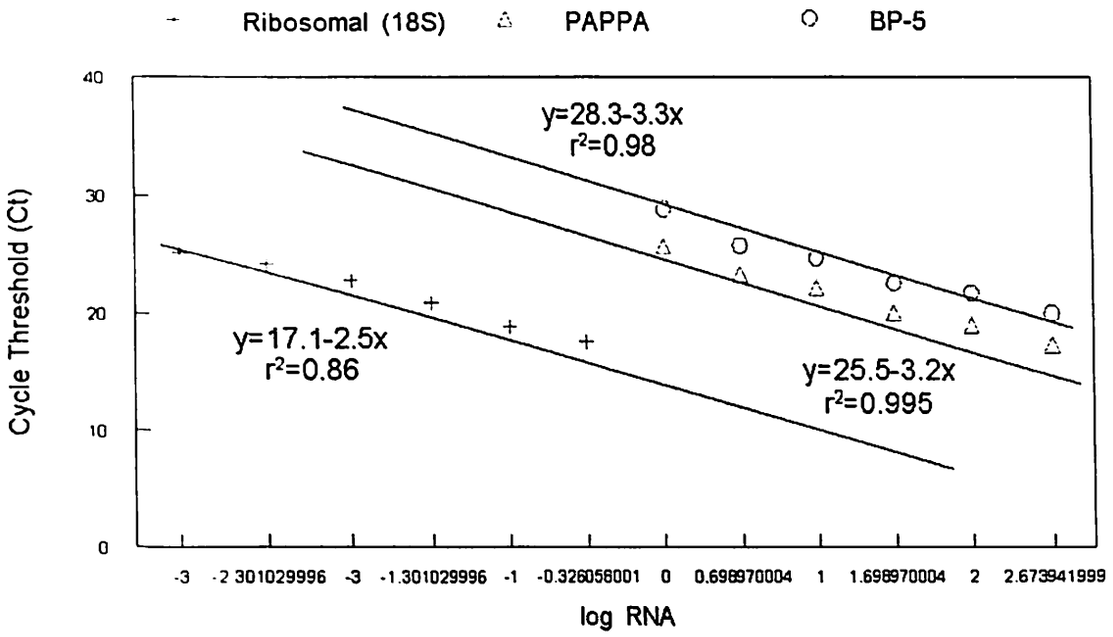

Figure 1. Parallelism between PAPP-A, IGFBP-5 and 18S rRNA. Different concentrations of the target (PAPP-A and IGFBP-5) and the normalizer gene (18S rRNA) were determined if fold changes in the target and normalizer gene are amplifying in a parallel fashion.

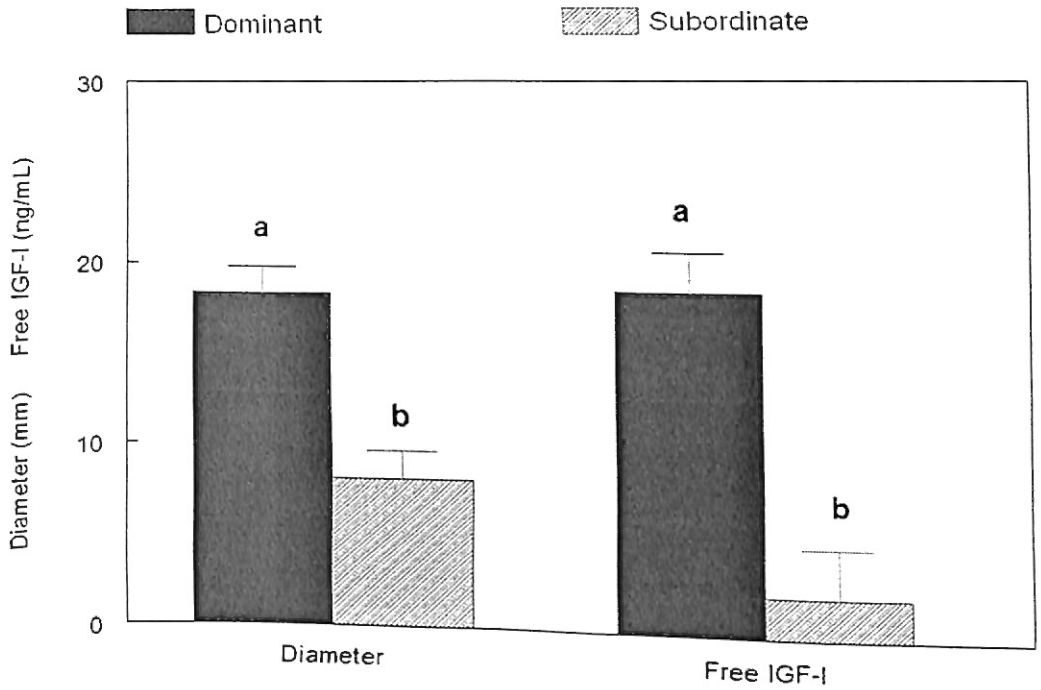

Figure 2. Mean diameters and Free IGF-I concentrations of dominant and subordinate follicles across time. [a,b] Within a variable, means differ ($P<0.05$).

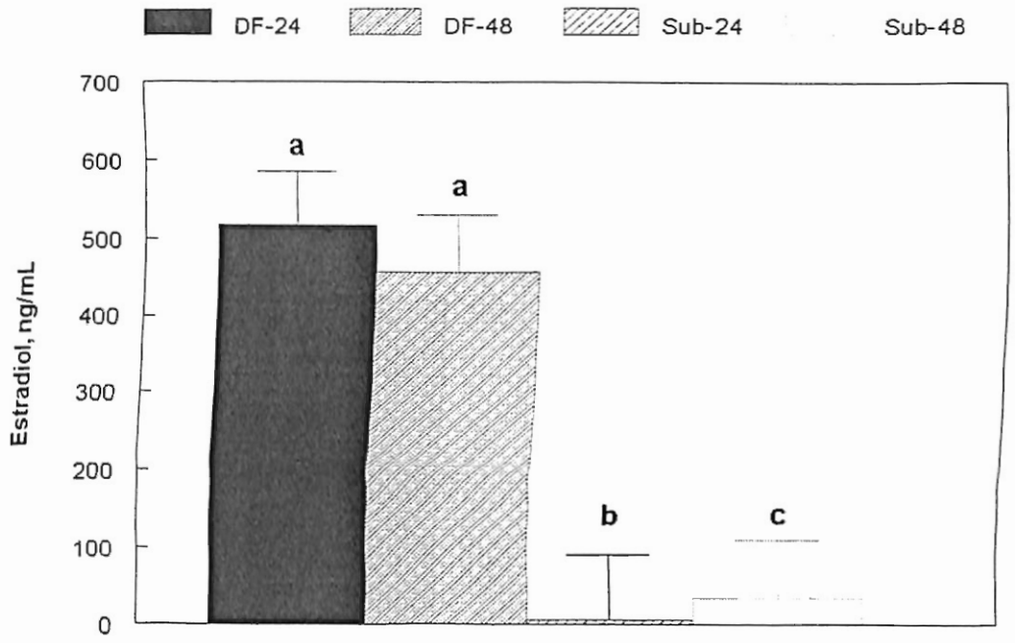

Figure 3. Estradiol concentrations in dominant and subordinate follicles collected at 24 or 48 h post PGF-2α. [a,b,c] Within treatment means differ ($P<0.05$).

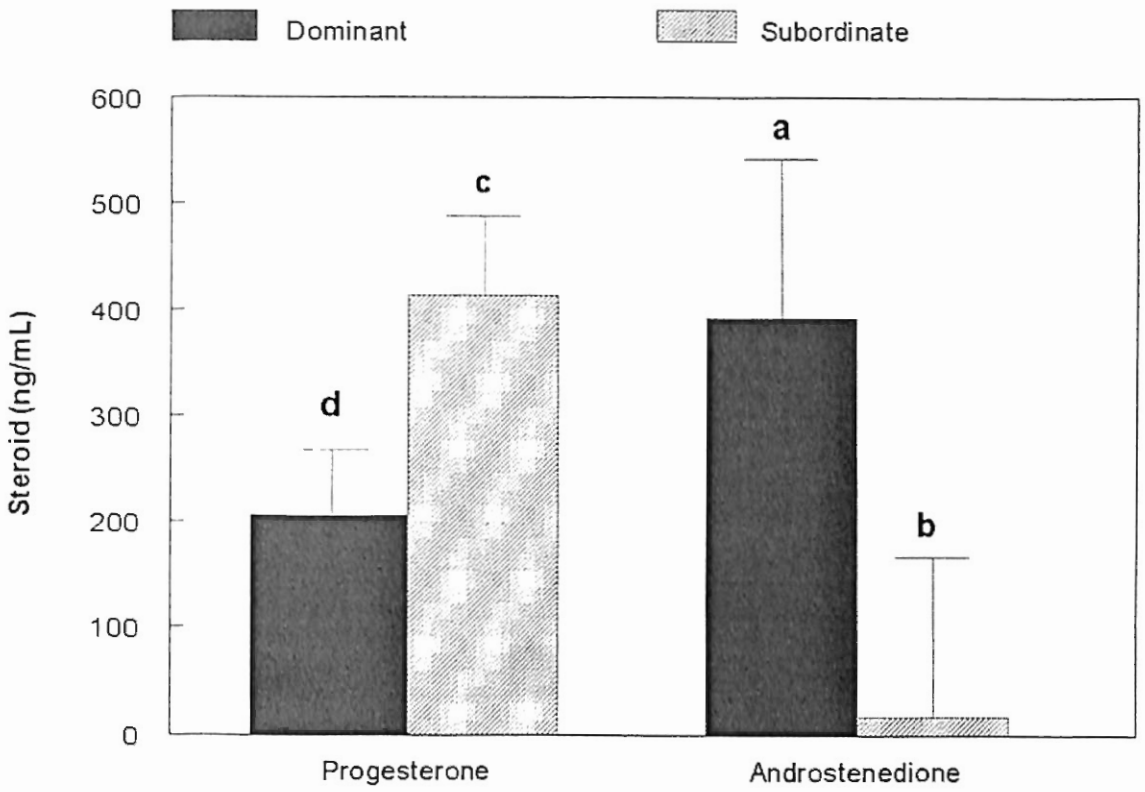

Figure 4. Progesterone and androstenedione concentrations of dominant and subordinate follicles averaged across time. [a,b] Within follicular fluid androstenedione, means differ ($P<0.05$). [c,d] Within follicular fluid progesterone, means differ ($P<0.08$).

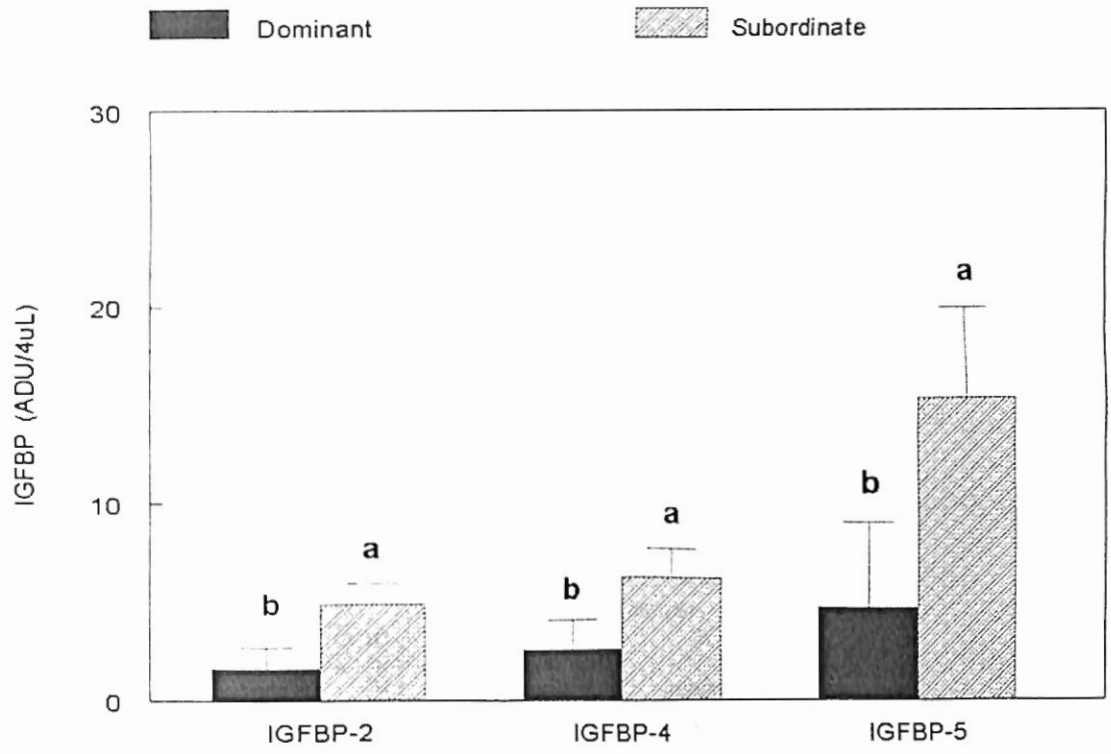

Figure 5. Binding activities of IGFBP-2, IGFBP-4 (total) and IGFBP-5 in follicular fluid of dominant and subordinate follicles. [a,b] Within a specific IGFBP, means differ ($P<0.05$).

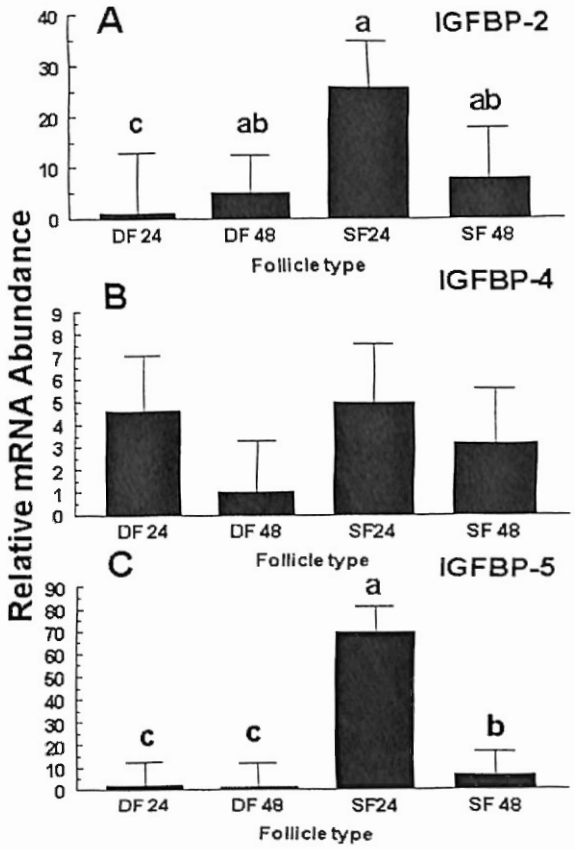

Figure 6. Relative mRNA abundance of IGFBP-2 (panel A), IGFBP-4 (panel B) and IGFBP-5 (panel C) in granulosa cells of dominant (DF) and subordinate (SF) follicles collected 24 or 48 h after PGF2α. [a,b,c] Means without a common superscript differ (Panel A, $P<0.10$; Panel C, $P<0.06$).

Table 2. Quantitative real-time PCR analysis of IGFBP-2,-3,-4, -5 and PAPP-A (PAP) mRNA expression in dominant and subordinate follicles collected at 24 or 48 h post PGF2α.

	BP-2 Ct	18S Ct	ΔCt	BP3 Ct	18S Ct	ΔCt	BP4 Ct	18S Ct	ΔCt	BP5 Ct	18S Ct	ΔCt	PAP Ct	18S Ct	ΔCt
Dom	27.7	18.8	8.7[a]	23.2	18.7	4.7	25.3	18.4	7.0	27.5	17.8	9.6[a]	22.5	18.0	4.5
Sub	25.3	19.8	5.3[b]	23.1	18.9	4.2	25.7	19.0	6.9	23.9	18.3	5.5[b]	24.2	19.0	5.3
24	26.7	19.1	7.4	22.0	18.6	3.8	24.6	18.6	6.1	24.1	17.8	6.2[b]	22.6	17.9	4.6
48	26.2	19.5	6.6	24.3	19.1	5.1	26.4	18.8	7.7	27.2	18.3	9.0[a]	24	18.8	5.1
D-24	28.8	18.4	10.5[a]	21.8	18.5	3.5	24.5	18.5	6.1	26.7	17.8	8.8	22.1	18.0	4.2
D-48	26.3	19.2	7.0[ab]	24.8	19.0	5.9	26.0	18.2	8.0	28.2	17.8	10.3	22.7	18.0	4.7
S-24	24.5	19.8	4.2[b]	22.3	18.7	4.1	24.6	18.6	6.2	21.5	17.8	3.6	23.1	18.0	5.0
S-48	26.2	20.0	6.2[ab]	23.8	19.2	4.2	26.8	19.3	7.6	26.3	18.9	7.4	25.2	19.7	5.5

[a,b] means within a column and horizontal grouping without a common superscript differ ($P<0.01$)

Table 3. Pearson correlation coefficients among diameter, follicular fluid hormones and IGFBPs and mRNAs of IGFBPs and PAPP-A.

		Hormones					Protein					mRNA				
	Di	E_2	P_4	IGF_T	IGF_F	A_4	BP_2	BP_3	$BP_{4\,T}$	BP_5	BP_T	BP_{2m}	BP_{3m}	BP_{4m}	BP_{5m}	PAP
Di		.75**	-.05	.25	.67**	.54*	-.11	-.001	-.01	-.15	-.07	-.29	.08	-.03	-.36	-.15
E_2			-.42*	.20	.73**	.75**	-.55*	-.02	-.46*	-.56*	-.26	-.23	-.52	-.18	-.73**	.19
P_4				-.26	-.19	-.25	.27	.02	.57*	.34	.18	.61*	.27	.13	.50*	-.19
IGF_T					.33	.47*	.19	.05	-.31	.05	.02	.07	-.04	.10	.11	.10
IGF_F						.56*	-.53*	-.21	-.51*	-.52*	-.36	-.35	-.20	-.31	-.67*	.22
A_4							-.14	.23	-.14	-.21	.12	-.19	-.41	-.25	-.52*	.15
BP_2								.37*	.57*	.91**	.68**	.41	.31	.23	.75**	-.21
BP_3									.12	.28	.91**	-.07	.23	-.13	-.02	.11
$BP_{4\,T}$										.71**	.43*	.66	.06	.12	.54*	-.07
BP_5											.63**	.55*	.19	.29	.81**	.26
BP_T												.23	.30	-.01	.30	.01
BP_{2m}													-.04	.78	.64*	-.31
BP_{3m}														-.06	.17	-.02
BP_{4m}															.51*	-.09
BP_{5m}																-.28
PAP																

*P<0.05
**P<0.001

Abbreviations:
Di, Diameter; E_2, estradiol; P_4, progesterone; A_4, androstenedione; IGF_T, IGF-I Total; IGF_F, IGF-I Free; $BP4_T$; Sum of N-glycosylated and non glycosylated IGFBP-4 BP_T; Sum of all binding protein activity; $BP2_m$, BP2 mRNA ; $BP3_m$, BP3 mRNA; $BP4_m$, BP4 mRNA; $BP5_m$, BP5 mRNA; PAP, PAPP-A mRNA.

CHAPTER IV

SUMMARY AND CONCLUSION

How the dominant preovulatory follicle manages to grow and differentiate while the rest of the cohort follicles regress and die can be attributed at least in part to differences in follicular fluid contents and (or) mRNA levels of IGF binding proteins.

Overall, the present study determined that dominant preovulatory follicles had 1) greater concentrations of free IGF-I, estradiol, and androstenedione than in subordinate follicles; 2) lower binding activities of IGFBP-2, -4, and –5 than subordinate follicles; 3) less IGFBP-2 and –5 gene expression than in subordinate follicles, and 4) similar IGFBP--3,-4, and PAPP-A mRNA levels to those of subordinate follicles.

As more free IGF-I becomes available for growth and estradiol and androstenedione synthesis in the dominant follicles, low molecular weight IGF binding proteins are reduced via proteolysis (as in the case of IGFBP-4), decreased mRNA levels (as in the case of IGFBP-2), or both (as in the case of IGFBP-5). PAPP-A may be just one of the many proteases found in the follicular fluid capable of IGFBP degradation, because PAPP-A mRNA did not differ among follicle types, and was not correlated with any follicular fluid hormones. Further research should be conducted to determine possible involvement of other proteases in the development of the dominant preovulatory follicle, as well as determine if PAPP-A proteolytic activity is regulated by hormones.

VITA (1)

Consuelo Amor T. Santiago

Candidate for the Degree of

Master of Science

Thesis: RELATIONSHIPS AMONG GRANULOSA CELL INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND PREGNANCY-ASSOCIATED PLASMA PROTEIN-A mRNA EXPRESSION AND FOLLICULAR FLUID CONTENTS IN DOMINANT AND LARGE SUBORDINATE FOLLICLES OF PREOVULATORY CATTLE

Major Field: Animal Science

Biographical:

Personal Data: Born in Manila, Philippines on December 27, 1976, the only daughter of Dr. Benedicto and Amelia Santiago.

Education: Finished the degree of Bachelor of Science in Agriculture at the University of the Philippines, Los Banos, Laguna, Philippines in March, 1998. Graduated *cum laude*. Completed the requirements for the Master of Science degree with a Major in Animal Science at Oklahoma State University, Stillwater in May, 2004.

Experience: Worked at as a junior faculty member (Instructor II) at the University of the Philippines, Los Banos, November, 1998 to present.

Professional Membership: Philippine Society of Animal Science, Philippine Journal for Veterinary and Animal Science, Gamma Sigma Delta Honor Society of Agriculture, Phi Sigma Biological Science Honor Society, American Society of Animal Science and Sigma Xi.