2019

Investigation Of Transforming Growth Factor-Alpha And Its Potential Role In Promoting Ovarian Follicular Dominance

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INVESTIGATION OF TRANSFORMING GROWTH FACTOR-ALPHA AND ITS POTENTIAL ROLE IN PROMOTING OVARIAN FOLLICULAR DOMINANCE

A Thesis Presented

by

Allie Lundberg

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Science
Specializing in Animal Science

August, 2019

Defense Date: May 13, 2019
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ABSTRACT

Intraovarian growth factors play a vital role in influencing the fate of ovarian follicles. They affect proliferation versus apoptosis of granulosa cells (GCs), and can influence whether small antral follicles continue their growth or undergo atresia. Transforming Growth Factor-alpha (TGFα), an oocyte-derived growth factor, is thought to regulate granulosa cell function, yet has been largely overshadowed by current interest in TGF-beta superfamily members, such as bone morphogenetic proteins (BMPs) and anti-Mullerian hormone (AMH). In the current study, effects of TGFα on bovine GC proliferation, intracellular signaling and cytokine-induced apoptosis were evaluated. Briefly, all small antral follicles (3-5mm) from bovine ovaries were aspirated and the cells were initially plated in T25 flasks containing DMEM/F12 medium, 10% FBS, and antibiotic-antimycotic, and incubated at 37 degrees Celsius in 5% CO_2 for 3-4 days. Once confluent, the cells were sub-cultured to 96-, 12- or 6-well plates in serum-free conditions (insulin 100 ng/mL; transferrin 55 ng/mL; sodium selenite 6.7 pg/mL). 24-hour treatment of bovine GCs with TGFα (10 and 100ng/mL) stimulated cell proliferation compared to control ($p<0.05$; $n = 7$ ovary pairs). Cell proliferation was accompanied by a concomitant increase in mitogen-activated protein kinase (MAPK) signaling within 2 hours of treatment, as measured by phosphorylated ERK1/2 expression ($p<0.05$, $n = 3$ ovary pairs). These effects were entirely negated, however, by the MAPK inhibitor, U0126 (10uM, $p<0.05$). Additionally, pre-exposure of the bovine GCs to TGFα (100ng/mL) failed to prevent Fas Ligand (100ng/mL)-induced apoptosis, as determined by caspase 3/7 activity ($P<0.05$, $n = 7$ ovary pairs). Collectively, the results indicate TGFα stimulates proliferation of bovine GCs from small antral follicles via a MAPK/ERK-mediated mechanism, but this action alone fails to prevent apoptosis, suggesting TGFα may be incapable of promoting the persistence of follicles during the process of follicular selection and deviation.

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge Dr. David H. Townson. I am so grateful for your mentorship, patience, and thoughtful approach to both science and life. You have taught me to approach obstacles with intention, to "trust in the process," and to be reflective. I will hold these lessons close and utilize them far beyond my educational experiences. Also, for letting me spend so much time with Zoey and Bella in the snow.

To Dr. Nicole Jaskiewicz who has been an inspirational role model and peer. Your passion and dedication to research and science is radiant, and it has been such an honor to have worked so closely with you in the Townson Lab. I must also acknowledge the many undergraduate and graduate students, faculty, and staff of the Department of Animal and Veterinary Sciences with which I have had the great honor of working alongside. Terrill has given me a sense of family and a home in Vermont.

I would also like to acknowledge my thesis committee members: Dr. Feng-Qi Zhao, Dr. Catherine Combelles, and Dr. Douglas Johnson. I have greatly valued the input and contributions that each one of you have dedicated towards enhancing my development as a scientist. It has been so humbling to have had your guidance and support throughout my career as a master's student.

Lastly, to Gerrit. Your support and encouragement has been a constant bright spot through the highs and lows of these past three years. Thank you for being a sense of stability and for always reminding me to be the best version of myself.

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CHAPTER 1: REVIEW OF LITERATURE

1.1 Bovine Folliculogenesis: Historical Perspectives and Understanding

Bovine folliculogenesis is a physiological process during which ovarian follicles are recruited in cohorts and grow over the course of the estrous cycle. Each cohort initially grows synchronously, through the integration of systemic gonadotropins that initiate the responsiveness of intraovarian cell populations. Specifically, the actions of hypothalamic gonadotropin releasing hormone (GnRH) and its action on the anterior pituitary gland to stimulate the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH) which have direct stimulatory effects on ovarian follicles. As its name suggests, FSH is responsible for stimulating follicular growth and recruiting primary follicle cohorts, which begin to grow synchronously and produce estradiol. Ultimately, these activities result in the selection of a dominant follicle from the cohort, which then potentially results in ovulation. With the advancement of ultrasonograpy and molecular techniques, our understanding of the bovine estrous cycle and folliculogenesis has accelerated immensely. Researchers can now visualize follicular wave patterns in animals non-invasively and in real-time, which has established the phases of follicular development, and has connected them to temporal changes in systemic gonadotropins. Molecular approaches have facilitated characterization of intraovarian mechanisms which respond to circulating gonadotropins. Collectively, these activities support local ovarian dynamics in and among the theca and granulosa cells (GCs) of the follicle, as well as the oocyte itself. Some of the major discoveries contributing to our current understanding of folliculogenesis in animals are highlighted in the following sections.

The estrous cycle of cattle has been very well characterized, including the behavioral, hormonal, and mechanistic aspects associated with ovarian function in cows and heifers [1]–[6]. In 1960, Erkki Rajakoski characterized the timing of follicular events in the bovine ovary in great detail through the use of comparative methods and slaughterhouse specimens [7]. Rajakoski concluded that primordial follicles develop into antral follicles and then Graafian follicles, and that their growth is influenced by the mitotic activity of GCs [7]. He also hypothesized the existence of two follicular waves during the estrous cycle, in which two dominant follicles develop (attaining a diameter of 8-13mm), but the first dominant follicle of the first wave undergoes atresia and the second dominant follicle of the second wave ovulates [7]. The ovulated follicle forms the corpus luteum of the next estrous cycle, that then regresses around seventeen days post-ovulation [7]. Thus, the two-wave hypothesis of follicular growth during folliculogenesis in cattle was conceived, and it had a profound effect that guided research in this area for many years to follow. For instance, 20 years later, in 1984, Pierson and Ginther published observations about bovine ovarian function in which ovarian structures within the cow were visualized using transrectal ultrasonography [8]. Although the study was primarily descriptive and did not test an experimental hypothesis, it transformed research in reproductive physiology by establishing transrectal ultrasonographic techniques to accurately image follicles within bovine ovaries and sequentially monitor them over the course of the entire estrous cycle [8]. Using this technique, the authors visualized follicles as small as 2-3mm, noting that follicles and corpora lutea have distinct echogenic patterns which make them easily distinguishable and identifiable [8]. The ultrasound data assimilated in this study also further supported the two-wave hypothesis because the

authors noticed that during each estrous cycle, an anovulatory midcycle dominant follicle is evident [8].

The emergence of ultrasonography was a defining moment for this area of reproductive physiology research as many investigators began to appreciate the value of monitoring bovine ovarian follicular dynamics non-invasively and in real-time. Additionally, ultrasonography has utility to examine follicular dynamics during pregnancy without harming the fetus, which is something unattainable using slaughterhouse sample approaches [9]. The knowledge acquired using this tool facilitated targeted investigations of hypotheses regarding the specific pattern of follicular emergence and development; including the two wave hypothesis [7] and the hypothesis that follicular growth and atresia is a continuous process [10], [11]. In 1988, for example, it was discovered that two, three, and even four wave patterns of follicular development occur in cattle [12]–[14]. Additionally, these studies were among the first to show that growth rate of the largest (termed dominant) follicle does not differ among follicular waves, and that the ovulatory follicle is consistently the dominant follicle on the day of estrus (standing heat). Measurement of plasma progesterone and LH confirmed that these two hormones are associated with atresia and the onset of ovulation of the dominant follicle, respectively [12]. Sirois and Fortune [11] were among the first to track follicles individually over the course of an estrous cycle, and they reported that three- and even four-wave patterns of follicular growth were possible. Conversely, Ginther et al. [15] and Knopf et al. [16] favored the two-wave hypothesis, suggesting that differences in wave patterns observed among various studies was due to the different cattle populations in varying geographical regions. Although their assertion did not deny the presence of three

wave patterns, it pointed to more complicated impacts of genetic and environmental influences on folliculogenesis among different herds. At the time of these studies, there was still considerable debate about the wave hypothesis altogether, as some researchers suggested that follicular growth occurs in continuous phases [13]. Eventually, a timeline and characterization of the temporal differences between two and three follicular waves of development during the estrous cycle emerged [14], which provided great insight about the diameters of follicles and the size of the corpus luteum throughout the estrous cycle [14]. Overall, the studies of the 1980s established new terminology, such as "subordinate" and "dominant" to describe the temporal relationships (size, growth rate) among the follicles within the cohort or wave as a proxy to predict their ultimate physiological fate.

The concept of follicular waves in cattle and other mammalian species predominates to this day, with the general acceptance that the number of waves can vary among individuals [17]. In light of this, investigative focus shifted toward examining hormonal regulation and defining the phases of follicular development: recruitment, selection/deviation, and dominance. Additionally, researchers sought physiologic mechanisms that dictate subordinate and dominant follicular phenotypes [18], [19]. For instance, the emergence of a newly recruited follicular cohort, as visualized using ultrasonography, occurs 1-2 days following the peak of an FSH surge [20], [21]. Cauterization of the first dominant follicle of the first wave 3 days post ovulation relieves the suppression of FSH concentrations, which delays regression of subordinate follicles and hastens recruitment of the next follicular cohort [20]. These concepts highlight the fact that the dominant follicle (of any wave) is responsible for inhibiting subsequent rises

of FSH [14], and this effect is due to its increased secretion of estradiol and inhibin [22], which ultimately inhibit pituitary FSH through negative feedback mechanisms [23], [24].

The ability of the dominant follicle to diverge and become self-sustaining in its growth has been coined "follicle deviation". Follicle deviation has since been adopted to encompass the dynamics involved in selection of follicles during folliculogenesis. Interestingly, if the dominant follicle is experimentally ablated, the second largest follicle of the wave continues its growth and becomes the new dominant follicle of the cohort [25]. Moreover, any follicle of the cohort that is 5 mm or larger has the potential to become the dominant follicle [26]. Mechanistically, follicular deviation occurs in tandem with the establishment of basal concentrations of FSH following the FSH surge [27]. Particularly, lower concentration of FSH encourage and maintain granulosa cell proliferation and production of estradiol by the dominant follicle, whereas higher concentrations impair cell division and stimulate progesterone production [28]. Thus, growth of the dominant follicle requires diminishing concentrations of FSH as it approaches maturity [29]. The importance of relative size of the follicle in establishing its dominance is further underscored by the observation that both LH and FSH receptor expression remains relatively unchanged during selection of the dominant follicle [30], despite the waning concentrations of available gonadotropins. Follicular dominance is generally established at ≥8.5 mm, within an 8 hour window, and only once the follicle secretes sufficient amounts of inhibin to impair further FSH secretion [25], [31]. As the dominant follicle matures to become pre-ovulatory, it switches from FSH- to LH-responsive [32].

In recent years, research focus in folliculogenesis has shifted from the temporal and hormonal regulation at the systemic level to mechanisms that regulate intraovarian dynamics, specifically intracellular signal transduction pathways that influence cell fate. Many types of signaling molecules exist in the follicular fluid and are secreted by cells of the ovarian follicle: these include insulin-like growth factor-1 (IGF-1), members of the transforming growth factor (TGF) -β superfamily, fibroblast growth factor (FGF), and members of the epidermal growth factor (EGF) family ligands [33]. These pathways have multiple functions within the ovary that invoke cellular activities and influence cell fate in response to hormonal stimulation. The establishment cell culture methodology to sustain bovine ovarian cells *in vitro* [34]–[36] has helped to characterize key intraovarian factors involved. For instance, through the use of a theca cell culture system, investigators have found that TGFα (an EGF family ligand) increases cellular proliferation; while TGFβ stimulates progesterone production in the presence of estradiol [37]. This suggests an inverse relationship, regulated by TGF signaling, possibly exists to influence growth and differentiation of follicles. Oocyte-secreted factors of the TGFβ superfamily, such as bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), protect granulosa cells from apoptosis in cultures of cumulus-oocyte-complexes [38]. Hence, not only do somatic cells contribute to follicular mechanisms, but the oocyte itself likely has an important regulatory role in determining follicular dynamics and follicular fate. Initial insight about mechanisms possibly triggering follicular atresia has also been obtained using granulosa and theca cell culture systems. For instance, Porter et al. demonstrated that Fas antigen mRNA is expressed in granulosa cells of follicles, and that granulosa cells of dominant follicles exposed to the LH surge *in*

vivo are resistant to FasL-induced apoptosis [39]. From these observations, Porter et al. concluded that GCs from subordinate (atretic) follicles are more susceptible to FasL-induced killing than those of healthy dominant follicles, suggesting a fundamental difference between GCs of the follicular phenotypes, and LH-induced differentiation. Collectively, contributions such as these have informed current understanding about the regulation of bovine folliculogenesis and the potential cellular mechanisms by which hormones and growth factors mechanistically exert their effects.

Among the more intensely-studied growth factors associated with the bovine ovary are FGF, EGF and TGFβ. Bovine granulosa cells express a gene encoding for FGF, which is hypothesized to develop the vascular system of the theca layer of the follicle through paracrine actions [40]. In culture, FGF and EGF help maintain the bovine GC phenotype; whereas absence of these factors causes cells to quickly differentiate and lose GC characteristics [41], [42]. The effects and interactions of these local growth factors can be complex, often altering the actions of other growth factors on cultured follicular cells. For instance, TGFβ produced by thecal cells inhibits the proliferative effect of EGF on cultured bovine granulosa cells in a paracrine manner [43]. As the field of growth factor research expands, growth factors are now classified into family groups based on similar receptor-ligand interactions. For example, TGFα is a member of the EGF family of ligands. It is expressed in bovine theca cells [44], [45], yet stimulates the proliferation of both theca and GCs *in vitro* [45]. The works of Gospodarowicz et al., Skinner et al., and others has affirmed the complexity of growth factor interactions and ligand-induced intracellular mechanisms as they pertain to follicular development [46], [47]. As an

example, FGF and EGF in combination induce a robust, proliferative response in bovine granulosa cells; but they have no proliferative effect independently [48].

With a clear, demonstrated presence within the ovarian follicle, growth factors are plausible local regulators of gonadotropin effects on ovarian cell populations. Among the various families of growth factors characterized, for instance, TGFα and TGFβ share an inverse yet complementary relationship that impacts proliferation and steroidogenesis of bovine thecal cells. TGFα increases bovine theca cell proliferation while decreasing androstenedione production; whereas TGFβ inhibits proliferation and stimulates progesterone production [37]. In terms of bovine granulosa cells, IGF-1 increases estradiol secretion, principally by increasing expression of cytochrome P450 enzymes that promote aromatase and steroidogenic activity [49]. As insight is gained about the actions of these various growth factors, focus has shifted towards determining the signaling pathways that mediate these regulatory actions and determining how these factors influence follicular fate. Very little is known at this point, however, about how these mechanisms translate to modulation of bovine follicular dynamics.

During the past 60 years or more in which research of bovine follicular dynamics has been studied, great advances in technology (e.g., ultrasonography, immunoassays, and molecular techniques) have led to greater understanding of the growth, maturation, and death of follicles, ranging from the systemic to the cellular levels. Folliculogenesis is a process requiring many temporally-orchestrated ovarian events; the majority of which are regulated both systemically and molecularly. Historical hypotheses and experiments put forward have clearly defined the follicular phases, providing opportunities for future

scientific pursuits, as well as the potential to manipulate and improve fertility. Continued exploration of these phenomena at the cellular and molecular levels will provide new insight, and perhaps bring new avenues to benefit agricultural production systems and our understanding of the ovarian system in other monovulatory species.

1.2 Hormonal Regulation of Follicular Dynamics

The bovine estrous cycle is approximately 21 days in length and is regulated through the endocrine actions of hypothalamic, anterior pituitary, ovarian, and uterine secreted hormones [17], [50]. The major endocrine hormones involved in this regulation are GnRH, FSH, LH, progesterone, and prostaglandin (PGF2α). These hormones are upregulated and down regulated over the course of the estrous cycle in cattle in response to positive and negative feedback systems. GnRH is produced by the hypothalamus and stimulates the anterior pituitary to secrete gonadotropins, FSH and LH [51]. Studies first linking the pituitary gland to the reproductive organs in animals were reported in 1910, when partial removal of the pituitary gland compromised the gonadal function of dogs. The gonadal organs of dogs shrank and deteriorated, and the sexual development of puppies was interrupted [52]. In 1929, Zondek postulated that the secretions of the pituitary gland stimulate the gonads. He observed the timing of their secretion was temporally associated with follicular growth and ovulation [53], [54]. These secretions, originally known as 'Prolan A'(later FSH) and 'Prolan B'(later LH), coordinated the rhythm of ovarian events and regulated endometrial function [53]. FSH and prolactin were also detected in isolates of the anterior pituitary gland of cattle in 1935 [55]. Whereas these hormones ultimately became a large focus of human medical research and

infertility treatment in those days, manipulation of their expression to control estrus in production animals was also viewed as potentially advantageous. Thus, the combined effort of many researchers over the past 100 years has led to considerable understanding of the bovine estrous cycle, specifically the behavioral, ovarian, and associated physiologic hormonal changes of the cycle.

Initially, hormonal concentrations of reproductive molecules were difficult to study in cattle, but the development and refinement of techniques such as liquid-gas chromatography and competitive protein binding techniques changed all this. Researchers extracted hormones from circulating blood plasma, and determined their relative abundances over the course of the estrous cycle [50], [56]–[58]. Since these initial observations, the hormonal regulation of bovine folliculogenesis has been well characterized. As previously stated, folliculogenesis consists of three defined phases of development that are regulated by these hormonal fluctuations: recruitment, selection/deviation, and dominance.

Recruitment of ovarian follicles is defined by the emergence of a follicular cohort from a primordial state. A transient increase in anterior pituitary-derived FSH activates a new follicular wave by binding to its receptors on GCs of primordial follicles [20], [59], [60]. Experimentally, injections of GnRH can synchronize FSH surges, which subsequently stimulate follicular recruitment in cattle [21]. Anti-Müllerian hormone (AMH) is secreted by GCs of primordial follicles [61] and is a marker that predicts gonadotropin-responsive primordial follicles [62], [63]. Thus, AMH potentially regulates follicular recruitment and FSH activation, ensuring that the entire ovarian reserve is not depleted in one follicular wave. FSH binding to granulosa cells stimulates proliferation

and aromatase activity, enabling the cells to synthesize estradiol [64]–[66] and inhibin [67], [68]. LH binds to receptors on theca cells and stimulates the conversion of cholesterol to androgen precursors for the granulosa cells to then convert to estradiol. These relationships establish an important coordination between the theca and granulosa cells in follicular development [69]–[72]. Estradiol secreted by the developing follicles asserts a positive feedback mechanism on the hypothalamus to enhance GnRH secretion. This effect triggers transient rises in FSH and LH, but FSH secretion is later suppressed by the negative feedback effects of follicular inhibin [28]. As the follicles reach 6 mm in diameter, a selection event occurs in which one follicle from the cohort continues to grow and increase in size while the remaining follicles regress and undergo atresia [73]. Ultimately, the largest follicle deviates from its subordinates and continues to grow (i.e., becomes dominant), becoming less reliant on FSH stimulation as it produces higher amounts of estradiol and inhibin [29].

Once the dominant follicle reaches ovulatory stage, its granulosa cells become LH responsive [74], [75] and estradiol secretion peaks. The estradiol positive feedback on the hypothalamic-pituitary axis generates a transient surge in LH secretion, which causes the dominant follicle to ovulate [76]–[78]. Following ovulation, both the theca and GCs of the ovulatory follicle luteinize to form the corpus luteum and begin to secrete progesterone [79], [80]. Although the corpus luteum is the dominant structure on the ovary during this time, follicular waves still emerge throughout the estrous cycle. However, the high concentrations of circulating progesterone prevents the LH surge necessary for ovulation [74], [81]. Dominant follicles of waves that emerge during this period of high progesterone secretion are unable to continue their growth and eventually

undergo atresia like their subordinates within the cohort. If fertilization and pregnancy is not established following ovulation, PGF2α secreted by the uterine endometrium exerts a luteolytic effect on the corpus luteum, causing tissue regression and the loss of progesterone production [82], [83].

1.3 Reproductive Challenges Associated with Dairy Cattle

Unique challenges exist in the reproductive management of dairy cattle. In order for dairy cows to remain productive and economically valuable, they must produce calves every year in order to meet the demands of profitable milk production. High producing dairy cattle often experience postpartum anestrus for a number of reasons [84], but the most notable reason is the partitioning of energy toward lactation rather than reproductive function [85]. Ideally, dairy cattle should become pregnant in early- to mid-lactation. However, at this point in time the cows are in negative energy balance metabolically and have insufficient energy reserves to support fertilization, let alone a fetus [86]. Energy is partitioned away from ovary function, thus negatively affecting follicular growth, ovulation, and overall estrous cyclicity [87]. The difficulty of attaining good fertility in dairy cattle is also complicated by the trend toward increased herd sizes. Many herds in the U.S. average 500 head or more in the milking group, complicating reproductive management strategies related to timed artificial insemination (AI) protocols that require specific and individual attention to detail [88]–[90]. A better understanding of the mechanisms influencing ovarian cyclicity, particularly at the cellular and molecular level, may offer insight to improve management strategies and timed AI protocols.

1.4 The Bovine Ovarian Follicle

The intraovarian environment of the follicle is composed of somatic cells (e.g., theca and GCs) and the oocyte that secrete a large variety of signaling molecules to coordinate processes during development and maturation. These somatic cells and the oocyte all have distinctive functions and secrete various autocrine and paracrine molecules that harmoniously impact the development and maturation of the follicle and its continued growth or atresia/regression. The two main somatic cell types of the ovarian follicle are GCs and theca cells. Theca cells are LH responsive and synthesize androgens for GC conversion to estrogens [69]–[71], [91]. Theca cells are also important in supporting angiogenesis of the follicle [92], [93] and antrum formation [94]. GCs are by far the most abundant cell type in the follicle and their proliferation in response to FSH is necessary for the initial growth of antral follicles (3-5 mm) [11], [95]. As mentioned above, GCs also have an important role in the conversion of androgens to estradiol in growing antral follicles [64], [70]. This compartmentalized aspect of steroidogenesis within the follicle is known as the "two cell, two gonadotropin hypothesis", where LH receptors and FSH receptors expressed on theca and GCs, respectively, and respond to the gonadotropins independently to collectively synthesize estradiol [96], [97]. The initial growth of primordial follicles is largely characterized by proliferation and formation of gap junctions between GCs, and the growth of the oocyte [98], [99]. Eventually, the zona pellucida surrounding the oocyte is formed, and the gap junctions extending from granulosa cells to the oocyte are formed. The gap junctions help to maintain the oocyte in meiotic arrest [98]. As the GCs proliferate and the follicle grows, estradiol secretion also

increases, exerting a positive feedback effect on the hypothalamus to stimulate GnRH release, an LH surge, and ultimately ovulation of the dominant follicle [31], [87]. Upon ovulation, the somatic cells of the follicle differentiate into luteal cells to form the corpus luteum and synthesize progesterone [80], [100].

1.5 Cellular Mechanisms of Ovarian Follicular Selection

Although the primary regulation of ovarian events is carried out by circulating gonadotropins and hormones, when it comes to follicular development and maturation in response to these hormones, local regulation within the microenvironment of individual follicles is imperative. Intrafollicular dynamics influence follicular growth by stimulating proliferation of GCs and thecal cells and by protecting the developing oocyte. Conversely, these same signaling factors can regulate follicular fate by triggering follicular atresia. Although multiple local signaling factors likely coordinate these follicular processes and influence follicular fate, IGF-1, through stimulation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway, is widely-believed to have the greatest impact on follicular fate and survival during follicular selection and deviation. IGF-1 was first suggested to have a regulatory role in the ovarian follicle based on its observed ability to stimulate cellular proliferation and differentiation in a wide variety of cell and tissue types [101]. Indeed, IGF-1 influences secretion of both progesterone [102] and estradiol [49], [103] by GCs. However, in the presence of LH, production of estradiol is attenuated [49], which suggests the stimulatory effect of IGF-1 on estradiol production by GCs is only relevant to small follicles [102] (i.e. before the follicles reach ovulatory stage). IGF-1 stimulates both mitogen activated protein

kinase/extracellular regulated kinase (MAPK/ERK) and PI3K/Akt signaling in GCs [104], [105]. However, only its effect on PI3K/Akt activation stimulates GC cell cycle progression to protect against FasL-induced apoptosis [105]. This observation has been put forth as a potential survival mechanim for GCs to protect against follicular atresia. IGF-1 also stimulates GC proliferation [103], [106], which supports an active mitotic environment for developing follicles. Interestingly, conditional knockdown of the IGF-1 receptor in GCs results in sterility, with an inability to develop antral follicles and a 90% decrease in estradiol production [107]. Thus, it is clear that IGF-1 plays a vital role in follicular growth.

Despite the previous assertion that IGF-1 stimulates both MAPK/ERK and PI3K/Akt signaling, the effect on MAPK is not consistent [49]. Moreover, when present in high concentrations, IGF-1 makes GCs more prone to apoptosis [49], contradicting the idea that IGF-1 alone supports follicular survival. Phosphorylated forms of both ERK and Akt (the downstream targets of MAPK and PI3K signaling, respectively) are upregulated in follicles destined to become dominant [108]. Both ERK and Akt activation in GC and theca cells by IGF-1 and gonadotropins stimulate cell growth and steroidogenic function [109]. Thus, although there is some uncertainty about the relative importance of MAPK/ERK activation in promoting GC function and follicular survival, it remains important to identify specific ligands that stimulate this pathway and evaluate their effects. Importantly, down regulation of ERK signaling is associated with apoptosis of GCs [110]. The EGF receptor ligands are prominent stimulators of MAPK signaling activity [111], therefore their effects are worthy of consideration when investigating the

fates of follicles. For instance, EGF and TGFα are hypothesized to prevent apoptotic cell death of GCs in ovulatory follicles [112].

1.5.1 Epidermal Growth Factor Family of Ligands

During the 1970s, growth factor discovery and classification was prominent; growth factors regulating cellular activity was a novel concept and researchers strove to improve cell culture systems through their use [113]–[115]. EGF was one of the first factors isolated and purified, and is a mitogenic factor in a number of different cells and tissues [116]. Growth factors of the EGF family of ligands are associated with tumorigenic activity, stimulating cellular growth and differentiation through receptor tyrosine kinase (RTK) signaling [117]. EGF family ligands include, but are not limited to, TGFα, heparin-binding EGF, amphiregulin, betacellulin, epiregulin, and epigen [118], [119]. These factors bind to a common receptor (EGFR), which is an RTK that heterodimerizes and is activated through autophosphorylation, and stimulates multiple signaling pathways within cells, including MAPK and PI3K [119]–[121]. MAPK activation through this receptor requires the recruitment of the Grb2 adaptor protein, and the formation of a Grb2/Sos complex to activate Ras; whereas PI3K signaling can be activated directly [122].

The molecule EGF, specifically, influences ovarian signaling and cellular regulatory mechanisms. For example, EGF stimulates GC proliferation in cells derived from large follicles, but once the cells luteinize the proliferative response is attenuated [123]. EGF also regulates steroidogenic functions, including the enhancement of progesterone production [46], which suggests it augments GC differentiation, possibly during post-ovulatory processes. In the pig, EGF is present in follicular fluid [124] and

promotes GC proliferation *in vitro* [106]. Furthermore, proliferation is synergized when combinations of TGFβ and IGF-1 are included in the treatments [106]. However, EGF was the only growth factor in the study to stimulate proliferation independently. TGFβ and IGF-1 failed to stimulate cell number and DNA synthesis [106], further supporting the concept that IGF-1 alone is insufficient to promote GC growth.

Since its original discovery and characterization in murine cells [125], [126] TGFα continues to be viewed as closely related to EGF, sharing a common membrane receptor. However, TGFα and EGF do not exert the same biological actions [127], and each has specific and unique functions in cellular activities. May and Shomberg speculated that TGFα and EGF have separate actions in ovarian function, with TGFα being more important in luteal formation due to its ability to stimulate angiogenesis and wound healing in other tissues [128]. Consistent with the observation that EGF induces progesterone production, TGFα prevents FSH-stimulated aromatase activity in rat granulosa cells [129]. Conversely, this same study demonstrated an augmentation of aromatase activity by TGFβ, and postulated that TGFβ may be the more influential transforming growth factor in the regulation of ovarian cyclicity [129]. TGFα is localized to the theca-interstitial cells of rat ovaries [130], and this is consistent with a study of the bovine ovary [44]. Together, these studies establish TGFα is theca-derived. A more recent study, however, indicates TGFα is also present in the oocytes of bovine follicles [131], which suggests multiple cellular sources of TGFα exist in the follicle.

In bovine GCs, TGFα inhibits the secretion of estradiol, inhibin A, activin A, and follistatin both independently and in the presence of FSH and IGF-1 stimulation [131].

Under these same conditions, TGFα also stimulates cellular proliferation [131], which suggests a modulatory role of TGFα during early follicular development. Others report that TGFα prevents spontaneous apoptosis of rat granulosa cells in culture [132]. Thus, TGFα exerts potent proliferative and somewhat protective effects in cultured GCs, which suggests a possible regulatory role in the selection and/or survival of the dominant follicle.

1.5.2 Transforming Growth Factor-alpha in the Bovine Ovary

Characterization of TGFα in the bovine follicle since the late 1980s and early 1990s is limited [47]. TGFα is secreted by bovine theca cells [44], [133] into the follicular fluid where it can act in a paracrine manner to decrease estradiol production by GCs from large follicles (>10 mm) [134]. Although it impairs bovine GC steroidogenesis, TGFα also stimulates cell proliferation [37], indicating that it may also prevent cellular differentiation. TGFα stimulates cumulus GC function, which in turn supports the maintenance of high quality oocytes [135]. No other literature about TGFα in the bovine follicle seemingly exists until the early 2000s, when Glister and colleagues localized TGFα to the bovine oocyte [131], and showed the paracrine action of TGFα to suppress estradiol and inhibin secretion by GCs in response to FSH and IGF-1 [131], [136]. These authors proposed that TGFα mediates the actions of oocytes on GCs. Additionally, this study initially established the novel and diverse functions of TGFα, as compared to EGF, by showing that EGF is undetectable in the same oocytes [131].

1.5.3 Mechanisms of Follicular Atresia and Apoptosis

The idea that cell death is an organized and regulated process through apoptotic signaling was first presented in the 1970s when Kerr et al. summarized evidence from multiple studies to characterize the process, and established it as a "basic biological phenomenon" that balances mitosis with cell turnover [137]. The process of programmed cell death occurs in two phases: the first entails condensation of the nucleus and cytoplasm, and their subsequent fragmentation into "apoptotic bodies", the second consists of phagocytosis of the apoptotic bodies by other surrounding cells. Based upon the criteria established by Kerr et al., the ovary soon became an attractive model to study these mechanisms due to its dynamic and constant cellular remodeling, particularly in the context of ovulation and luteal function. With regard to follicular dynamics, apoptosis is an extremely important process because the majority of follicles within the ovary fail to become dominant, and instead undergo atresia [138]. This newly defined apoptotic phenomenon was characterized in ovine follicles based upon observations of cellular and nuclear fragmentation, and the subsequent phagocytosis of these fragments [139] [140]. Granulosa cells of the follicle are among the most severely affected.

By the early 1990s, apoptosis became widely accepted as a common process by which follicular atresia ensued, and that granulosa cells were the main cell type targeted [112], [138], [141]–[143]. A shift from identifying the cell types undergoing apoptosis within the follicle to determining the mechanisms of atresia occurred. Several locally produced cytokines are associated with apoptotic events during follicular atresia. Among these, tumor necrosis factor-α (TNF-α) invokes apoptosis in cultured rat antral follicles and decreases aromatase activity in cultured granulosa cells [144]–[146]. TNF-α-induced

effects are mediated through the secondary messenger, ceramide [144]. Fas ligand (FasL), a member of the TNF family of ligands, is also thought to contribute to follicular atresia. Its receptor, Fas, is a member of the TNF family of cellular membrane receptors [147]. Abundant amounts of Fas expression are seen in cells of mouse ovaries [148], [149], in bovine theca and GCs [39], [150], and in hen GCs [151], particularly during episodes of follicular atresia. In cultured bovine GCs, FasL-induced apoptosis is enhanced by serum withdrawal [152], and is modulated by the presence of growth factors [153]. For these reasons, Fas is commonly considered the mediator of GC apoptosis and follicular atresia [154].

1.6 Hypotheses, Aims, and Objectives

In the current study, our working hypothesis was that growth factors, through increases in MAPK/ERK signaling, provide resistance to FasL-induced apoptosis in bovine GCs during folliculogenesis. Particularly, GCs fundamentally support the early growth of small ovarian follicles through their proliferation and cellular signaling. This proliferation is largely supported by the actions of intrafollicular endocrine molecules which act to stimulate and support GC proliferation, and, ultimately, follicular survival. The specific objective was to determine if an enhancement of ERK signaling in bovine GCs provides resistance to Fas-mediated apoptosis as a possible cellular mechanism for follicle persistence and selection.

The specific aims were to:

(1) Evaluate the effects of growth factor stimulation on MAPK/ERK signaling in bovine GCs,

(2) Reproduce the apoptotic effects of FasL on cultured bovine GCs, and

(3) Demonstrate that MAPK/ERK signaling mediates the protective effect of growth factors on FasL-induced apoptosis

CHAPTER 2: PRELIMINARY EXPERIMENTS

2.1 Introduction

To investigate the stated objective and aims, it was imperative to first survey and profile which growth factors stimulate bovine GCs in our unique, serum-free culture system established in the Townson laboratory. Many of the previous growth factor studies reported in the literature utilized culture systems in which GCs are isolated from follicles, pooled together from several animals, and then treated with growth factors in which the culture medium contains varying amounts of fetal bovine serum (FBS). In cell culture systems, FBS is utilized to support successful cellular growth, differentiation and maintenance in *in vitro* environments and has been used to promote cellular adhesion of GCs to culture flasks following isolation [155], [156]. Without question, FBS inherently contains of variety biomolecules and cell growth promoting constituents at undefined concentrations, which could confound effects of added growth factors. Conversely, in our culture model, the cells are maintained in a serum-free system in which the constituents of the culture medium are defined, and the effects of growth factors can be individually or collectively evaluated. Additionally, the cultures of GCs are established using ovary pairs collected from an individual cow (i.e., the cow is the experimental unit) to avoid any confounding effects attributable to pooling of cells from several animals. For these reasons, the preliminary experiments described herein were intended to evaluate the effects of several growth factors individually and collectively to ensure that any stimulatory effects were consistent with those previously reported in the literature.

Additionally, the purpose of this screening was to identify particular growth factors that induced a proliferative response mediated by MAPK/ERK stimulation.

2.2 Materials and Methods

2.2.1 Review of Literature

A review of the literature, summarized above, was completed to gain understanding and an introductory perspective about the various growth factors that exist and that potentially impact the ovarian follicular microenvironment. The growth factors that were identified as plausible candidates for the thesis work, and that were further discussed among the thesis committee members for further profiling were FGF, EGF, TGFα , TGFβ, and IGF-1. These growth factors were then tested in dose-response and time-course experiments to evaluate their efficacy in stimulating bovine GC proliferation, and as a lead-in to examining growth factor-induced MAPK/ERK signaling.

2.2.2 Cell Culture and Reagents

Pairs of bovine ovaries were obtained from Champlain Beef (Whitehall, NY) and transported to the laboratory in 0.9% NaCl solution supplemented with antibiotic-antimycotic at room temperature. All ovaries were collected and processed within 4-5 hours of slaughter. All small (3-5mm) antral follicles were aspirated to obtain bovine GCs. Small (3-5mm) follicles were selected due to follicular dominance not being achieved until a follicle has reached a diameter of 8-10mm, indicating that granulosa and follicular fate would not be decided at a small follicular size. The GCs obtained from each ovary pair were kept separate, rather than pooled, for the culture experiments. Hence, a given culture consisted of cells obtained from a single pair of ovaries, making an individual cow the experimental unit (n = 3). The retrieved cells were pelleted,

resuspended, and seeded into individual T25 flasks. Cells were cultured in Dulbecco's Modified Eagle Medium/Ham's F12 Nutrient Mixture (DMEM/F12) medium initially containing 10% FBS and antibiotic-antimycotic (complete culture medium) and incubated in a humidified incubator at 37°C with 5% CO_2 until the cultures reached confluency (~3-4 days). Once confluent, the cells were passaged, enumerated, and subsequently seeded into culture vessels for further testing. The day after seeding, the cultures were switched to serum-free DMEM/F12 medium supplemented with insulin-transferrin-selenium (ITS) (10 ng/ml insulin, 5.5 ng/ml transferrin, 0.67 pg/ml sodium selenite), without or with specific concentrations of growth factors. The growth factors tested were recombinant human epidermal growth factor (EGF; Gibco), recombinant human transforming growth factor-β1 (TGFβ; R&D Systems), recombinant human transforming growth factor-α (TGFα; R&D Systems), and human insulin-like growth factor-I (IGF-1; Cell Signaling Technology). The growth factors selected were determined based on materials used throughout previous studies reported in the literature.

2.2.3 Growth Factor Effects on Cellular Proliferation

Bovine GCs were seeded into clear-walled, 96-well plates (5,000 cells/well for individual growth factor tests and 10,000 cells/well for combined growth factor tests) using complete culture medium. The following day, the cells were switched to serum-free medium containing individual or combined concentrations of the growth factors (0, 1, 10, and 100 ng/mL) and incubated for 24 or 48 hours. Complete culture medium (containing FBS) was used as the positive control for proliferation, and staurosporine (a chemotherapeutic agent that induces apoptosis) was used as the negative control and

successfully abolished any proliferative effect of all treatments and killed the cells, detected both observationally and by no detectable level of MTS absorbance (data not shown). Following the time of treatment, relative proliferation of bovine GCs was assessed using an MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) in which the absorbance at 490 nm was measured and recorded using a BioTek Synergy HT plate reader (BioTek).

2.2.4 Growth Factor Effects on Cellular Proliferation

All experiments were conducted using a minimum of three biological replicates from an independent pair of ovaries (i.e., cow is the experimental unit). Data were analyzed by one-way analysis of variance (ANOVA), followed by a Student's T-Test. Differences among means were considered significant at $p<0.05$.

2.3 Results

2.3.1 Effects of Individual Growth Factors on Bovine Granulosa Cell Proliferation

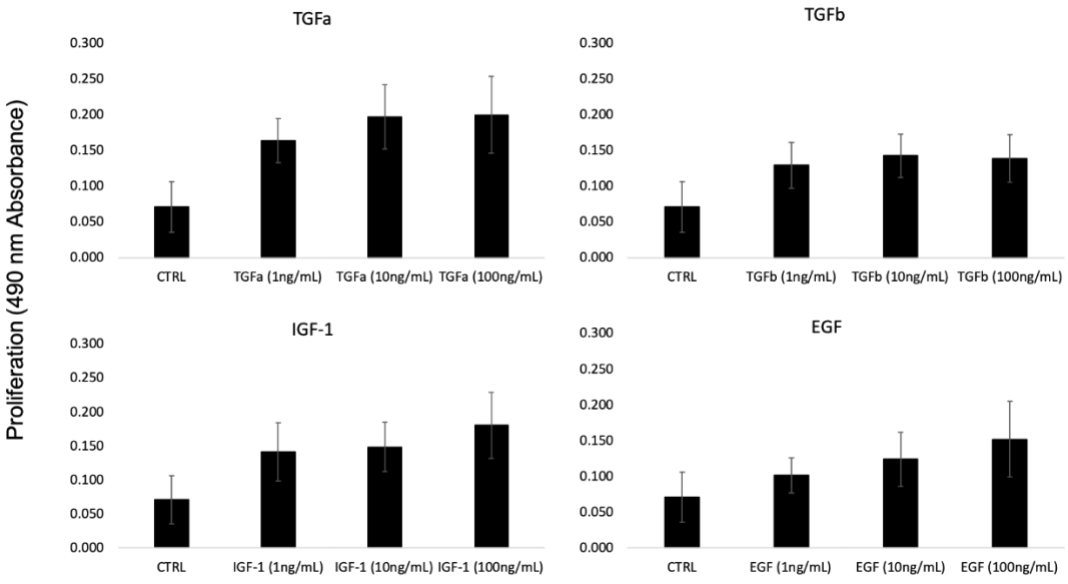

Figure 1. MTS Assay analysis of bovine granulosa cell proliferation in response to growth factor treatment (TGFα, TGFβ, IGF-1, and EGF). Bars represent means of recorded absorbance values at

490 nm ± SEM (n = 3 ovary pairs) for bovine granulosa cells exposed to different concentrations of each growth factor (0, 1, 10, or 100 ng/mL) for 24 hours . The same absorbance value patterns were seen after 48 hours of treatment, with the recorded values raised slightly.

All growth factors examined suggested an induced a proliferative response in comparison to control treatment (Figure 1). No statistical difference among treatments or doses was identified, but this was most likely attributed to the low number of ovary pairs utilized for the experiments. Among the 4 growth factors tested, TGFα was qualitatively the most potent, reaching a maximal response at 10 ng/mL (Figure 1). TGFβ and EGF were the least potent qualitatively (Figure 1), with EGF being the only growth factor to suggest a dose-dependent trend. Effects of IGF-1 treatment were intermediate to TGFα and the other growth factors (Figure 1). Following 48 hours of treatment, the same proliferation trend was observed, and all treatments showed a similar amount of increase in absorbance values (data not reported).

2.3.2 Combined Growth Factor Effects on Bovine Granulosa Cell Proliferation

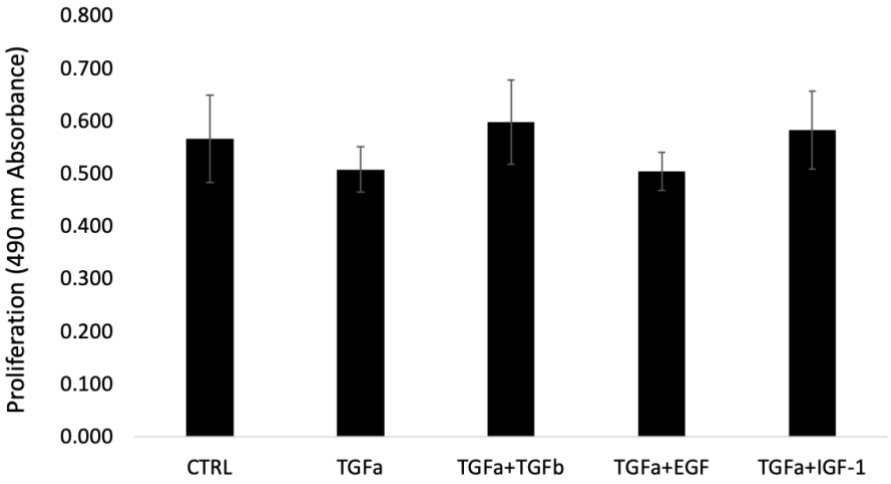

Figure 2. MTS Assay analysis of bovine granulosa cell proliferation in response to growth factor treatment combinations including TGFα. Bars represent means of recorded absorbance values at 490 nm ± SEM (n = 3) for bovine granulosa cells exposed to a 10 ng/mL concentration of each growth factor combination for 24 hours.

All possible treatment combinations of the growth factors were evaluated at multiple doses (0, 1, 10, and 100 ng/mL). However, for ease of interpretation, only the growth factor combinations that included TGFα at the 10 ng/mL dose are depicted here. Overall, and unexpectedly, the proliferative responses here were not as robust as in previous experiments (Compare Figures 1 and 2). This was attributed to higher seeding densities (10,000 cells/well) established in these experiments, which presumably predisposed the cells to premature contact inhibition. Nevertheless, TGFα, when combined with either TGFβ or IGF-1, somewhat augmented the proliferative response, but overall there were no statistical differences among the various treatment groups

2.4 Discussion

Based upon the observed effects of growth factor stimulation on bovine GC proliferation, TGFα was selected as the candidate stimulator of proliferation among the four growth factors tested. Seeding methods between the two experiments (individual and combined growth factor experiments) differed and were inconsistent because the investigator had not yet developed consistent seeding techniques. Nevertheless, based upon the results of the individual growth factor experiments, and the apparent lack of published studies describing TGFα effects in bovine GCs, TGFα was selected as the growth factor of consequence for further study. In addition, there was evidence in the literature at the time that TGFα induces an upregulation of phosphorylated ERK1/2 expression in the granulosa-like, KGN cells [157]. We therefore hypothesized that TGFα has similar actions in bovine GCs, which warranted further testing.

CHAPTER 3: EFFECTS OF TGFα ON PROLIFERATION, MAPK/ERK SIGNALING AND CYTOKINE-INDUCED APOPTOSIS IN BOVINE GRANULOSA CELLS

3.1 Introduction

Ovarian folliculogenesis is a dynamic process in which cohorts of follicles are recruited and develop during selected periods of the estrous cycle [13], [14], [17], [158], [159]. The process involves highly-coordinated and regulated interactions between the systemic, pituitary-derived gonadotropins and their cellular targets at the ovarian level (i.e., granulosa and theca cells and the oocytes). Cohorts of antral follicles emerge within the ovary following an episodic surge in follicle stimulating hormone (FSH) [20]. These follicles continue to grow and develop in synchrony due to proliferation of granulosa and thecal cells and the development of the antrum [94], [160]. However, as the follicles mature, a selection or deviation event occurs in which one follicle continues its growth to become dominant, whereas the remaining follicles of the cohort become subordinate and are vulnerable to atresia [73]. The dominant follicle then either ovulates or undergoes atresia similar to its subordinates. Its fate depends upon on the phase of the estrous cycle and the relative concentration of circulating progesterone [161]–[163]. The coordination of the fates of these follicles is influenced by a vast array of autocrine and paracrine regulators, including growth factors. In particular, growth factors are viewed as being critical for selection of the dominant follicle and its deviation in growth from the remaining follicles [33], [163].

There are a variety of growth factors within the ovary that not only stimulate the growth of follicles, but also influence follicular fate (i.e., continued growth or atresia). These include, insulin-like growth factor-1 (IGF-1; [101], [164]), epidermal growth

factor (EGF; [44], [165], [166]), fibroblast growth factor (FBF; [132], [167]**)** and the superfamily of transforming growth factor-beta (TGFβ; [168]**)**. The cellular actions of these growth factors are invoked principally through stimulation of multiple intracellular signaling pathways, including receptor tyrosine kinase (RTK; [120], [169]), protein kinase B (PI3K/Akt; [49], [170]), mitogen-activated protein kinase (MAPK/ERK; [26], [27], [30]) and the Smad pathways ([173]). In recent years, oocyte-derived TGFβ molecules, such as growth differentiation factors (GDF; [174], [175]) and bone morphogenic proteins (BMP; [176], [177]) have attracted increased attention because of their influence on granulosa cell (GC) and cumulus cell function, including proliferation, steroidogenesis, differentiation, and apoptosis [177]–[179]. The concept that the oocyte influences its own fate is particularly intriguing. However, TGFβ superfamily members are not the only oocyte-derived factors that can influence follicular fate.

The EGF family of growth factors can also impact follicular fate by affecting GC function [111]. Most notably, EGF family growth factors stimulate differentiation of GCs and regulate steroidogenic function through the attenuation of GC production of estradiol and inhibin, particularly in response to luteinizing hormone (LH) [111]. One such family member, TGFα, is a potent mitogen that stimulates cell growth through rapid activation of MAPK/ERK and PI3K/Akt signaling [157]. Strong mitogens also likely promote GC survival in the midst of follicular selection and deviation. EGF family members have similar structure to one another, and bind to a common receptor (EGFR) to stimulate a variety of ligand-specific cellular functions; including activation of RTK signaling, which can simultaneously trigger both PI3K/Akt and MAPK/ERK pathways, and influence cellular proliferation and differentiation [180]. EGFR ligands have diverse functions in

multiple tissue types, and are particularly important factors involved in ovarian follicle maturation and ovulation [118].

Stimulation of MAPK/ERK and PI3K/Akt signaling in GCs is particularly important in oocyte-GC communications within the follicle. MAPK/ERK signaling is activated by both follicle stimulating hormone (FSH) and LH stimulation in porcine GCs, with LH stimulating the more rapid response [181]. In rat GCs, FSH activates MAPK/ERK signaling and contributes to follicular growth and differentiation [182]. PI3K/Akt signaling promotes follicular survival through stimulation of cell proliferation, transcription of FOXO3a (protecting cells from oxidative stress), and phosphorylation of BAX (which prevents apoptosis) [170]. Phosphorylation of ERK and Akt in these signaling pathways is higher in dominant follicles than subordinate follicles [171], and their expression is upregulated in GCs of future dominant follicles before follicle selection has occurred [108].

In the bovine ovary, TGFα, which is known to stimulate both MAPK/ERK and PI3K/Akt signaling [157], is expressed in both the oocyte and surrounding thecal cells [44], [131]. It is thought to promote follicular growth through paracrine stimulation of GC proliferation [45]. Thus, TGFα and its actions through intracellular signaling may be an important mechanism influencing follicular fate and the establishment of follicular dominance. Effects of TGFα on GC steroidogenesis is both stimulatory [183] and inhibitory [134], but there is a general lack of knowledge about the direct effects of TGFα on bovine GC intracellular signaling and downstream effects like cytokine-induced apoptosis. In the current study, the objective was to evaluate these effects of TGFα on GCs obtained from bovine small (3-5mm) antral follicles. Cells were obtained

from small follicles to ensure that a selection event had not yet occurred. TGFα was hypothesized to stimulate bovine GC proliferation and survival via a MAPK/ERK-mediated mechanism.

3.2 Materials and Methods

3.2.1 Cell Culture and Reagents

Pairs of bovine ovaries were obtained from Champlain Beef (Whitehall, NY) and transported to the laboratory in 0.9% NaCl solution supplemented with antibiotic-antimycotic at room temperature. All ovaries were collected and processed within 4-5 hours of slaughter. All small (3-5 mm) antral follicles were aspirated to obtain bovine GCs. The GCs obtained from each ovary pair were kept separate, rather than pooled, for the culture experiments. Hence, a given culture consisted of cells obtained from a single pair of ovaries. The retrieved cells were pelleted, resuspended, and seeded into independent T25 flasks. Cells were cultured in Dulbecco's Modified Eagle Medium/Ham's F12 Nutrient Mixture (DMEM/F12) medium containing 10% FBS and antibiotic-antimycotic (complete culture medium) and incubated in a humidified incubator at 37°C with 5% CO_2 until the cultures reached confluency (~3-4 days). Once confluent, the cells were passaged, enumerated, and subsequently seeded into culture vessels for further testing. The day after seeding, the cultures were switched to serum-free DMEM/F12 medium supplemented with insulin-transferrin-selenium (ITS) (10ng/ml insulin, 5.5ng/ml transferrin, 0.67pg/ml sodium selenite), without or with specific concentrations of growth factor, cytokines, and/or inhibitors (see details below) depending upon the experiment.

3.2.2 Cell Treatment and Cell Proliferation

Bovine GCs were seeded into clear-walled, 96-well plates (5,000 cells/well) using complete culture medium. The following day, the cells were switched to serum-free medium containing recombinant human TGFα (R&D Systems; 1, 10, or 100ng/mL) and incubated for 24 hours. Following 24 hours of treatment, relative proliferation of bovine GCs was assessed using an MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) in which the absorbance at 490 nm was measured and recorded using a BioTek Synergy HT plate reader (BioTek).

3.2.3 Cell Treatment and Western Blot Analysis

Aliquots of bovine GCs were seeded in 6-well plates (150,000 cells/well) using complete culture medium. The following day, the cells were serum-starved for 4 hours, and then treated with recombinant human TGFα (TGFα)(R&D Systems; 10ng/mL) for 2 hours. MAPK inhibitor, U0126 (Millipore; 10μM), was applied 1 hour prior to TGFα treatment. Following treatments, the cells were washed twice with ice-cold PBS solution and lysed using RIPA buffer and scraping. The cell lysates were then transferred to 1.5mL tubes and passed through a 27-gauge needle. Protein concentration of each sample was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher). 10μg of total protein was loaded into the wells of a Mini-PROTEAN® TGX Stain-Free Precast Gel (BioRad) and separated using electrophoresis. Separated proteins were then transferred to a PVDF membrane (BioRad) using a semi-dry transfer unit (Hoefer TE70XP), and the resulting blot was blocked with TBST containing 5% BSA and then probed with primary monoclonal antibodies for pERK1/2 (rabbit; cat# 4370) and ERK1/2 (mouse; cat# 4696). Goat-anti-rabbit and rabbit-anti-mouse antibodies, respectively, conjugated with HRP

were used in conjunction with Clarity Western ECL Substrate (BioRad) for imaging using the BioRad ChemiDoc Imager. All antibodies were obtained from Cell Signaling Technology. Immunoblots consisted of at least 3 biological replicates that were run either in duplicate or triplicate.

3.2.4 Fas Ligand-induced Apoptosis

To evaluate the effects of TGFα on the incidence of FasL-induced apoptosis, bovine GCs were seeded either in 24-well plate (50,000 cells/well) or white-walled 96-well plates (5,000 cells/well) in complete culture medium. The following day, the cells were switched to serum-free medium containing either DMSO (0.1%), or U0126 (10μM) and incubated for 1 hour. Following inhibitor pre-treatment, growth factor treatments were applied, and cells were incubated for an additional 24 hours. The growth factor tested was TGFα (100ng/mL). Dosing was based upon preliminary experiments (data not shown) and dosing used in previous publications [157]. Following growth factor treatment, recombinant human Fas Ligand (FasL) (EMD Millipore; 100ng/mL) was applied and the incidence of apoptosis was determined visually (24-well plates – photographic images taken at hour intervals for 8 hours) or by using a caspase assay (96-well plates - Caspase-Glo® 3/7 Assay, Promega; detection run after 6 hours of FasL treatment). 6-hour FasL treatment was determined through time-course observation of sufficient induction of cell death and anticipated caspase activity before the administration of the Caspase Assay. RLU values from the caspase assay were recorded using a BioTek Synergy HT plate reader. Cells cultured in complete medium (i.e. 10% fetal bovine serum) throughout the experimental period were used as the positive control to test for protection against

cytokine-induced apoptosis. These experiments were conducted using 3-7 biological replicates.

3.2.5 Statistical Analysis

All experiments were conducted using a minimum of three biological replicates from an independent pair of ovaries (i.e., cow is the experimental unit). Data were analyzed by analysis of variance (ANOVA), followed by a Student's T-Test. Differences among means were considered significant at $p<0.05$.

3.3 Results

3.3.1 TGFα stimulates bovine granulosa cell proliferation

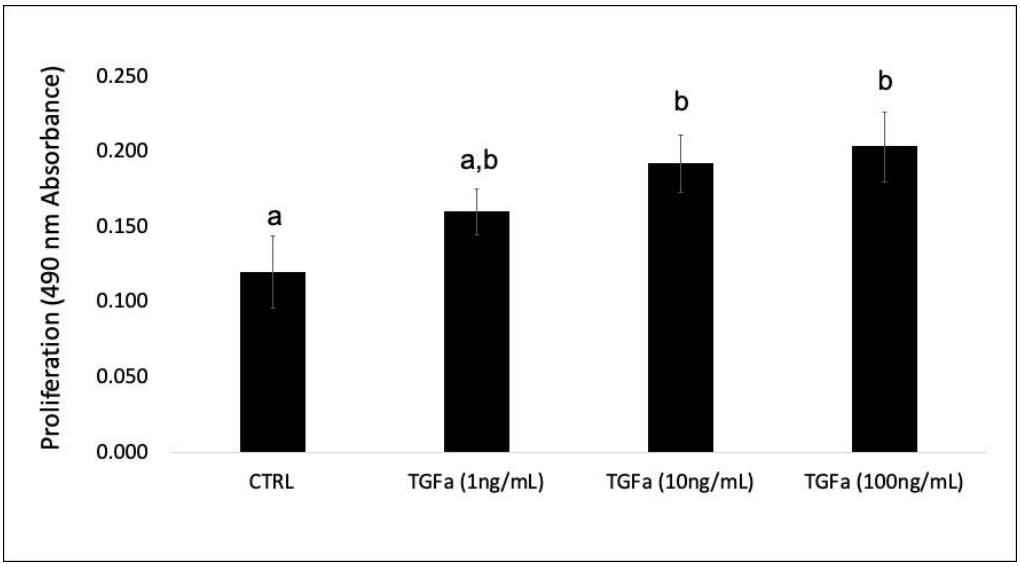

Figure 3. MTS Assay analysis of bovine granulosa cell proliferation. Bars represent means of recorded absorbance values at 490 nm ± SEM (n = 7 ovary pairs) for bovine granulosa cells exposed to different concentrations of TGFα (0, 1, 10, or 100 ng/mL) for 24 hours. Different letters denote differences in proliferation induced as a result of TGFα treatments ($p<0.05$).

Dose-response experiments evaluating the effect of TGFα on bovine GC proliferation during a 24-hour culture indicated that cell numbers increased at the 10 and 100ng/mL doses (Figure 3). Conversely, TGFα had no effect at the 1ng/mL dose. Based on these observations, the higher doses of TGFα were used in all subsequent experiments.

3.3.2 TGFα effects in bovine granulosa cells occur via stimulation of MAPK/ERK signaling

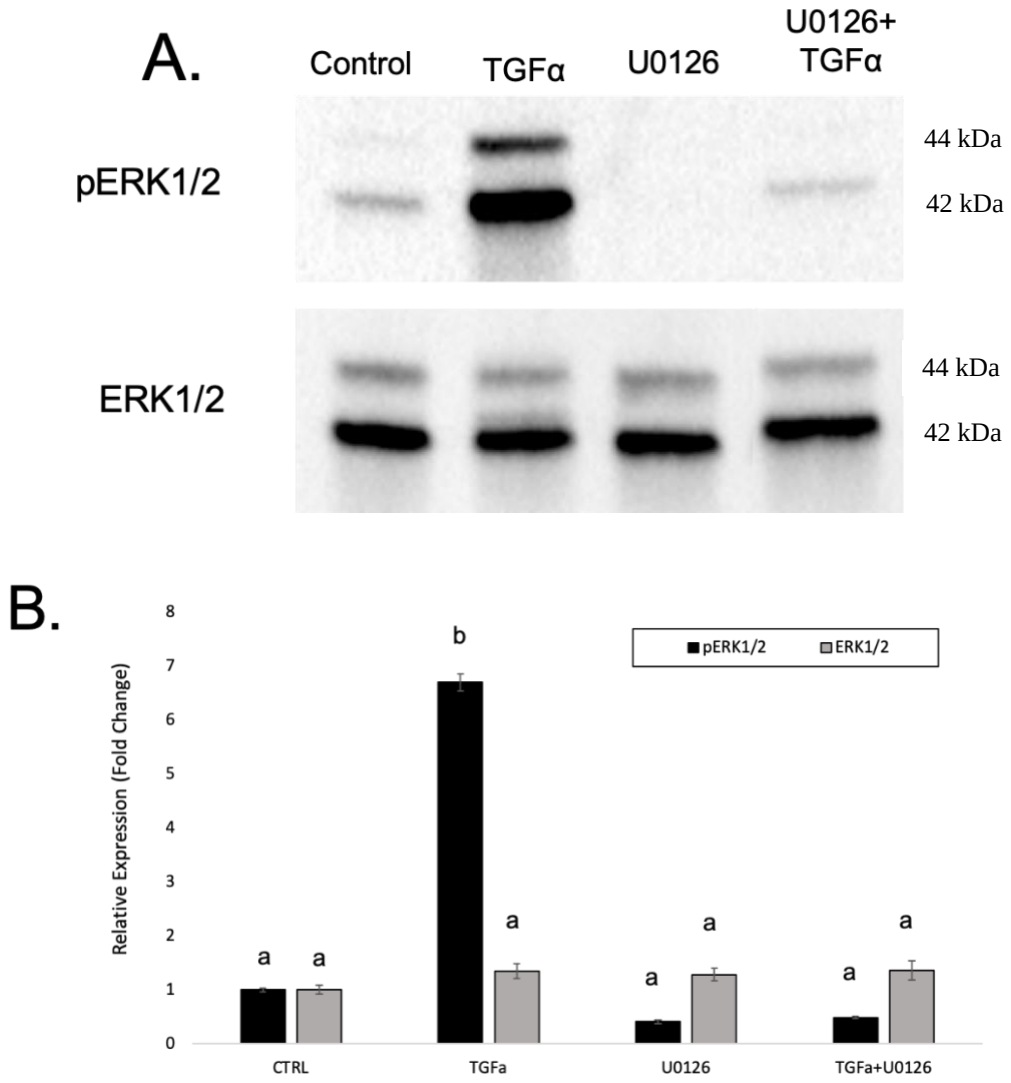

Figure 4. Immunoblot detection of pERK1/2 and ERK1/2 in bovine granulosa cells following exposure to TGFα (10 ng/mL) and/or MAPK inhibitor, U0126 (10 μM). a.) Representative immunoblot of pERK1/2 and ERK1/2 in response to treatments. b.) Bars representing mean densitometry results of immunoblots ± SEM (n = 3 ovary pairs). Relative expression is normalized to total protein, and values have been transformed to represent fold change. Different letters denote

differences in protein expression among the treatments ($p<0.05$). Treatment and inhibitor effects were evaluated on relative protein expression quantified through the densitometry analysis. Data are representative of 3 biological samples run in duplicate or triplicate.

MAPK signaling was stimulated in bovine GCs following TGFα (10 ng/mL) treatment, based upon the upregulation of phosphorylated ERK1/2 expression (Figure 4A). Densitometry analysis confirmed the upregulation to be 7-fold higher than that of control samples ($p<0.05$; Figure 4B). This upregulation was attenuated, however, by the MAPK inhibitor, U0126 (10μM), which effectively reduced phosphorylated ERK1/2 expression to a level comparable to control ($p<0.05$; Figures 4A and B).

3.3.3 TGFα fails to prevent Fas ligand-induced apoptosis of bovine granulosa cells

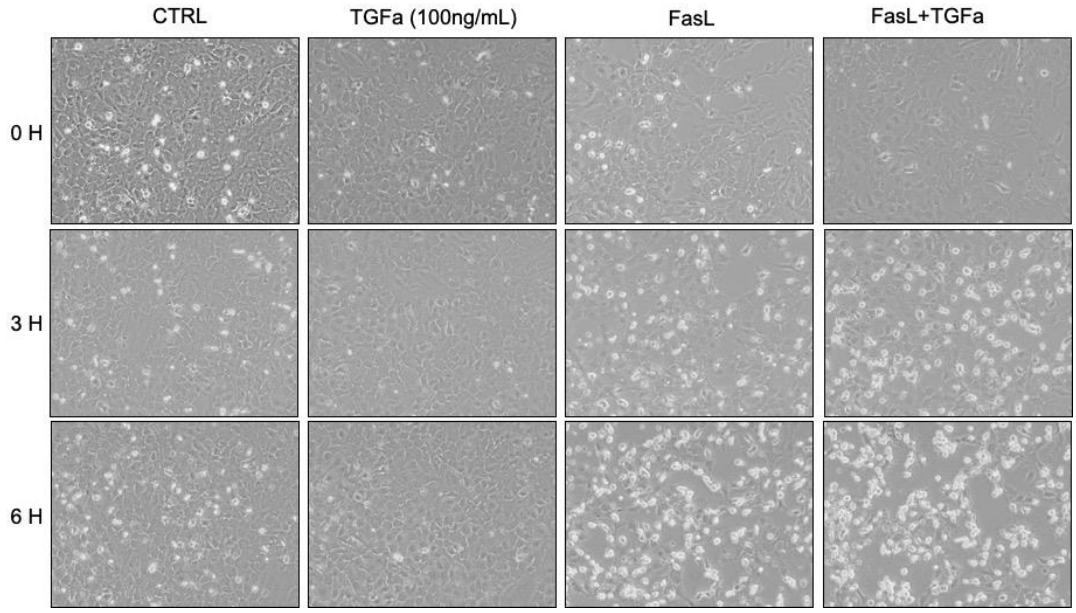

Figure 5. Representative images of bovine granulosa cells treated with FasL either with or without TGFα (100 ng/mL). Cells were imaged on an hourly basis for 8 hours, images up to 6 hours are shown. The presence of refractive and detached cells is indicative of cell death in response to FasL (n = 3 ovary pairs).

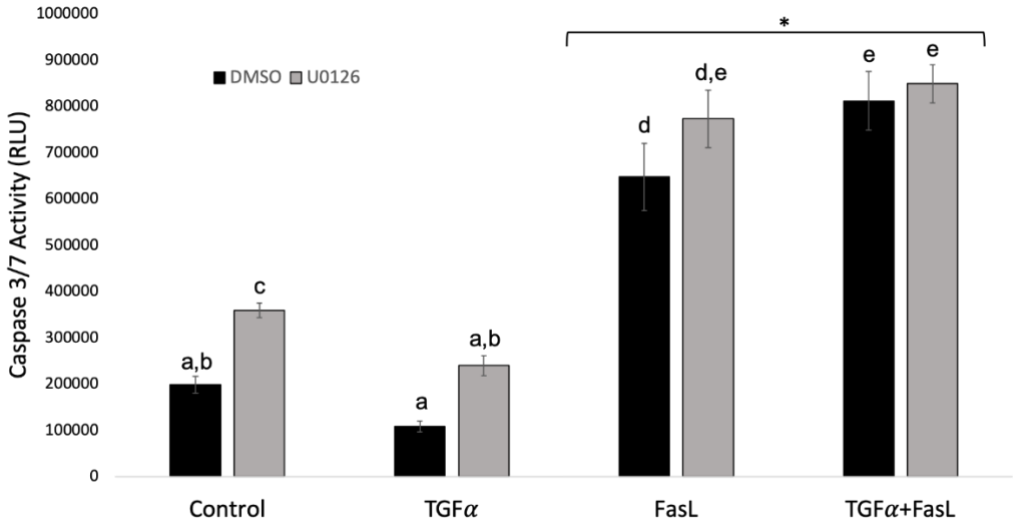

Figure 6. Caspase 3/7 activity assay analysis of bovine granulosa cells following 24-hour treatment with TGFα (100 ng/mL), followed by a 6-hour exposure to FasL (100 ng/mL). The MAPK inhibitor, U0126 (10 µM) or vehicle control, DMSO (0.01%) was applied to the cells 1 hour prior to TGFα treatment. Treatment groups denoted with an asterisk (*) are different from the control group ($p<0.05$**). Bars having a different letter are statistically different (**$p<0.05$**; n = 7 ovary pairs).**

24-hour pre-treatment of bGCs with TGFα (100ng/mL) failed to provide any protective effect against FasL-induced cell death (Figure 5). Signs of cell death in the presence of FasL (i.e., refractive and detached cells in the culture) were observed as early as 3 hours post-treatment (Figure 5.).. By 6 hours, most of the cells were dead or dying and had become detached from the culture vessel (Figure 5). Comparatively, cells treated with TGFα had greater viability over the course of the culture period compared to cells in serum-free control medium (Figure 5). In fact, the cells looked similar to those cultured in FBS-containing, complete culture medium (images not shown). FasL-induced cell death via apoptosis was evidenced by the increased expression of caspase 3/7 activity (Figure 6). Interestingly, and unexpectedly, exposure of the cells to TGFα enhanced

susceptibility to FasL-induced apoptosis as compared to FasL alone (Figure 6); this effect was observed regardless of MAPK inhibition (U0126), suggesting that TGFα-induced MAPK stimulation provided no protection against FasL-induced apoptosis. Conversely, FBS included in the culture medium abolished the FasL killing effect and protected against the induction of apoptosis. (data not shown).

CHAPTER 4: GENERAL DISCUSSION

In the work presented here bovine GCs were exposed to TGFα to test the hypothesis that TGFα, through increases in MAPK/ERK signaling, provides resistance to immune-mediated apoptosis. In so doing, it was determined that TGFα stimulates a proliferative response in bovine GCs and does this through activation of MAPK/ERK signaling, as evidenced by an increase in the phosphorylation of ERK proteins. The use of the MAPK inhibitor, U0126, showed the specificity of TGFα-induced MAPK/ERK signaling by completely abolishing the expression of phosphorylated ERK. In our serum-free culture system, it was also determined that Fas Ligand induces apoptosis of bovine GCs, as evidenced by an increase in caspase 3/7 activity, and the effect is augmented by prior exposure of the cells to TGFα. Lastly, although TGFα induced bovine GC proliferation and stimulated MAPK/ERK signaling, there was no evidence to support the hypothesis that MAPK/ERK signaling mediates a protective effect of TGFα on FasL-induced apoptosis. These observations collectively cast doubt on the likelihood that TGFα, and MAPK/ERK signaling in particular, support granulosa cell survival and follicular persistence during follicle selection and deviation.

A substantial amount literature exists in which effects of various intrafollicular signaling molecules have been investigated. However, TGFα has seemingly been overshadowed by these and its better-known family member, EGF. Nevertheless, the current study contributes to the existing literature and adds to our current understanding of the molecular dynamics influencing ovarian physiology. For instance, results from the current study support the concept of direct effects of TGFα on bovine GCs, which include stimulation of cell proliferation and the upregulation of MAPK/ERK signaling. In

work of others, TGFα induced a proliferative response in primary cultures of GCs from cattle [131] and rats [184], as well as in immortalized GC lines [157], but the mechanistic effects were not entirely elucidated. TGFα induces proliferation of KGN cells (a granulosa cell tumor line) through EGFR stimulation and activation of multiple pathways, including mTOR, PI3K/Akt, and MAPK/ERK [157]. TGFα is more potent than EGF as a mitogen, but the effect are attributed to PI3K/Akt and mTOR, rather than MAPK/ERK, activation [157]. Conversely, in current study, the proliferative effect of TGFα on bovine GCs appeared to be attributable to the stimulation of MAPK/ERK signaling. The MAPK inhibitor, U0126, effectively negated TGFα-induced effects on ERK1/2 phosphorylation. In both the current and previous study [157], the TGFα-induced phosphorylation of ERK occurred within 10 minutes of treatment and was sustained for up to three days of culture. These observations suggest TGFα is indeed a potent activator of MAPK/ERK signaling, and lends credibility to the idea that interactions between MAPK/ERK and PI3K/Akt signaling occur and promote cell survival [185], [186].

The current results also support the previous findings of Glister and co-workers [131], who demonstrated that TGFα is expressed in bovine oocytes and stimulates GC proliferation when co-cultured with oocytes [131]. GCs do not express TGFα mRNA [45], so there is no evidence that TGFα is a GC-derived molecule. Beyond stimulating GC proliferation, TGFα also inhibits estradiol, inhibin, activin, and follistatin secretion by GCs, but has no effect on progesterone production [131]. From these observations, Glister and colleagues suggested a role for TGFα in GC-oocyte interactions. However,

as has become evident from the current study that the role of TGFα may be more selective to the dominant follicle, particularly during ovulation and subsequent luteal function, because TGFα fails to prevent FasL-induced apoptosis and most likely would fail to protect against follicular atresia. The effects of TGFα on bovine GC and theca cells occur through EGFR stimulation [45]. Another growth factor, EGF, is also a potent stimulator of GC proliferation [42]. Related molecules of the EGF family are expressed and stimulate follicular growth in multiple species including chickens [187], goats [188], pigs [124], and humans [189]. However, TGFα is considered the major EGF ligand within cow follicles [131], and may have a unique, mechanistic role in the follicle that has yet to be characterized and understood.

Theca cells and/or oocytes are the principal sources of EGF and TGFα secretion in the ovary and possibly have paracrine effects on GCs through the EGFR [45], [165], [190], [191]. In bovine GCs, the activation of both MAPK/ERK and PI3K/Akt signal transduction promotes cell growth and viability, which is thought to translate to development and differentiation of follicles [108]. Differences in the expression of MAPK/ERK and PI3K/Akt proteins are evident between large and small follicles, but more importantly, the difference in their expression occurs just after the emergence of the "putative" dominant follicle [108]. Dominant follicles have higher expression of the phosphorylated forms of ERK and Akt than subordinate follicles [171]. Elevated expression of pERK and pAkt are detectable in the putative dominant follicle, and are detectable before any other distinguishable markers, such as changes in follicular size or estradiol production, become evident [108]. Thus, MAPK/ERK and PI3K/Akt signal transduction may join forces to provide a selective advantage to follicles as they attain

dominant status. In the current study, TGFα clearly upregulated pERK expression in bovine GCs, and we suggest that this effect occurs through MAPK/ERK activation. Indeed, pERK expression is completely abolished by the MAPK inhibitor, U0126, which confirms that the effects of TGFα in bovine GC is mediated, at least in part, through the MAPK/ERK pathway. Despite this, stimulation of MAPK/ERK signaling failed to provide any protective effect against cell death in the FasL experiments. The lack of protective effect was somewhat surprising because others report that a reduction in ERK expression leads to apoptotic signaling [192]. In porcine GCs, for example, ERK expression aids in cell survival during times of oxidative stress [172]. Although considerable crossover exists between MAPK/ERK and PI3K/Akt signaling [185], [193], and the expression of these two pathways differs between putative dominant and subordinate bovine follicles [108], the question of which of these signaling mechanisms ultimately impacts granulosa cell fate during follicle selection remains unresolved.

Perhaps the most compelling evidence, to date, to suggest that PI3K/Akt signaling, rather than MAPK/ERK signaling, is most important to GC survival comes from studies of IGF-1 effects. Briefly, Quirk and colleagues investigated the effects of IGF-1 on FasL-induced apoptosis of GC in a variety of ways [105], [153], [194]. In particular, IGF-1 exerts a protective effect against FasL-induced apoptosis in a manner similar to fetal bovine serum (i.e., 10% FBS) [153]. Interestingly, EGF and basic FGF (also found in FBS) also provide these effects, and EGF stimulates GC proliferation more effectively than IGF-1 [153]. The protective effects occur through the activation of PI3K/Akt signaling, and results in the inactivation of pro-apoptotic molecules, BAD and caspase-9 [153]. However, others have found MAPK/ERK signaling has protective

effects, coordinating extensively with PI3K/Akt signaling, by mediating cell survival [185], reducing BAD expression [185], and preventing other apoptotic effects [110], [172]. Use of IGF binding protein-3 (IGFBP-3) restores FasL-induced cell death in serum-containing conditions [58], which suggests that IGF-1 alone in FBS-containing culture medium is responsible for the protective effect. However, IGFBP-3 modulates not only IGF-1-related signaling, but also the effects of TGFβ/Smad and EGFR signaling [195], which further stimulate a variety of intracellular pathways. All of these factors, including IGF-1, are likely components of FBS, so the conclusion that PI3/Akt activation alone mediates cell survival is debatable. Of note, in most of the aforementioned experiments, the culture medium often contained up to 5% FBS, and interferon gamma was used to augment FasL-induced cell killing. In the current study, bovine GCs were cultured using serum-free conditions, and FasL-induced apoptosis of bovine GCs in the absence of interferon gamma. These precautions diminish the possibility that any confounding growth factors, other than TGFα alone, influenced the outcome of the FasL experiments. Nevertheless, TGFα failed to prevent FasL-induced apoptosis. Observing that effects of TGFα in bovine GCs are mediated, in part, through MAPK/ERK signaling, the case for such signaling offering protection against cytokine-induced cell death is weakened. Conversely, a compelling case can be made for the involvement of PI3K/Akt signaling in GC survival during follicular selection in cattle [153], [196]. Furthermore, inhibition of PI3K/Akt signaling stimulated by IGF-1 and TGFα in chicken GCs results in enhanced apoptosis, whereas inhibition of MAPK/ERK stimulation by these same factors does not [197]. Although TGFα induces phosphorylation of Akt, its actions are less potent than IGF-1 [197], which further supports the concept of extensive crosstalk

between MAPK/ERK and PI3K/Akt signaling in granulosa cells. Rather than influencing follicular selection, perhaps TGFα is more critical to differentiation of GCs within the ovulatory follicle, augmenting the process of luteinization.

Based upon the results presented here and the findings of others [111], [131], we now speculate that TGFα is an oocyte-derived factor that induces MAPK/ERK signaling in bovine GCs to prepare the dominant follicle for ovulation while simultaneously preparing the oocyte for final maturation. In bovine GCs, EGF/TGFα suppress estradiol production [131], [134]. In humans, other EGF ligands (amphiregulin and epiregulin) augment luteal steroidogenesis through activation of both ERK and Akt phosphorylation [198]. Further evidence supporting this role of TGFα in GC differentiation stems from work performed in human granulosa cells, wherein LH stimulated an upregulation of mRNA and protein expression of EGF family members, amphiregulin and epiregulin, and treatment with these factors, in turn, induced progesterone biosynthesis [198]. As the follicle begins to prepare to ovulate, the cumulus GCs surrounding the oocyte undergo rapid proliferation, with EGF stimulation of MAPK signaling being required for cumulus expansion [199]. MAPK signaling is similarly important for oocyte maturation [200], which clearly shows that connections between the oocyte and GC are mediated through EGF ligands. The LH-induced breakdown of gap junctions between cumulus GC and the oocyte triggers resumption of meiosis, and the activation of MAPK signaling is required for this [200].

In conclusion, the results of the current study show that TGFα is a potent activator of bovine GC proliferation, in which the actions of TGFα are mediated principally by MAPK/ERK signaling and the upregulation of phosphorylated ERK

expression. Despite its growth-promoting properties, TGFα failed to prevent FasL-induced apoptosis, which casts doubt on its capacity to promote GC survival and follicle persistence during bovine follicular selection and deviation. An alternative role for TGFα is suggested, in which its actions via MAPK/ERK signaling facilitate differentiation of GC within the follicle as ovulation becomes imminent.

Due to the eliminative culture methods utilized for the experiments herein, there are a number of experimental pitfalls which could be addressed. One such pitfall being that as more substances are removed from the culture media, the less likely the culture environment mimics the *in vivo* conditions from which the cells are derived. Adding to this, no phenotypic screening was pursued to confirm that the bovine granulosa cells in culture had maintained their granulosa cell-like characteristics (e.g., the ability to respond to FSH stimulation and produce estradiol). Because of this, the possibility remains that the cells luteinized over the course of the culture period. Additionally, many of the isolated growth factors utilized were of human origin. This problem of species-specificity call into question whether human-derived biomolecules and growth factors exert the same effects on bovine cells. It is conceivable that cow-derived growth factors with different sequence homologies and binding specificities differ biologically from their human isoforms. Any observed effects induced by the human-derived TGFα, for example, might not exhibit an identical biological action to that of cow-derived TGFα.

Broader the impacts of this work could extend to feeding the world more sustainably, and by understanding the reproductive mechanisms of cattle more clearly, help to produce animal-sourced food in an economical manner. By improving our understanding of bovine folliculogenesis at the cellular and molecular levels, estrus

synchronization protocols may be further refined and applied in the context of improved fertility in cattle. The United Nations Food and Agriculture Organization (FAO) predicts that global food shortages will occur by 2050 as global human populations exceed the rate of sustainable food production [201]. Many countries affected by population growth have a growing middle class economy in which people earn more money and desire to purchase animal-based food sources [202]. This presents a challenge to animal agriculture, which has an ongoing goal to improve animal fertility in an efficient and sustainable manner.

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