# FIBROBLAST GROWTH FACTOR SIGNAL REQUIREMENT FOR BOVINE OOCYTE MATURATION AND DEVELOPMENTAL COMPETENCE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2011

To my parents and my family

ACKNOWLEDGMENTS

The completion of this work has been made possible due to the excellent guidance of Dr. Alan Ealy, who is my major advisor. I was deeply impressed when I met Dr. Ealy for the first time after coming to the US. He is so easygoing that I feel like one of my close friends. As an excellent mentor, Dr. Ealy is always ready to help and give suggestions on my projects. I have achieved a lot from communications with him and because of the great freedom he provided us in the lab. Dr. Ealy is very supportive of my professional development and has given me plenty of wonderful advices. As an international student, I have also felt warmth every time he gave me praise and encouragement after finishing each oral presentation and earning even a little progress in work. Dr. Ealy is an exceptional PI who make everyone happy in the lab. I will never forget my wonderful experience in his lab.

I would like to extend my sincere thanks to my committee members: Dr. Peter Hansen, Dr. Sally Johnson and Dr. James Resnick for their mentorship and suggestions throughout my PhD study. Dr. Hansen is one of the best mentors that I have ever seen in my life. He is the role model of what a good scientist and good mentor should be. I am truly grateful for the opportunity he offered me to complete the majority of my project in his IVF laboratory and specially thank him for the advice when I had to switch laboratories after the first year of my PhD study.

Dr. Johnson is extremely supportive of my projects and career development. I am truly grateful of Dr. Johnson's insightful suggestions and comments on my project. I have learned a lot by communicating with Dr. Johnson and have been especially impressed by her intelligence and knowledge.

It is my great pleasure to have the opportunity to get advice from Dr. Resnick throughout my PhD training. I will always remember Dr. Resnick's intelligent feedback and insightful

questions. Appreciation is also extended for his kind suggestions during my postdoctoral job searching. Overall, I feel proud to have worked with this talented group of people.

Special thanks are also extended to Dr. Karen Moore, who was my first mentor during my PhD training. I am sincerely grateful for the opportunity to join the AMCB program with the help of Dr. Moore. During the huge transition from China to the US, Dr. Moore gave me tremendous help and suggestions to help me overcome the culture shock and get used to the new environment. I am also deeply thankful of the help from Ewa Hughes and Joe Kramer in Dr. Moore's lab.

I would like to thank Dr. William Thatcher for his care about my projects and PhD study. It will be wonderful to remember that the many times I talked with Dr. Thatcher over lunch. I have learned a lot from his insightful questions and comments during seminars and have been impressed by his hard-working approach. I feel fortunate to have been able to learn from him.

The molecular part of the work was completed in Dr. Ealy's lab. I would like to thank all the labmates for their diverse help during my stay in the lab, including Dr. Qi-En Yang, Dr. Kathleen Pennington, Flavia Cooke, Susan Rodgers, Dr. Manabu Ozawa and Paula Morelli. It has been my pleasure to work with this enthusiastic and talented group of young scientists.

Sincere thanks are also extended to members of Dr. Hansen's lab. All the oocytes and embryos used in the work were collected in Dr. Hansen's IVF lab. The IVF group is very friendly, productive and highly efficient. I am truly grateful for Justin Fear's patience in teaching me how to do bovine IVF. The completion of the work would not be made possible in so short time without the high efficient team work for sharing media and doing IVF. The team includes Dr. Aline Bonilla, Luciano Bonilla, Dr. Manabu Ozawa, Dr. Miki Sakatani, Sarah Fields, Kyle

Dobbs, James Moss, Dr. Silvia Carambula, Dr. Jeremy Block, Dr. Barbara Loureiro and William Rembert.

I would like to express grateful thanks to all the friends I met at UF. I have spent an enjoyable time in the Department of Animal Sciences. Thank all my Chinese friends, Dr. Ju Li, Sha Tao, Dan Wang, Wenli Sun, for their encouragement and sharing ideas.

Last but not least, I am extremely grateful of the endless support and encouragement from my wife, Dr. Shaohua Wang. I thank her for the understanding and sacrifice during my pursuit of the Ph.D. It is so fortunate for me to work and live with this intelligent colleague and life partner.

Throughout my study so far, I have been deeply indebted to my brother, Dr. Yunhai Zhang, for his great help and support.

No words can be found to describe my extreme thankfulness to my parents, who are ordinary peasants in the rural area of China. Being illiterate persons, they taught me ‘hard work always pays off’ and did their best to support my elder brother and me to finish high education. It is rarely seen in rural China. Therefore, it is always one of my motivations to make them proud of me.

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| | |
|---|---|
| AMH | Anti Mullerian Hormone |
| ART | Assited Reproductive Technology |
| BMP | Bone Morphogenetic Protein |
| BSA | Bovine Serum Albumin |
| BSS | Bovine Steer Serum |
| CL | Corpus Luteum |
| COC | Cumulus Oocyte Complex |
| dbcAMP | dibutyryl cyclic AMP |
| DNMT | DNA Methyltransferase |
| dpc | day post coitum |
| EGA | Embryonic Genome Activation |
| EGF | Epidermal Growth Factor |
| ERK | Extracellular signal-Regulated Kinase |
| FGF | Fibroblast Growth Factor |
| FSH | Follicle Stimulating Hormone |
| GDF9 | Growth Differentiation Factor 9 |
| GLM | General Linear Model |
| HPG | Hypothalamus Pituitary Gland |
| ICM | Inner Cell Mass |
| IGF | Insulin-like Growth Factor |
| IVF | *In vitro* Fertilization |
| IVM | *In vitro* Maturation |
| LH | Luteinizing Hormone |
| MAPK | Mitogen-Activated Protein Kinase |

| | |
|---|---|
| mSOF | modified Synthetic Oviduct Fluid |
| NEB | Negative Energy Balance |
| OMM | Oocyte Maturation Medium |
| PGC | Primordial Germ Cell |
| PI3K | Phosphoinositide 3-Kinase |
| PKA | Protein Kinase A |
| PR | Progesterone Receptor |
| PVA | Polyvinyl Alcohol |
| PVP | Polyvinyl Pyrrolidone |
| RA | Retinoic Acid |
| SNP | Single Nucleotide Polymorphism |
| StAR | Steroidogenic Acute Regulatory protein |
| TCM | Tissue Culture Medium |
| TE | Trophectoderm |
| ZP | Zona Pellucida |

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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By

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August 2011

Chair: Alan D. Ealy
Major: Animal Molecular and Cellular Biology

Poor reproductive efficiency in cows impacts profitability in the dairy industry. Inferior oocyte quality is one major contributor to reproductive failure in cows. Superior oocyte quality relies on an optimal ovarian follicle environment, which is regulated by various endocrine, paracrine, and autocrine factors. Accordingly, a series of studies have been conducted to evaluate the fibroblast growth factor (FGF) signal requirement for bovine oocyte maturation and developmental competence.

The first study was to determine if FGF10 is an oocyte competence factor. FGF receptor 1 (FGFR1) transcripts predominated in cumulus cells whereas FGFR2 was most abundant in oocytes. FGF10 addition during *in vitro* maturation improved the nuclear maturation rate, promoted cumulus expansion, and increased developmental potential. These characteristics were compromised by addition of FGF10 antibody. In addition, FGF10 influenced the expression of *CTSB*, *SPRY2* in cumulus cells and *BMP15* in the oocyte.

The second study was to describe how FGF2 may facilitate oocyte maturation and improve subsequent embryo development. FGF2 (≥0.5 ng/ml) increased the percentage of blastocysts at day 7 post-IVF. FGF2 increased cumulus expansion index scores and nuclear maturation rate

after 21 h. Also, 0.5 and 5 ng/ml FGF2 reduced the proportion of apoptotic cumulus cells after 21 h culture.

The third study evaluated the role of comprehensive FGFR signal in oocyte maturation and developmental potential. FGFR1 and R2 were dynamically expressed throughout *in vitro* maturation process and responsive to FSH. Meiotic maturation and cumulus expansion was inhibited by the addition of a FGFR inhibitor, SU5402. This effect was associated with reduced MAPK activity in oocytes. SU5402 and another FGFR inhibitor, PD173074, both inhibited the developmental potential of oocytes.

The last study was completed to determine if FGF2 and FGF10 were still able to increase embryo development when using serum-containing maturation medium. The percentage of embryos with ≥8-cells was improved by FGF10 but not by FGF2. At day 7 and 8 post-IVF, the blastocyst rate and the advanced blastocyst (expanded and hatched) rate were increased by FGF10. The percentage of blastocysts at day 7 was increased by oocyte supplementation with FGF2.

Overall, results indicate that FGFR signaling is involved in regulating oocyte maturation, cumulus expansion and subsequent embryonic development. The characterization of this signaling could lead to the identification of potential markers of oocyte quality and the development of strategies to control female fertility in mammals.

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# CHAPTER 1
# LITERATURE REVIEW

The general public is somewhat knowledgeable of several reproductive technologies, including *in vitro* fertilization (IVF), and the advent of several provocative discoveries, such as the cloning technologies that gave us Dolly. These achievements were made possible due to substantial progress achieved in our basic understanding of oocyte maturation, sperm capacitation, fertilization and manipulation of gametes *in vitro* over the past 40 years.

Oocyte quality plays an important role in determining embryonic fate (survival or death). The contribution of the oocyte and the sperm to the formation of the zygote is not equivalent. Importantly, the oocyte provides the majority of cytoplasmic factors to nurture early embryonic development until embryonic genome activation (EGA). However, our understanding of oocyte maturation, especially in regards to cytoplasmic maturation, is far from complete. This lack of knowledge compromises our ability to adequately mature human and bovine oocytes *in vitro* and limits the overall fertility of domestic animals, and oocyte quality may be especially important for the lactating dairy cow because poor oocyte quality is considered a main contributing factor for the declining fertility over the past sixty years.

Based on these and other observations that will be described in this literature review, it is imperative that new discoveries in the regulation of oocyte growth, maturation and acquisition of developmental competence be completed. The knowledge gained in this field will conceivably lead to improved efficiencies of assisted reproductive technologies (ART) in humans, cattle, and other mammals, including endangered and extinct animals. These insights also may be used to reduce embryonic and fetal losses in various species.

## Subfertility in High Producing Dairy Cows

### The Situation

Dramatic improvements in milk yield per cow have been achieved over the past six decades. However, this achievement has come at the cost of declining fertility [1-5]. Reductions in fertility of dairy cattle have been observed in several countries and regions regardless of the production system and irrespective of continuous or seasonal calving schemes [1, 6]. Accordingly, one of the greatest challenges for animal reproduction biologists is to understand what has gone awry during the reproductive process in these lactating animals.

### Factors Contributing to Declining Fertility

Various factors are linked with infertility of high producing dairy cows. These include genetics, physiology, developmental programming, management, and environment. What follows is a brief overview of how each of these factors impact dairy cow fertility.

Genetics: During the past sixty years, high producing cows have been selected primarily based on milking merit and limited attention has been placed on reproductive capability [1]. In addition, there has been an increase in the inbreeding coefficient, an estimate of the percentage of two identical alleles inherited by an animal [7, 8]. Inbreeding can increase the chances of offspring being impacted by deleterious genes, which may account for the reduction of fertility in high yielding cows [8, 9]. Therefore, selection for reproductive traits (e.g. daughter pregnancy rates) has been used to minimize infertility even though the heritability of the reproduction merit is low [7]. Furthermore, there are indications that embryonic fate is influenced by specific genes. For example, *STAT5* and *FGF2* single nucleotide polymorphisms (SNPs) are correlated with embryo survival in cattle [10, 11]. These facts collectively implicate the importance of genetic background on the reproductive performance in cows.

Physiologic factors: During the early postpartum period, high producing cows undergo negative energy balance (NEB), and during this time they are susceptible to various metabolic (e.g. ketosis, parturient paresis) and pathogenic (e.g. mastitis, retained placenta) disorders, any of which can reduce fertility [12, 13]. Notably, postpartum cows under NEB have increased concentrations of non-esterified fatty acids [12], and low glucose as well as insulin-like growth factor 1 (IGF1), all of which are found to be correlated with poor oocyte developmental competence [4].

Developmental factors: After fertilization, the presumptive zygote is subject to a series of developmental achievements and maternal tests it must meet for pregnancy to continue to term. Only ~35% of bred cows will be pregnant by day 28 post-breeding, and a large portion of pregnancy losses occurs in this first month of gestation because of problems associated with fertilization and pre- and peri-implantation embryo development [5]. The root causes of these failures are numerous and may include problems associated with oocyte and follicle development and suboptimal uterine conditions [3-5].

Managerial influences: The reduced fertility of high producing cows may also be contributed by inappropriate management [14]. High producing herds have better reproductive performance than low producing herds [14]. This may be linked with better nutrition and reproductive management in high producing cows. The maintenance of uterine health is also critical for the cow fertility. The uterus is susceptible to bacterial contamination, especially at parturition or in following days. A meta-analysis of 23 studies shows that cows with endometritis have increased mean days open and decreased pregnancy rate [15]. Moreover, superovulation and synchronization using hormones are also correlated with poor embryonic survival in cows [16, 17].

Environmental factors: The reduced fertility of cows has been associated with environmental stress, especially heat stress. Heat stress affects the reproductive system weeks before ovulation by damaging follicular function and oogenesis [18]. Additionally, oocyte maturation, fertilization and preimplantation embryos are very susceptible to heat stress [19]. Therefore, various attempts have been made to prevent embryo loss during periods of elevated temperatures in lactating dairy cows [20-22]. Included among these strategies are embryo transfer and pharmacological treatment of embryo with antioxidants or IGF1 for improving thermotolerance [22, 23].

**Contribution of Oocyte Quality to Cow Fertility**

Oocyte competence refers to the capability of an oocyte to resume meiosis, undergo fertilization and complete early embryo development and develop to healthy offspring [24]. *In vivo,* oocytes acquire their developmental competence during the course of folliculogenesis, which is controlled by endocrine and paracrine factors [25]. This acquisition can be achieved *in vitro*, albeit at a lower efficiency [26].

Oocyte incompetence is a major contributing factor for infertility in cattle [27]. One study implicates the developmental competence of the oocyte is reduced in modern, high producing dairy cows [13]. One reason for such incompetence likely reflects oocyte maturation during high production [28]. Outcomes appear to be exacerbated with extensive NEB in post-partum period [3]. After parturition, energy output surpasses the energy input rapidly due to milk production, and therefore NEB is established. During this period, nutrients ingested may preferentially be diverted to other biological functions such as milking other than reproduction, and hence limit the growth and development of the oocyte [29].

Stressful environmental conditions, such as heat stress, undoubtedly contributes to oocyte incompetence [19]. *In vivo* evidence has shown that reduced proportion of viable embryos is

seen in superovulated heifers that exposed to heat stress (above 41.0 °C) during estrous [30]. Meanwhile, direct effects of elevated temperature to reduce oocyte competence are observed *in vitro* [31-33]. This is likely mediated by disruption of nuclear maturation and reorganization of cytoskeleton elements (i.e. microtubules) as well as induced apoptosis in the oocytes [32, 34].

Hormonal regiment used to synchronize estrous and ovulation also conceivably impact oocyte competence. For example, GnRH administration to induce ovulation of follicles ≤11mm is associated with decreased pregnancy rates and increased late embryonic/fetal mortality [17]. One potential explanation is that oocyte development is incomplete when small follicles (≤11mm) are ovulated as cow oocyte competence is positively associated with the follicle diameter [35].

Oocytes matured *in vitro* are less competent than their *in vivo* counterparts. Holm et al. has shown that the development of bovine zygotes produced from *in vitro* maturation (IVM)-derived oocytes is reduced relative to *in vivo* controls [36]. Studies utilizing IVM-derived embryos often contain low pregnancy rate and abnormal fetal development (Large Offspring Syndrome) [37-42]. Hence, IVM conditions conceivably impact the short and long term development of IVM-derived embryos. Consequently, it is imperative that IVM conditions be optimized

The acquisition of competence for the oocytes is a series of systematic events occurring from the specification of germ cell to the extrusion of oocyte from the ovarian follicle. To better learn and potentially manipulate oocyte quality, it is important to understand how a fertilizable oocyte is generated *in vivo*.

## Regulation of Female Germ Cell Development

Oogenesis, or the process of generating fertilizable oocytes, is a dynamic process that is controlled by a variety of physiological, cellular and molecular events. The oocyte grows, matures and acquires developmental competence in the follicle. This niche dictates oocyte

quality and competence. One important feature of oocyte quality relates to resumption of meiosis and the preparation of the oocyte cytoplasm for early embryo development. Although great progress has been made in describing these developmental events, numerous questions remain, including understanding how paracrine factors impact oogenesis and oocyte competence. Herein, the major events that occur during folliculogenesis and oogenesis are reviewed. Descriptions will include germline formation and their migration to the genital ridge, factors that drive folliculogenesis and oogenesis, and our current understanding of meiotic and cytoplasmic maturation.

**Germline Specification and Migration**

The oocyte is originated from the primordial germ cell (PGC). Our current understanding of the mechanism of early oogenesis events such as PGC formation and migration has been generated primarily from mice. The PGCs originally are located in the proximal epiblast and can be distinguished from somatic cells by responding to inductive signals from the embryonic ectoderm [43, 44]. For example, bone morphogenetic protein 4 (BMP4) and 8b are major extrinsic factors that stimulate the formation of PGC precursors at E6.5 in the mouse [45, 46]. Several other members of the TGF-β family also play an important role in regulation of PGC mitosis [47]. BMP2 and 4 are capable of promoting the proliferation of PGC cultured *in vitro* [48]. *BMP* deleted mice have decreased number of germ cells after 11.5 dpc [47].

Between E6.5-7.5, the PGC is specified towards germline expression. This commitment is thought controlled by proteins PRDM1 and 14 [49-51]. PRDM1 and 14 are transcriptional regulators that contain a PRDI-BF1-RIZ domain, the structural relative of histone methyltransferase. They are both expressed as early as E6.5 in PGC precursors in mice and dependent on BMP4 signaling. PRDM1 heterozygotes have reduced number of PGCs while

homozygotes have no PGCs at E7.5. PRDM14 null mice are infertile due to arrested PGC development in the absence of PRDM14.

During PGC specification, several major events occur, including repression of somatic cell gene expression, induction of PGC-enriched gene expression and pluripotency gene expression [49, 51, 52]. Particularly, *Hoxa1* and *Hoxb1*are predominant in somatic epiblast cells but undetectable in PGCs. However, they are expressed in PGC-like cells collected from *PRDM1* null mice. *PRDM14* mutant mice have reduced expression of lineage restricted *DPPA3* and the pluripotency gene, *Sox2*. Also, *PRDM1* mutant mice have no expression of PGC-specific *Nanos3*. Thus, PRDM1 and PRDM14 regulate PGC specification by manipulating the gene expression program.

Next, PGCs enter the tissue that will develop into the gonads and the ovary or testis forms soon thereafter. PGC migrates to gonad and colonizes the ovary at E10.5 in mice. It is poorly understood how migration is initiated and modulated but it appears that KIT ligand and SDF1 function as chemoattractants during migration [53-55]. PGCs express c-KIT receptor whereas hindgut cells, neighboring somatic cells during migration, contain the KIT ligand [56]. The c-KIT/KIT ligand signaling is believed to promote the proliferation and migration of PGCs [56, 57]. *In vitro* migration assays show that KIT ligand is an effective chemoattractant for the PGCs from E10.5 and 11.5 mice embryos. During this process, phosphatidylinositol 3-kinase (PI3K)/AKT and SRC kinases are involved in mediating KIT ligand effects on PGCs [54].

During migration and entry of PGCs into the genital ridge, dramatic epigenetic reprogramming events occur, including global DNA demethylation, removal of imprinting and reactivation of inactivated X chromosomes in female fetuses [58, 59]. The biological relevance of epigenetic regulation is under intensive investigation. DNA methylation is the addition of a

methyl group in the 5' position of cytosine residues in CpG islands located near or within promoters of genes. The modification, catalyzed by the family of DNA methyltransferases (DNMT), primarily causes the transcriptional silencing of genes. It is dynamic as two waves of DNA demethylation/methylation pattern occur throughout early embryogenesis [58, 59]. At the morula stage, DNA methylation status is erased for all but a few genes (i.e. imprinted genes). Then *de novo* DNA re-methylation occurs before implantation. Shortly after that, a second wave of demethylation takes place. Again this demethylation is genome-widely and occurs only in germ cells during migration and colonization. However, it remains unclear how global DNA demethylation is completed during PGC development [60, 61].

**The Onset of Meiosis Initiation and Meiotic Arrest**

Upon entry to the genital ridge, PGCs undergo several rounds of proliferation before beginning meiosis. Millions of germ cells are generated after these mitotic divisions are complete and meiosis begins. The mitosis-meiosis switch occurs at E13.5 in mice and around 30 days of gestation in cattle [62, 63]. Signaling from neighboring somatic cells is required for this initiation. One meiotic inducing molecule is retinoic acid (RA) [64], which is expressed by the gonadal neighboring somatic cells of both sexes but it only induces meiosis of germ cells in the fetal ovary. Germ cells in the fetal testis are protected from entering meiosis because several factors that degrade RA, including CYP26, are produced in the testes.

The patterns of oocyte meiotic progression appear similar among mammals. Oogonia arrest at the diplotene stage of meiosis I and remain at this stage until after puberty, which can be months or years away. During this early period of meiosis, homologous recombination occurs on 13-14 dpc in mice and in the 10-11th week of gestation in humans. These events are liable to the regulation of diverse proteins involved in DNA double strand breaks, synapsis and crossover (reviewed by [65]). Thus, mis-regulation of these events during meiosis could result in

aneuploidy that is one of the major factors conduce to low oocyte quality in humans as they age [65].

**Formation of the Primordial Follicles**

A series of events precedes follicle formation after meiotic arrest. During prenatal development, oocytes are clustered to form germ cell nests and are connected by intercellular bridges with each other. Coinciding with the breakdown of germ cell nests, massive germ cell loss occurs perinatally, [66, 67]. Members of BCL protein family are primarily involved in this process that is achieved by programmed cell death [68, 69]. However, the biological relevance of this massive loss of germ cells has not been described.

For unknown reasons, the breakdown of germ cell nests occurs after birth in mice and in the second trimester of human gestation, and the individual oocyte is enclosed by a single layer of pre-granulosa cells to form the primordial follicles. It appears that TNFα signaling is important for germ cell nest breakdown and the formation of primordial follicles [70]. Additionally, maternal hormones and certain transcription factors are involved in the formation of primordial follicles [71-73].

**Activation and Recruitment of Primordial Follicles**

During the remainder of prenatal development and early postnatal life, oocytes and follicles remain dormant. Upon puberty, subsets of activated primordial follicles are recruited to fulfill the mission to produce a viable oocyte. Oocyte-specific expression of transcription factors: Nobox, Lhx8, Sohlh1/2 are critical for the transition from primordial to primary follicle [74-76]. Mice lacking any of these factors are sterile. The progression to primary follicle is disrupted despite the fact that these mutants contain a similar number of primordial follicles. Furthermore, these phenotypes have been linked with reduced KIT/KITL signaling. Oocyte expression of KIT and pre-granulosa KIT ligand production is crucial for the transition from primordial to primary

follicle stage in mice. The interaction of this ligand with its receptor potentially changes granulosa cell shape from squamous to cuboidal [77-79]. This effect is likely mediated by the induction of the PI3K/AKT signaling pathway and subsequent inactivation of forkhead box O3 (FOXO3) [80]. In FOXO3 mutant mice, follicle growth is widespread and these mice lack primordial follicles [81]. Anti-mullerian hormone (AMH) [82] and the chemokine (SDF-1) and its receptor (CXCR4) also mediate recruitment of primordial follicles [83, 84].

Several additional paracrine factors also appear to function as mediators of primordial follicle development. The recruitment of primordial follicles is promoted by various factors including BMP4 [85], LIF [86], PDGF [87], GDNF [88], FGF2 [70] and FGF7 [89]. In one study, increases in the proportion of growing follicles and decreases in numbers of primordial follicles (i.e. not growing) were found in ovaries from 4-day-old rats cultured *in vitro* with FGF2, BMP4, LIF, PDGF, GDNF or FGF7 [70]. Administration of BMP7 to the rat ovaries also stimulates follicle devleopment [90].

In cattle, there is a lack of consistency for when primordial follicles first appear, although recent evidence suggests that primordial follicles are first observed around the beginning of the second trimester of pregnancy [91]. Several of the same mediators found to be important for mediating primordial follicle development in mice also appear important for this process in cattle [92].

Steroids, namely estrogen and progesterone, show inhibitory effect on activation of primordial follicles in cattle [91, 93]. Yang and Fortune have shown that maximum production of steroids by ovaries is detected at day 80 of gestation when meiosis has not yet been initiated, and decreased to undetectable level by day 160 when meiosis begins and follicle formation takes place [91]. Hence, it has been hypothesized that maternal hormones are negative regulators of

follicle development. Indeed, treatment of fetal ovarian pieces collected between day 90 and 140 of gestation with estradiol and progesterone reduced the proportion of activated (primary) follicles [92]. This evidence is consistent with the findings on *in vivo* pattern of secretion of steroids by the ovaries.

**Preantral Folliculogenesis**

After primordial follicles are established at 1-2 d after birth in mice and *in utero* in cattle, a subset of follicles will develop into preantral follicles. This process involves oocyte growth (i.e. increase in oocyte diameter), proliferation of granulosa cells into multiple layers, and the development of theca cells. The major mediators of these processes are derived from the oocyte and somatic cells (granulosa and theca cells). Gonadotropins do not appear important for preantral follicle development. For example, normal preantral folliclulogenesis is seen in mice deficient in the FSH receptor or the FSHβ subunits despite disrupted antral follicle growth [94, 95].

Extensive studies over the past decade reveal that the oocyte plays an active role in controlling preantral folliculogenesis [96-99]. One well-known experiment was carried out by Eppig to determine if the oocyte impacts the follicle developmental rate. In this study, secondary oocytes were collected and mixed with somatic cells from the primordial follicles to form 're-aggregated ovaries', which were then grafted beneath the renal capsules of ovariectomized host females [100]. Finally, accelerated folliculogenesis is observed in re-aggregated ovaries while the development of follicles is normal in re-aggregated control ovaries consisting of oocytes and somatic cells both from primordial follicles. This result clearly indicates oocyte derived factors are critical for follicle development [100].

A host of oocyte signals is found within the TGF-β superfamily of paracrine factors. Notably, BMP15 and growth differentiation factor-9 (GDF9) play very important roles in

regulating the folliculogenesis. These factors are produced by the oocyte and act on the neighboring cumulus and granulosa cells to regulate glycolysis, amino acid transportation, cholesterol synthesis and ultimately maintain the metabolism and development of the oocyte [96]. GDF9 knockout mice are infertile with follicles arrested at primary follicle stage [101]. Mouse BMP15 targeted disruption causes subfertility with a defective cumulus phenotype [102]. Also, BMP15 overexpression in mice reveals the growth-promoting role of BMP15 in ovarian follicles [103].

Linkage analysis shows that spontaneous BMP15 mutations also affects ovulation rate in sheep [104]. Interestingly, the homozygotic mutation results in ovarian failure, but, the heterozygotic mutation causes increased ovulation rate. It is possible that more antral follicles are responsive to LH in the presence of 50% of the normal level of BMP15 by compromising proliferation of granulosa cells and reducing production of steroids and inhibin in follicles. It is also likely that reduced level of BMP15 affect the actions of other oocyte secreted factors, such as GDF9, on granulosa cell proliferation and differentiation. Thus, these changes in the follicles may result in the increase of ovulation rate in the heterozygotic mutant.

**Antral Follicle Development**

Dramatic morphological and biochemical changes take place during antral follicle development. From the primary to preovulatory stage, the meiotically-arrested oocyte grows dramatically from 30 to 125 μM in diameter in cattle [105]. The fluid-filled structure, known as the antrum, is established within the follicle. The sensitivity of follicles to gonadotropin becomes apparent although paracrine and autocrine factors, notably IGF1 and epidermal growth factor (EGF)-like molecules, still function. Thus, follicle development is modulated by a more dynamic and complicated network of signaling to ensure the production of viable oocytes and steroids.

The antrum consists of a fluid-filled space that is established by fluid derived from serum and by induction of an osmotic gradient between the follicle and the serum in the thecal vascular [106]. Recent evidence has shown that aquaporin, a membrane channel protein, is involved in promoting passive movement of fluids through granulosa cells to establish the antrum [107]. *In vivo*, follicles that have antra shown increased expression of aquaporin 7 and 8 [108]

After formation of the antrum, follicles require FSH and LH for normal development [25]. FSH and LH are pituitary gonadotropins that are heterodimeric glycoproteins containing a unique β subunit and a common α subunit. FSH not only promotes proliferation and differentiation of granulosa cells, but prevents apoptosis in granulosa cells, stimulates estradiol production and LH receptor expression in granulosa cells, and suppresses follicular atresia [109]. Various positive/negative feedback loops in the hypothalamic-pituitary-gonadal (HPG) axis manipulate follicle development and selection as well as pregnancy establishment. Estradiol negatively feedbacks on the HPG axis to decrease secretion of FSH. Additionally, growth factors (i.e. inhibin, activin) derived locally from the ovary also regulate production of FSH [109].

Estradiol production is one of the milestone events in folliculogenesis. Its production is crucial for the growth, differentiation and survival of ovarian follicles and in triggering the surge of gonadotropins surrounding estrus and ovulation [110]. Estradiol production is stimulated by FSH in follicles. Intrafollicular estradiol production is linked to variation of follicle numbers during follicular waves, which is positively associated with improved oocyte quality [111]. This result implies that appropriate estradiol production is important for the oocyte to acquire competence during folliculogenesis.

The regulation of gonadotropin-induced estradiol production (steroidogenesis) in granulosa cells occurs in cooperation with the theca cells through a process known as the “two-cell, two-

gonadotropin" concept [52]. Thecal cells produce ample amounts of androgens but lack aromatase (CYP19A1), the key enzyme responsible for the conversion of estradiol precursor (androstenedione) to estrogens. In contrast, granulosa cells contain CYP19A1 but are deficient of CYP17A1, the key enzyme necessary to produce the estradiol precursor. Thus, theca and granulosa cells rely on each other to produce estradiol in granulosa cells. These key steriodogenetic genes are responsive to gonadotropins. LH induces the expression of CYP17A1 and StAR in theca cells whereas FSH stimulates CYP19A1 in granulosa cells.

Oocyte secreted factors are also involved in the regulation of antral follicle development. Several BMPs appear crucial for development. Notably, BMP15 and BMP6 prevent spontaneous apoptosis occurring in bovine cumulus cells by inducing the expression of anti-apoptotic gene, *Bcl2* [112]. Moreover, DNA synthesis in mural granulosa cells of antral follicles is promoted by oocyte signal, namely GDF9 [113].

Oocytes modulate several activities of cumulus cells. Oocyte-derived paracrine factors are required for the ovarian follicular response to LH, which is mediated by EGFR signaling [114]. It has been hypothesized that oocyte stimulates the expression of EGFR in the cumulus cells and thus enables them responsive to LH-induced EGF in mural granulosa cells. This mechanism has been validated by a recent report that oocyte factors, namely BMP15, stimulate EGFR level in cumulus cells by a SMAD2/3 dependent pathway [115, 116].

Growth factors secreted from follicular somatic cells also play a critical role in antral follicle development. One factor that has been characterized is IGF1. Although derived primarily from an extraovarian source, it is also produced locally in ovarian somatic cells and stimulates steroidogenesis and granulosa cell proliferation in cooperation with gonadotropins [117]. Mice lacking IGF1 are infertile with follicles arrested at the preantral stage [118]. Both IGF2 and type

1 IGFR mRNA are detected in follicles as early as preantral stage in cattle [119]. *In vitro*, IGF1 induces bovine preantral follicle growth [120]. The complete IGF system is functional during early antral stage development [121]. IGF binding proteins (IGFBP) derived locally are probably responsible for the regulation of functions of IGFI and II [122]. IGFBP2 is expressed by granulosa cells whereas IGFBP4 mRNA is detected in theca cells in cattle [123]. Fortune et al. has shown that IGFBP level in follicular fluid is negatively associated with emergence of the dominant follicle [124].

**Final Oocyte Growth and Maturation**

Due to the acquisition of LH receptors in granulosa cells as well as the increased production of estradiol, LH program of gene expression becomes predominant in the follicle and consequently the FSH program is turned off. Then, the majority of the follicles in the growing pool will undergo atresia, which is induced primarily by the reduction of FSH secretion. Only a dominant follicle or a few selected follicles (dependent on species) reach the preovulatory stage and are prepared for ovulation. Finally, the LH surge triggers the termination of preovulatory follicle development and initiates the ovulation, cumulus expansion, the meiotic resumption and luteinization.

**Preovulatory Follicles**

Preovulatory follicles are characterized by their large volume size and the stage-specific gene activation of the ovarian somatic cells. The increase of the follicle size is contributed to the proliferation of the granulosa cells in response to FSH and the expansion of the antral compartment. In terms of gene expression, more than 7000 genes are upregulated in either theca or granulosa cells in bovine preovulatory follicles [125]. In particular, high concentration of LH receptor is achieved in granulosa/cumulus cells in preovulatoy follicle so that it can enable the follicle responsive to the LH surge and initiate the downstream events.

**Follicle Selection**

There is generally one dominant follicle selected to undergo final differentiation and ovulation in cattle. Other unselected follicles will die by atresia. It is still poorly understood how the follicle is selected and how dominance is determined.

The follicle continues to grow after recruitment into the growing pool and becomes responsive and dependent on FSH after the early antral stage with a diameter of 1-3 mm. Then, a rapid increase of the volume is observed in a cohort of antral follicles due to the cyclic release of FSH. As noted above, the increased production of estradiol and inhibin feedback on the HPG axis to suppress the secretion of FSH. When the reduction of FSH reaches a certain extent, growth of subordinate follicles are arrested and the atresia is trigged in these follicles.

One follicle from the growing pool acquires the dominance due to unknown reasons.This dominant follicle grows under the control of LH pulses, controlled by progesterone concentrations [126]. If this selection occurs during the luteal phase of the estrus cycle (with high progesterone), dominance will disappear due to inadequate LH pulsatility [127]. Accordingly, secretion of estradiol and inhibin in the follicles is decreased, which release the inhibition on FSH production. Therefore, a new wave of follicle development will follow in response to a new FSH surge [126, 128]. Conversely, if dominance is acquired in the follicular phase of the estrous cycle, the high frequency of LH pulse will dictate the dominant follicle to continue growing and undergo the process of ovulation.

**Ovulation**

Triggered by increased serum estradiol concentration, the LH surge rapidly acts on granulosa cells to turn off follicular gene expression and initiate the expression of genes necessary of ovulation and luteinization [129]. The process of ovulation involves large amounts of prostaglandin production, synthesis of a hyaluronan-rich extracellular matrix, proteolytic

degradation of the follicular wall and final extrusion of the oocyte [129]. Based on these characteristics, ovulation is very similar to an inflammation-like process. For example, like during ovulation, expression of matrix-associated genes is promoted at sites of inflammation [130]

Several transcriptional regulators are required for LH-induced ovulation. One of these factors is progesterone receptor (PR) [131]. In response to the LH surge, PR expression is rapidly initiated in the mural granulosa cells of preovulatory follicles [132]. This evidence implicates the importance of progesterone signaling during the process of ovulation. Indeed, PR null mice show normal cumulus expansion but do not undergo ovulation [133]. Similar results are observed when PR are blocked with pharmacological drugs [25].

**Cumulus Expansion**

Expansion (mucification) of cumulus cells is required for normal ovulation and fertilization [134]. Cumulus expansion *in vivo* is induced in response to the LH surge. Likely, this process is also induced by the presence of FSH or EGF in the maturation medium *in vitro*. The chemical reaction that causes this involves the secretion of hyaluronan that disperses into the extracellular matrix and makes the cumulus cells sticky. There are various factors involved in the regulation of this process.

Several genes, namely *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip6*, are critical for production, stabilization and linkage of hyaluronan [52]. Hyaluronan synthase 2, the product of *Has2*, is responsible for the production of hyaluronan, the backbone of the cumulus matrix. TNFα-induced protein 6, the product of *Tnfaip6*, is required for the formation of chemical crosslinks between hyaluronan and the serum-derived molecule, inter-α-trypsin inhibitor. Pentraxin 3, the product of *Ptx3*, is important for the interaction between hyaluronan and IαI. Prostaglandin synthase 2, the product of *Ptgs2*, catalyzes the synthesis of prostaglandin and acts upstream of

TNFAIP6. Mice deficient in each of these genes have abnormal, a lack of cumulus expansion (*Ptgs2*, *Tnfaip6* and *Ptx3*) or are embryonic lethal (*Has2*) [135-138]. It is still not completely known how these proteins act with each other to achieve the formation of the matrix.

These genes are up-regulated by the LH surge through action mediated by EGF-like molecules. Conti et al. have found that the LH surge results in a rapid increase of EGF-like molecules, *Areg*, *Ereg* and *Btc*, which encode amphiregulin, epiregulin, and betacellulin, respectively in granulosa cells [114]. Indeed, all these molecules alone are sufficient to induce cumulus expansion *in vitro* through stimulating the expression of *Ptgs2*, *Has2* and *Tnfaip6*.

Downstream of LH surge-EGF-like molecules is mitogen-activated protein kinase (MAPK) signaling. Cumulus expansion is suppressed in follicles of MAPK conditional knockout mice [139]. Consistently, gonandotropin and EGF induced cumulus expansion *in vitro* is prevented by pharmacological blockade of MAPK signaling with U0126 [140]. Both LH and EGF-like molecules also result in the activation (phosphorylation) of MAPK3/1 [140].

The oocyte also plays an active role in the process of cumulus expansion [52]. To define the role of oocyte on the cumulus expansion, the classical oocytectomized (OOX) model has been widely used, in which the oocyte is removed via microsurgery. The expansion of cumulus cells in OOX complex is inhibited in the presence of expansion-inducing molecules (i.e. FSH, EGF, cAMP analog) *in vitro* [141]. However, inhibition is released when the OOX complex is cocultured with denuded oocytes. As oocyte secreted factors, BMP15 and GDF have been identified to restore the cumulus expansion in OOX complex.

**Resumption of Meiosis During the LH surge**

The oocyte resumes meiotic maturation after induction by the LH surge. The sequence of events occurring during this process includes germ vesicle breakdown (GVBD), chromatin condensation, spindle formation and ultimately the extrusion of the first polar body. The final

asymmetric cell division, which allows more distribution of maternal factors in oocytes, is achieved by a cortical migration of spindle that is dependent on actin microfilaments and actin binding protein formin-2 [142-145]. Meiotic maturation is required for normal development as immature oocytes are incapable of generating repeated $Ca^{2+}$ response upon fertilization [146].

The LH-dependent resumption in meiosis is mediated by EGF-like molecules [114]. As discussed above, the LH surge results in a rapid increase of EGF-like molecules, *Areg*, *Ereg* and *Btc*, in granulosa cells [114]. These EGF-like molecules are sufficient for meiosis reentry and required for LH action in preovulatory follicles [114]. It has also been shown that EGF supplementation improves cumulus expansion and nuclear maturation in cattle *in vitro* [147]. Hence, EGF is recognized as a regular supplement to the cattle oocyte maturation medium (OMM) *in vitro*.

As addressed above, MAPK is critical to mediate the role of LH surge in downstream events, such as cumulus expansion and meiosis reentry. After EGFR is activated upon binding with mature EGF-like molecules, MAPK (3/1) is stimulated. Defects in meiosis resumption, ovulation, cumulus expansion and luteinization are seen in mice with MAPK3/1 conditional knockout in granulosa cells [139]. Although oocyte MAPK is not the only mechanism that leads to GVBD, MAPK in follicular somatic cells is required for the gonadotropin-induced reentry to first meiosis division [139]. Specifically, MAPK3/1 (ERK1/2) activity in oocytes is increased throughout oocyte maturation *in vitro* and *In vivo* [148]. One of MAPK upstream kinases in vertebrate oocyte is MOS. MOS, a germ-cell specific Ser/Thr protein kinase, is the product of proto-oncogene c-mos [149]. It has been shown that increased MAPK activity could be observed by injection of mos-RNA to the oocyte [148]. Also, this results in a higher oocyte maturation rate

in bovine [148]. This observation highlights the importance of MAPK signaling in meiotic maturation.

The maintenance of meiotic arrest and its resumption after the LH surge are regulated primarily by maturation-promoting factor (MPF). This factor is responsible for the $G_2$/M transition of the cell cycle. MPF consists of cyclin-dependent kinase 1 (CDK1) and cyclin B. In preovulatory follicles, oocyte MPF is maintained at a low activity to prevent the breakdown of the nuclear envelope and chromatin condensation, that are required for the entry into the first meiotic division [150, 151]. However, removing an oocyte from the antral follicle will induce these processes to reinitiate the meiosis Thus, there are inhibitory factors in the mural granulosa cells that maintain the low level of MPF that achieve the meiotic arrest [152, 153].

Wee1b and Cdc25b are two direct upstream regulators of MPF activity. They are responsible for phosphorylation/dephosphorylation of CDK1, respectively [65]. Specifically, CDK1 is inhibited by phosphorylation on Thr14 and Tyr15. Genetic and *in vitro* evidence show that Wee1b and Cdc25b regulate the activity of CDK1 in oocyte [154, 155]. In addition, the regulation of Cyclin b1 is also important for meiosis arrest at MI. It is regulated by APC, which is negatively regulated by Emi1 [65].

Cyclic AMP is a major player in the regulation of the meiotic progression and functions upstream of MPF. Meiosis arrest is closely associated with the higher level of cAMP in oocytes. Reduced concentration of oocyte cAMP leads to meiosis resumption [156]. Thus, the mechanisms underlying meiosis arrest and resumption are generally surrounding how cAMP is regulated in oocytes and the downstream and upstream of cAMP.

The oocyte has endogenous machinery to maintain intracellular cAMP homeostasis. PDE3A, an oocyte-specific phoshpodiesterase, is responsible for reduction of cAMP in oocytes

after activation by LH surge [157]. Oocyte PDE3A activity is suppressed by the cGMP signaling initiated from granulosa cells, which thus maintain the meiotic arrest of oocytes [158]. Recently, natriuretic peptide precursor type C (NPPC) and natriuretic peptide receptor 2 have been found to be expressed in mural and cumulus granulosa cells, respectively [159]. NPPC increases cGMP concentration in both oocyte and cumulus cells through NPR2, which is a guanylyl cyclase. Meanwhile, NPPC inhibits meiotic resumption *in vitro*. NPPC or NPR2 mutant mice have precocious meiosis resumption [159]. It suggests NPPC might be the long-sought inhibitory molecule that derived from mural granulosa cells that line the wall of follicles.

The inhibitory effect of cAMP on meiosis resumption is mediated by the activation of PKA signaling pathway and then followed by the inactivation of MPF. However, it remains unclear whether PKA directly regulate the two direct regulators of MPF, Wee1b and Cdc25b.

**Cytoplasmic Maturation**

Cytoplasmic maturation is required for oocyte competence. Major components of this process involves reorganization in cytoplasmic organelles, changes in mRNA transcription and protein translation as well as post-translational modification of proteins [160]. The regulation of cytoplasmic maturation is poorly known as well as its markers.

One of the main features of oocyte maturation involves mitochondria remodeling. This probably helps to ensure sufficient energy supply. The mitochondria are redistributed in clusters around the pronuclei of zygotes [42]. The distribution of mitochondria is believed to impact embryonic development. Mitochondria are important for oocyte survival. Microinjection of mitochondria to oocytes reduces oocyte apoptosis [161]. Therefore, the membrane potential of mitochondria, indicative of mitochondria health, and number of mitochondria are thought to be potential predictors of oocyte competence [42].

The location of cortical granules also changes during final oocyte maturation. These organelles are generated from the Golgi complex and consist of a plasma membrane containing various proteins, structure molecules and glycosaminoglycans [162]. The cortical granule migrates to the inner surface of the ooplasm membrane in preparation for fertilization [163]. The role of this shift is primarily to block polyspermy [162]. Once the penetration of sperm occurs, the cortical granule will be released to the perivitelline space between the oocyte outer membrane and the zona pellucida. This process is induced by a cascade of events that triggers the increased intracellular calcium concentration. The released cortical granule functions on zona pellucida to modify the zona sperm receptor and results in zona hardening and hence block the penetration of more sperms.

One other dramatic change during oocyte maturation is mRNA transcription. The genome wide synthesis of mRNA is increased in growing oocytes and then ceases around the time of GVBD [164, 165]. After this point, some mRNA is degraded whereas other RNA is not degraded quickly but rather is stored and utilized for several days as the oocyte is fertilized and prepares to begin the synthesis of new embryonic mRNA. The stabilization of RNA is achieved by selective deadenylation of the 3' poly (A) tail [166]. Upon fertilization, more than 90% of maternal transcripts disappear before the first cleavage in mouse [167]. Some critical mRNA is stored for supporting fertilization and/or early embryogenesis. The translation of these stored mRNA is regulated by the polyadenylation, cis-acting CPE, CPE binding protein and/or maskin (reviewed by [168]). Along with the activation of the embryonic genome soon after fertilization, the translation of maternal RNA will be suppressed, which is believed to be regulated by deadenylation and/or binding with MSY2, a Y-box protein [169, 170]

It is likely that the stage-specific translation during oocyte maturation and early embryogenesis is regulated by the polyadenylation in bovine. *In vitro* evidence shows that polyadenylation and translation activation of mRNAs encoding proteins necessary for GVBD and chromatin condensation take place within 6 h after culture of the bovine cumulus oocyte complexes (COCs) [171]. Indeed, gonadotropin-induced meiotic maturation is prevented when the bovine COCs are treated with adenosine analogue cordyceptin, which inhibits the polyadenylation [171].

Several maternal factors have been identified that play important roles in early embryogenesis [172]. In cattle, several oocyte molecules have been found important for oocyte maturation and developmental competence. The bovine oocyte-specific gene, *JY-1*, promotes progesterone production in granulosa cells and is required for successful early embryogenesis [173]. Another important oocyte maturation factor is follistatin. Knockdown of locally-produced follistatin in the bovine oocyte by microinjection of siRNA to zygote reduces the number of zygotes developing to 8-16 cell stage and inhibits blastocyst formation [174]. Using the similar approach, several other factors have been linked with oocyte maturation. These include *Importin α8* [175] and *NOBOX* [176].

The dynamics of gene transcription throughout oocyte maturation, fertilization and early embryogenesis is also subject to the epigenetic regulation. The epigenetics is defined as "heritable changes in gene function that cannot explained by the changes in DNA sequence" [177]. Epigenetic modification involves the DNA methylation, histone modification as well as small non-coding RNA. Various lines of evidence have shown that these modifications are relatively dynamic during oogenesis and early embryogenesis [60].

As mentioned above, DNA methylation is a major epigenetic modification that involved in the transcriptional silencing of genes. There are several DNMTs. Among these, DNMT1 is the one responsible for maintaining methylation in DNA and DNMT3a and 3b are responsible for the *de novo* synthesis of DNA methylation. The oocyte has a specific isoform of DNMT1, DNMT1o, which is predominantly located in the oocyte cytoplasm. DNA methylation is associated with the developmental potential of the oocytes and embryos. It is reported that higher level of DNA methylation in the cloned embryos may account for the low development efficiency in animal cloning [178].

The dynamic pattern of DNA methylation is observed in the transition from oocyte maturation to early embryogenesis [60]. After fertilization, genomic DNA of paternal origin undergoes active demethylation while the maternal genome is passively demethylated. An intriguing question is how two different demethylation patterns occur in the same zygote. In addition, the methylation status of some sequences is well maintained during preimplantation development such as imprinting centers [179] and some repetitive sequences [180]. It is still a mystery what the mechanism is for distinguishing sequences to be demethylated and those to be maintained. *De novo* and maintenance DNA methylation throughout the whole genome is recovered during the period of perimplantation to build tissue-specific methylation status.

Histone modification is also very important for the oocyte and early embryogenesis. Various modifications of histone undergo dramatic change hours after fertilization in mice. In terms of the function, for example, high level of histone arginine methylation, catalyzed by CARM1, in 4-cell stage embryos will predispose blastomeres to contribute to the pluripotent cells of ICM [181].

**Potential Markers of Oocyte Quality**

Markers of oocyte quality have been sought out to improve our selection of oocytes that will develop into embryos and provide viable offspring. Microscopic evaluation of the COC is used to assess whether oocytes have the potential to mature normally in culture. Specifically, the intactness of the cumulus cells and integrity of the ooplasm is examined in many IVM systems [182]. Post maturation assessments also are useful markers of oocyte quality. Most attempts have been made by using cumulus cell expansion and polar body extrusion as non-invasive markers of oocyte potential.

Glutathione has been thought as a potential marker of cytoplasmic maturation. Its concentration in oocytes increases throughout final maturation [183-185]. Glutathione is the major cytosolic molecule that protect cells from oxidative damage by reducing reactive oxygen species (ROS) within mitochondria metabolism [183]. Attempts have been made to use low-molecular-weight thiols such as cysteamine to induce the production of glutathione and thereby improve embryonic competence in development [42].

Several molecules are linked with the developmental potential of oocytes in different species. The identification of these predicable markers will enable us to improve the reproduction efficiency in both human clinics and agricultural industry. In human, Cdc42, Hsd3b1, Fdx1, Serpine2 and Cyp19a1 are more highly expressed in follicular somatic cells associated with clinical pregnancies [186] and mRNA abundance of Pck1, Nfib and Bcl2L11in cumulus cells is indirectly associated with oocyte developmental competence [187]. In cattle, two independent laboratories determined that cathepsin B (CTSB) expression in cumulus cells is negatively associated with the oocyte developmental competence [188-190]. CTSB belongs to the cathepsin family of lysosomal cysteine proteinase, which has been involved in diverse biological activities [191-195]. Genome-wide gene expression analyzes have generated several

potential markers for oocyte competence [196-198]. Among these differentially expressed transcripts are *EGFR*, *TNFAIP6*, *HAS2*, *CD44* [197]. However, no firm causal relationship has been determined for any of these candidate genes.

## *In Vitro* Maturation of Bovine Oocytes

Maturation of oocytes *in vitro* is an important technology for human and animal ART. In this section procedures of IVM/IVF/IVC will be briefly reviewed as well as the factors affecting the IVP efficiency and potential solutions.

### IVM

This technique refers to the maturation of oocytes in culture. The starting materials, bovine ovaries, are generally derived from ovaries obtained at the slaughterhouse and transported to IVF laboratories in saline solution. Such transportation can take hours to complete. Oocyte used in IVM procedures generally are collected from mid-sized follicles [199]. These follicles contain germinal vesicle-stage oocytes and are appropriate for *in vitro* culture. Oocyte developmental competence is positively correlated with the size of the follicle [200], therefore, small-sized follicles are avoided. Meantime, large-sized follicles are presumably too old to culture *in vitro* in large animals. The oocytes used for IVM can also be obtained by ovum pick-up (OPU).

### Factors Impacting IVM Efficiency

A variety of factors are considered when developing an IVM system [42, 201]. In cattle, tissue culture medium (TCM) 199 is commonly used as the basic maturation medium. Various supplements are also used to mimic the *in vivo* follicular environment, and many laboratories use serum (e.g. bovine steer serum [BSS]), FSH and selective growth factors (like EGF) [202]. Successful IVM can be achieved under serum-free maturation conditions [203]. Several defined conditions include bovine serum albumin (BSA), PVA, or PVP in place of serum [204]. In our

laboratory, the day 8 blastocyst rate is 20-50% when the oocytes are cultured in serum free conditions.

**IVF and IVC**

Fertilization and culture of embryos *in vitro* (IVF and IVC) are widely applied in human clinics and agriculture industry [201]. For example in cattle, after maturation, the COCs are co-incubated with Percoll-purified spermatozoa in a culture dish with medium that induces sperm activation *in vitro* [199]. A pool of frozen semen from three different bulls is used to reduce the developmental variation between replicates, after 8-10 hours of incubation, the putative zygotes are removed from the cumulus cells by vortexing in hyaluronidase [199]. Then they are cultured in potassium simplex optimized medium (KSOM) or synthetic oviductal fluid (SOF)-based medium for another 7-8 days before transferring to the uterus or used in research [205].

**Low IVM Efficiency and Potential Solutions**

As mentioned above, the developmental competence of IVM/IVF-derived embryos is poor compared with when oocyte maturation and fertilization occurs in utero [40, 206]. IVP blastocysts have somewhat lower pregnancy rates at day 32 and increased pregnancy loss after first diagnosis than embryos collected from superovulated cattle [26, 40]. One cause is likely due to the gamete sources and, at least in part, to inadequacy of *in vitro* environment during IVM, IVF and IVC. One outcome is that the cytoplasmic and molecular maturation of oocytes is relatively low. This problem likely accounts for most of the embryo loss and pregnancy failure observed after embryo transfer [26]. Thus, development of optimal oocyte maturation conditions is critical for improving *in vitro* production efficiency.

Attempts to improve IVM efficiency have focused on manipulating maturation conditions so they more closely resemble the follicular niche environment. One way to do this is by supplementing factors that exist in the follicle but may be absent in COCs maturing *in vitro*.

Some supplements include cAMP analogs ( i.e. dibutyrul cyclic AMP (dbcAMP)), and PDE3 inhibitors (i.e. hypoxanthine) [207, 208]. These are used to regulate the cAMP level of oocytes. Their addition successfully improves embryo cleavage and developmental potential probably because increased cAMP delays spontaneous maturation and leaves more time for the oocyte to achieve cytoplasmic maturation.

Examining follicular fluid-containing factors is also of interest. Midkine, one heparin-binding factor, improves oocyte developmental potential when added to the IVM medium. More attention has been paid to those "positive factors" such as growth factors that have stimulatory role in folliculogenesis and/or oogenesis For example, the presence of EGF or IGF1 in the maturation medium improves the cytoplasmic maturation of bovine oocytes and promotes oocyte developmental potential [147, 209].

In summary, the efficiency of IVM of oocytes is critical for the entire IVP efficiency for use in human clinics and agriculture industry. However, the current IVM protocols still limit the developmental competence of oocytes. A growing body of evidence has demonstrated that IVM efficiency could be improved through optimization of various conditions, such as the search of beneficial supplements to the maturation medium. One recent outcome of such search is the family of fibroblast growth factors (FGFs).

## Fibroblast Growth Factors

At least 22 genes encode various FGFs that function as important paracrine regulators of proliferation, morphogenesis and angiogenesis in various tissues [210, 211]. Five FGF receptor (FGFR) genes are found and four of these gene products (FGFR1-4) belong to the tyrosine kinase family of receptors [211]. FGFR1-R3 generates two major isoforms: b and c, and several other minor subtypes by alternative splicing events. Some FGFs bind to multiple FGFR while FGFRs can bind to multiple FGFs. FGFs are well known for binding heparing or hepran sulfate

proteoglycans to stabilize their interaction with their receptors and protect from proteolysis [212].

## FGF and Reproduction

Various FGFs and their receptors are expressed within oocytes and follicular somatic cells in cows, mice and pigs [213-215]. Over the past twenty years, the FGFR signaling has been linked with reproductive functions in mammals such as sex specification, primordial follicle activation and granulosa cell differentiation. Accumulative evidence implicates the FGF system as an important regulatory system for folliculogenesis and oogenesis.

FGF2 is well studied for its role in follicle development. FGF2 is dramatically up-regulated in theca cells and granulosa cells during bovine follicle final maturation *in vivo* [216]. Gilbert et al. showed that FGF2 is upregulated by GnRH challenge *in vivo* [217]. These data indicate FGF2 is important for follicle final maturation or ovulation. One other study showed that FGF2 and its cognate receptor are dynamic expressed in the oocyte during maturation *in vitro* [218]. Granulosa cell production of FGFs, most notably FGF2 [219, 220] are linked to granulosa cell proliferation, LH receptor expression and primordial follicle development [221-224]. FGF2 SNPs were associated with bovine oocyte developmental competence [11].

In an earlier study, heparin-binding fractions screened from bovine follicular fluid were demonstrated to be beneficial for oocyte competence when supplemented to IVM medium [225]. It was later confirmed that midkine, one heparin-binding factor potentially exists in follicular fluid, stimulates embryo development after IVF when supplemented during IVM [226].

Another FGF that has been examined for its role in oogenesis is FGF7. This protein is detected in theca cells of primordial follicles in rats and localized in granulosa cells in bovine preantral follicles. FGF7 promotes activation of primordial follicles, enhances growth of preantral follicles and inhibits apoptosis in growing follicles in rats [89, 227]. It was also shown

that FGF7 modulates the KITL/KIT signaling, which is a crucial stimulator of early stage follicles.

Another member of the FGF7 subfamily of FGFs is FGF10. This factor is of particular interest as a candidate of oocyte competence factor. FGF10 mRNA is detected in both theca cells and oocytes in cattle [228]. Interestingly, FGF10 mRNA concentration is associated with health status of follicle. Specifically, FGF10 mRNA abundance is obviously higher in healthy follicle than atretic follicles [228]. The cognate receptor for FGF10 and FGF7, FGFR2b, is dynamically regulated by FSH in granulosa cells [228]. Recently, it was determined that FGF10 inhibits FSH-induced estradiol production and another FSH signaling target (angiotensin type 2 receptor) in bovine granulosa cells cultured *in vitro* [228, 229]. This result suggests FGF10 could inhibit FSH's actions on granulosa cells. Collectively, it is reasonable to expect that FGF10 is another heparin-binding factor that promotes oocyte competence.

Another subfamily of FGFs that have been examined recently is the FGF8 subfamily. This grouping consists of FGF8, 17 and 18, all of which have been linked with mammalian follicle development. FGF8 is produced by the oocyte in mice and acts cooperatively with BMP15 to promote glycolysis in cumulus cells [141, 230]. This fact is important as the mouse oocyte is incapable of glycolysis and requires cumulus cells to provide energy substrate such as pyruvate to support oocyte growth. FGF8 mRNA is also detected in follicular somatic cells in cattle, indicating its paracrine and autocrine function during folliculogenesis [231].

FGF18 mRNA is detected in theca cells and absent in oocytes in cattle [232]. The level of FGF18 mRNA in theca cells is greater in subordinate follicles than dominant follicles [232]. The expression of steroidogenic genes in granulosa cells are inhibited by FGF18 [232]. This suggests FGF18 may be associated with the atresia. FGF18 enhanced apoptosis of granulosa cells cultured

*in vitro* [232]. FGF17 mRNA is located primarily in oocytes rather than follicular somatic cells in cattle [233].

The expression of FGFRs is critical for the function of FGF signaling. FGFR1, 2 and 3 mRNA are detected in both theca cells and granulosa cells whereas FGFR4 mRNA is only found in theca cells in cattle [216, 234]. FGFR3c mRNA is also detected in bovine oocytes [234]. However, FGFR2b and R4 mRNA are absent in bovine oocytes [234, 235]. FGF2b and R3c mRNA abundance are increased along with follicle development [234]. Nonetheless, FGFR4 mRNA abundance is reduced in theca cells when the follicle size increases [231]. All of these evidences indicate the potential functional role of FGFR signaling in folliculogenesis.

Although the expression profiles of FGFs and FGFRs have been described extensively in rodents and ruminants, there are few reports of functional roles for these signals during the final stages of folliculogenesis. A more complete description of FGF-dependent signaling is, therefore, needed to understand how these paracrine factors function during the later stages of folliculogenesis and determine if this insight may be used to reduce the incidence of infertility in cattle and other mammals.

## Working Hypothesis

Ever-increasing evidence suggests that FGFs and their receptors participate in regulating follicle development [141, 228, 231]. The expression profile of most FGFs in ovarian cells has been characterized but no efforts have been made to use this information to improve oocyte competence, especially in regards to IVM systems. We propose that theca-, granulosa- and oocyte-derived FGFs can improve oocyte maturation and subsequent competence. These FGF effects likely impact cumulus cells. Thus, morphological and biochemical changes induced in cumulus cells may communicate with the oocyte to regulate the oocyte maturation, therefore influencing developmental competence.

Our overall hypothesis is that follicular and oocyte sources of FGFs take part in regulating granulosa and cumulus function and contribute to the follicular conditions that determine oocyte competence.

## Rationale and Significance

The decline of reproductive efficiency has a profound negative impact on the profitability of dairy cattle industry. The economic value of pregnancy in dairy industry can be calculated by the added value of lifetime milk production and value of the offspring in pregnant cows compared with the costs of re-breeding, feeding, and replacing cows that are not pregnant. A recent study determined that an average added value of $278 can be achieved when lactating dairy cows are pregnant [236]. Each pregnancy loss results in an average loss of $640 [237]. Therefore, strategies that aim to improve fertility or reduce pregnancy loss are needed. Completion of the proposed research may be used to develop schemes that enhance fertility in dairy cattle.

New knowledge may be gained regarding the regulation of oocyte maturation and the acquisition of developmental competence in cattle. Understanding of this biological basis of oocyte competence during IVM could lead to new methods to improve oocyte quality in cows by manipulating cellular and molecular mechanism of follicle development and oocyte growth and maturation. It may be possible to manipulate the embryonic developmental environment (reproductive tract) in the first days after insemination to ensure reproductive success. Second, a FGF-based strategy of genetic selection for fertility may be developed to improve reproductive performance in modern, high producing dairy cows. The use of genetic selection based on milk production has led rapid gains in milk yield. However, performance for other traits, notably female fertility, has declined [2]. Recently, several reports indicate FGF2 SNPs affect cow fertility [10, 11]. Lastly, FGFs may be supplemented to conventional OMM to improve *in vitro*

production efficiency in dairy cattle. Oocyte maturation *in vitro* is one important technology for genetic improvement and reproductive management of dairy cows and other animals [26]. Many technologies, such as *in vitro* production and somatic cell nuclear transfer, are dependent on oocyte maturation. Fully competent oocytes are determinants of the entire efficiency of these *in vitro* technologies. FGFs may improve the oocyte maturation and/or embryonic development to enhance reproductive performance.

# CHAPTER 2
# FGF10 ENHANCES BOVINE OOCYTE MATURATION AND DEVELOPMENTAL COMPETENCE *IN VITRO*

Oocyte competence is defined as the intrinsic ability of oocytes to resume meiosis, accept spermatozoa for fertilization, cleave after fertilization, and facilitate proper embryonic development that leads to the production of healthy offspring [24, 238]. Proper follicle development is vital for oocyte competence, and folliculogenesis is controlled by a variety of endocrine and intraovarian factors [25, 98, 238].

The oocyte plays an active role in regulating folliculogenesis. Specific members of the transforming growth factor-β superfamily of paracrine factors, most BMP15 and growth GDF9, are produced within the oocyte and act on cumulus and granulosa cells to regulate folliculogenesis and oogenesis. GDF9 is vital for folliculogenesis. GDF9-null mice are infertile and follicles fail to develop past the primary follicle stage [101]. Targeted disruption of BMP15 yields a subfertile phenotype in mice characterized by poor oocyte competence [102]. BMP15 over-expression promotes follicular development in mice [103]. Also, specific BMP15 mutations in sheep improve ovulation and lambing rates [104].

Another large class of paracrine-acting factors that has received some recent attention for their abilities to regulate follicular development and oocyte maturation are the FGFs. At least 22 genes encode various FGFs that function as important paracrine regulators of proliferation, morphogenesis and angiogenesis in various tissues [239, 240]. Several FGFs are expressed within oocytes and follicular somatic cells. FGF8 is produced by the mouse oocyte and acts cooperatively with BMP15 to promote glycolysis in cumulus cells in antral follicles [141, 230]. Granulosa cell production of FGFs, most notably FGF2 [219, 220] is linked to primordial follicle development, granulosa cell proliferation and LH receptor expression [221-224].

There also is evidence for FGFs providing a paracrine link between theca cells and granulosa/cumulus cells. FGF7 and FGF10 are produced by thecal cells in cattle [228, 235], and their primary receptor partner, FGFR2b, presides on granulosa cells [228]. FGF10 also is detected within the oocyte [228]. Studying the roles of these thecal- and oocyte-derived molecules is of particular interest since *in vitro* oocyte maturation systems lack thecal cells and their products, and the lack of these molecules may contribute to poor oocyte competence outcomes in IVP systems for cattle, humans and potentially other mammals [38, 98, 238].

The importance of FGF10 as a paracrine-acting mediator of oocyte competence was examined in this work. FGF10 is an important mediator of mesenchymal-epithelial communication in various organs and tissues [241-243]. Also, intraovarian FGF10 concentrations are associated positively with health status of bovine follicles [228]. Studies presented herein determined that FGFRs utilized by FGF10 are expressed in cumulus cells and oocytes and that providing FGF10 during IVM promotes bovine oocyte maturation, cumulus expansion and the rate of embryo development *in vitro*. Further studies were completed to provide insight into mechanisms controlled by FGF10 during oocyte maturation.

## Materials and Methods

Unless stated otherwise, reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All studies were completed in accordance and with the approval of the institutional animal care and use committee at the University of Florida.

### Bovine IVM, Fertilization, and Embryo Culture

Bovine oocyte IVM, IVF and IVC were completed as described previously [38, 124]. In brief, ovaries from beef and dairy cattle were obtained from Central Beef Packing Co. (Center Hill, FL, USA) and washed several times with 0.9% [w/v] sodium chloride supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin. COCs were collected, and groups of 10-12

COCs were cultured in 50 µl drops of OMM (TCM199 containing Earle's salts [Invitrogen Corp., Carlsbad, CA, USA] supplemented with 25 µg/ml bovine FSH [Bioniche Life Sciences, Belleville, Ontario, Canada], 2 µg/mlestradiol 17-β, 22 µg/mlsodium pyruvate, 50 µg/ml gentamicin sulfate, 1 mMglutamine, 1 mg/ml polyvinyl alcohol (PVA). Maturation medium was supplemented with varying concentrations of recombinant human FGF10 (Invitrogen Corp.; 0.5 to 50 ng/ml prepared in TCM199 containing Earle's salts) or immunoglobulin (IgG; anti-FGF10 polyclonal, IgG-purified [SC-7375] or control IgG [SC-2028], Santa Cruz Biotechnology Inc., SantaCruz, CA, USA). In one study, IVM was completed using denuded oocytes. For this, COCs were vortexed for 4 min to remove cumulus before maturation.

After 21-22 h at 38.5°C in 5% CO2 in humidified air, COCs were transferred to fertilization medium and exposed to Percoll gradient-purified bovine spermatozoa[38, 124]. The same pool of semen from three bulls was used throughout the studies. After 8 to10 h at 38.5°C (5% CO2 in humidified air), cumulus cells were removed by vortexingin 1000 U/ml hyaluronidase and denuded putative zygotes were placed in groups of 25-30 in 50 µl drops of synthetic oviduct fluid (mSOF; Millipore, Billerica, MA, USA) containing 25 µg/ml gentamicin sulfate, 0.4 mM sodium pyruvate, 2.77 mM myo-inositol, 0.5 mM sodium citrate, 1 mM alanyl glutamine, 5.3 mM sodium lactate syrup, 10 µl/ml non-essential amino acids, 20 µl/ml essential amino acids and 4 mg/ml fatty acid-free BSA). Drops were covered with mineral oil and maintained at 38.5°C in 5% CO2, 5% O2 and 90% N2 for 8 days. The proportion of cleaved zygotes and proportion of embryos containing 8-16 blastomeres was recorded on day 3 post-IVF. The proportion and stage of blastocysts (early, regular, expanded, hatching & hatched) was recorded at days 7 and 8 post-IVF.

**Nuclear Maturation, Cumulus Expansion and Apoptosis Following Oocyte Maturation**

After maturation, the degree of cumulus expansion in COCs were scored visually by phase-contrast microscopy on a 1 to 3 scale (1=poor expansion characterized by few morphological changes compared with before maturation; 2=partial expansion characterized by fair expansion but notable clusters lacking expansion; 3=complete or nearly complete expansion) as described previously [244].

To assess progression through meiosis after IVM, oocytes were denuded by vortexing for 4 min in saline after 6 or 21 h of maturation. Chromatin condensation status was determined at 6 h and meiotic staging and 1st polar body extrusion was completed at 21 h. At both time-points, oocytes were fixed with 4% (w/v) paraformaldehyde (Polysciences Inc., Warrington, PA, USA), permeabilized with 0.1% Triton X-100 and stained for 15 min with 1µg/ml Hoechst 33342(Invitrogen Corp.). Chromatin status and meiotic staging was determined with epifluorescence microscopy as described previously [32, 245]. The presence of 1st polar body extrusion was determined under stereomicroscopy.

In some studies, COCs were processed after IVM to determine the percentage of TUNEL-positive cumulus cells. For this, COCs were washed in 0.01 M PBS (pH 7.2) containing 1 mg/ml PBS-PVP and fixed in 4% (w/v) paraformaldehyde. COCs were permeabilized in 0.5%(v/v) Triton X-100, 0.1% (w/v) sodium citrate for 30 min at RT in a humidified box. Positive and negative controls were incubated in 50µl RNase-free DNase (50 U/ml; New England Biolabs, Ipswich, MA, USA) at 37°C for 1 h. COCs were incubated in 25µl drops of the TUNEL reaction mixture containingFITC-conjugated dUTP and terminal deoxynucleotidyl transferase (Roche Applied Sciences, Indianapolis, IN, USA) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of the enzyme terminal deoxynucleotidyltransferase. COCs were counterstained with 1 µg/ml Hoescht 33342, mounted on slides and analyzed with

epifluorescence microscopy. The proportion of TUNEL-positive cumulus cells was calculated by dividing the number of TUNEL-positive nuclei with total nuclei numbers in each of four fields under 200-fold magnification.

**Differential Staining in Blastocysts**

Differential staining for trophectoderm (TE) versus inner cell mass (ICM) cells were completed as described previously [21]. In brief, blastocysts obtained at day 8 post-IVF were incubated with 100µg/ml RNase A (Qiagen, Valencia, CA, USA) for 1h and transferred in as little solution as possible into 100µg/ml propidium iodide (PI; Invitrogen Corp.), 0.2% Triton X-100 and 1 mg/ml polyvinyl pyrrolidone (PVP) in PBS for 30 sec. After three washes in PBS-PVP, embryos were placed into a solution containing 10 µg/ml Hoechst 33342, 4% paraformaldehyde and 1 mg/ml PVP in 0.01 M PBS (pH 7.2) for 15min. After a final series of washes, blastocysts were placed into glycerol drops on microscope slides and TE and ICM cells were counted by using epifluorescence microscopy.

**Real-time Quantitative (q) RT-PCR**

Cumulus cells and oocytes (n=25 to 30/group) were separated from each other by vortexing either immediately after isolating COCs from follicles (0 h; no maturation) after 6 or 21-22 h after beginning IVM. Denuded oocytes were removed and washed thrice in PBS-PVP. Cumulus cells were transferred to microcentrifuge tubes and centrifuged at 700 × g for 2 min at room temperature to remove residual solution. Both oocytes and cumulus cells were snap-frozen in liquid nitrogen and stored at -80°C.

tcRNA was extracted from cumulus cells using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and from oocytes using the PicoPure tmRNA Isolation Kit (MDS Analytical Technologies, Sunnyvale, CA, USA). RNA concentrations were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA (10 ng/reaction for

cumulus samples; entire RNA sample for oocyte samples) was incubated in RNase-free DNase(New England Biolabs Inc.) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA). Primer sets (Table 2-1)were used in combination with a SYBR® Green detection system and a 7300 Real-Time PCR System (Applied Biosystems Inc.) to provide relative quantities of specific transcripts in cumulus and oocyte transcripts. Primers were used at a concentration of 200 nM and were mixed with RT products and SybrGreen PCR Master Mix (Applied Biosystems Inc.). After an initial activation/denaturation step (50°C for 2 min, 95°C for 10 min), 40 cycles of a 2-step amplification protocol (60°C for 1 min, 95°C for 15 sec) were completed. A dissociation curve analysis (60 to 95°C) was used to verify the amplification of a single product. Amplicons derived from newly synthesized primer pairs were sequenced (UF DNA Sequencing Facility) to ensure correctness of amplification. Each sample was run in triplicate and a fourth reaction lacking exposure to reverse transcriptase was included to verify the absence of genomic contamination. Relative amounts of 18S RNA was used as an internal control for quantifying relative gene expression. In one study, the ratio of target to reference RNA was used to determine relative expression (2-CT[target]/2-CT[reference]). The remaining studies used the comparative threshold cycle (CT) approach to determine relative abundance [246]. The average ΔCT value for each transcript was calculated (target CT– 18SCT) and used to calculate the fold-change (2-ΔΔCT).

**Statistical Analyses**

All analyses were completed with least-squares analysis of variance (LS-ANOVA) using the general linear model (GLM) of the Statistical Analysis System (SAS for Windows, version 9.0; SAS Institute Inc., Cary, NC, USA). Statistical analyses used arcsin-transformed percentage data generated from each replicate (experimental unit = average percentage within each

replicate). Differences in individual means were separated further by completing pair-wise comparisons (probability of difference analysis [PDIFF]; SAS Institute Inc.). Percentage data were graphed using non-transformed values and SEMs. Differential staining data were analyzed using embryo as the experimental unit, and data were analyzed to describe the effect of FGF10 treatment on TE and ICM cell numbers and proportion of cells that were ICM (ICM/Total). COC expansion was analyzed in two ways: 1) The mean COC expansion index was calculated for each treatment within each replicate and 2) the proportion of COCs observed within each of the non-parametric rankings (1, 2 and 3). When analyzing qRT-PCR data, either log-transformed ratios or ΔCT values, depending on the study was, used for the statistical analyses. Data were presented either as the ratio of target to reference RNA or as fold differences from control values.

## Results

### FGFR Transcript Profiles in Cumulus Cells and Oocytes

Four genes encode FGFRs in mammals (FGFR1-4), and alternatively spliced-variants for three of these FGFRs provide an extensive diversity of extracellular domains that bind various FGFs [247, 248]. Some FGFs bind to multiple FGFRs (e.g. FGF1, FGF2) whereas others, like FGF10, associate primarily with only a few receptor subtypes. FGF10 associates primarily with one spliced variant of FGFR2, termed R2b, and one spliced variant of the FGFR1 group, termed R1b [247-249]. An initial study was completed to describe the relative expression of these and other FGFRs in cumulus cells and oocytes. COCs were harvested and cumulus and oocytes were separated without IVM. Quantitative RT-PCR was completed to assess the relative expression of the b and c spliced variant forms of FGFR1 and R2 as well as FGFR3 (primers recognized both R3b and R3c) and R4 (no splice variants exist). In cumulus cells (Figure 2-1A), amounts of R1b transcripts were greater ($P<0.05$) than either of the R2 isoforms and R3 and R4. The abundance

of R1c was intermediate between R1b and the other FGFRs. In oocytes (Figure 2-1B), R2b and R2c mRNA levels were markedly greater ($P<0.05$) than the other FGFR transcripts, and R3 transcripts were barely detected.

**FGF10 Supplementation During IVM Improves Embryo Development**

The role of FGF10 during oocyte maturation initially was examined by collecting cumulus-oocyte complexes from slaughterhouse-derived ovaries and completing *IVM*, fertilization and culture. Methods described previously were used [38, 124] with the exception that OMM lacked serum (instead it contained 1% [w/v] PVA). FGF10 supplementation during oocyte maturation did not impact cleavage rates (Figure 2-2A), but supplementation with 0.5 ng/ml FGF10 increased ($P<0.05$) the percentage of cleaved embryos at the 8-16 cell stage by day 3 post-IVF (Figure 2-2B). In addition, 0.5 ng/ml FGF10 increased ($P<0.05$) the percentage cleaved embryos that developed to blastocysts on day 7 post-IVF (Figure 2-2C). The percentage of cleaved embryos that formed expanded, hatching or hatched blastocysts (collectively termed as advanced blastoycsts) on day 8 post-IVF also were greater ($P<0.05$) for oocyte cultures containing 0.5 ng/ml FGF10 than non-treated controls (Figure 2-2D). A biphasic response to FGF10 supplementation was evident throughout the study. Specifically, effects on percentage of 8-16 cell embryos, blastocysts on day 7 and advanced blastocysts on day 8 were not observed when 5 or 50 ng/ml FGF10 was provided (Figure 2-2B, C, D).

Differential staining was completed on a subset of blastocysts (n=17-28 blastocysts) to investigate whether FGF10 supplementation during oocyte maturation affects numbers of inner cell mass (ICM) and trophoblast cells. Exposing COCs to 0.5 ng/ml did not affect the numbers of ICM, trophoblast, overall cell numbers or ICM:trophoblast ratio at day 8 post-IVF (37.2 ± 4.1 vs. 42.8 ± 2.3 ICM/blastocyst, 134.8 ± 7.8 vs. 122 ± 8.7 trophoblast/blastocyst for controls and 0.5 ng/ml treatments, respectively).

**Blocking Endogenous FGF10 Action During Oocyte Maturation Reduces Subsequent Embryo Development**

Thecal cells are the predominant source for FGF10 in bovine follicles but the oocyte also produces FGF10 in antral follicles [228]. Anti-FGF10 IgG was used to determine the importance of oocyte-derived FGF10 during IVM. In the first study, the effectiveness of anti-FGF10 IgG at blocking FGF10 actions was examined. Providing an excess of anti-FGF (0.1 μg/ml; 25-fold excess compared with 0.5 ng/ml FGF10)did not affect cleavage rates or rates of early embryonic development (data not shown) but it effectively blocked FGF10 (0.5 ng/ml) from increasing ($P<0.05$) blastocyst rates on day 7 post-IVF (Figure 2-3A). Anti-FGF10 also reduced ($P<0.05$) the percentage of cleaved blastocysts when compared with non-treated controls (Figure 2-3A).

A second study was completed to further define the developmental events impacted by anti-FGF10 treatment in the absence of supplemental FGF10 (Figure 2-3B). Anti-FGF10 treatment did not affect cleavage rates, but there was a tendency for rates of cleaved embryos reaching the 8-16 cell stage on day 3 post-IVF to be decreased ($P=0.08$) in response to anti-FGF10 (45 ± 8.8% vs. 37.2 ± 13.4% for control vs. anti-FGF10 treatment).On day 7 post-IVF, fewer ($P<0.05$) blastocysts resulted from oocytes exposed to anti-FGF10 (0.1 μg/ml). In addition, providing a 3.9-fold molar excess of FGF10 (50 ng/ml) reversed the negative effect of anti-FGF10 on day 7 blastocyst rates.

**Effects of FGF10 on Oocyte Maturation**

The physiological basis for the newly described FGF10 effects was explored by determining if FGF10 supplementation affects oocyte maturation and cumulus expansion. The percentage of oocytes reaching telophase I or metaphase II (TI/MII) after 21-22 h of maturation also was influenced by FGF10 supplementation (Figure 2-4A). Supplementation with 0.5 or 5 ng/ml FGF10 did not affect the percentage of TI/MII oocytes but exposure to 50 ng/ml FGF10

increased (P<0.05) the percentage of TI/MII oocytes compared with controls. The percentage of oocytes extruding their first polar body was influenced by FGF10 supplementation (Figure 2-4A). Supplementation with 0.5 and 50 ng/ml FGF10 but not 5 ng/ml FGF10 increased (P<0.05) first polar body extrusion rates as compared to the control. A related study was completed to determine if endogenous sources of FGF10 impact bovine oocyte maturation(Figure 2-4B). Supplementing anti-FGF10 (0.3 μg/ml) during IVM did not affect the percentage of TI/MII oocytes and first polar body extrusion rates atv21h post-maturation.

The ontogeny of oocyte maturation was examined by describing how FGF10 affects chromatin condensation during IVM (Figure 2-4C). Adding 50 ng/ml FGF10 increased (P<0.05) the proportion of oocytes containing condensed chromatin after 6 h. Supplementation with 0.5 ng/ml FGF10 or anti-FGF10 did not affect the percentage of oocytes with condensed chromatin.

In a final study, oocytes devoid of cumulus cells were matured in the matured *in vitro* in the presence or absence of FGF10 or anti-FGF10 to examine the site of FGF10 action on oocyte maturation (Figure 2-5). Providing 50 ng/ml FGF10 decreased (P<0.05) the percentage of TI/MII oocytes at 21 h post-IVM. Adding 5 ng/ml FGF10 or anti-FGF10 did not affect the percentage of TI/MII oocytes. Also, none of the treatments affected first polar body extrusion rates.

**Effects of FGF10 on Cumulus Expansion**

FGF10 also impacted cumulus expansion rates during IVM (data not shown). The first study examined the dose response effect of FGF10 supplementation on the degree of cumulus expansion (rank scoring from 1 to 3). A biphasic response to increasing FGF10 concentrations was observed. When examining the mean cumulus index score for each treatment, cumulus expansion was increased (P<0.05) in oocytes matured in medium containing 0.5 ng/ml but not greater concentrations of FGF10. Data also were analyzed to determine the proportion of COCs in each rank category. The proportion of oocytes with fully or near fully expanded cumulus

(index score=3) was greater ($P<0.05$) in oocytes supplemented with 0.5 ng/ml FGF10 than controls but not when greater amounts of FGF10 were supplemented. TUNEL analysis was completed on a subset of the COCs used in these experiments, and supplementation with FGF10 did not affect percentage of apoptotic cumulus cells at 21 h post-maturation (data not shown).

The effect of endogenous FGF10 on cumulus expansion rates was examined by anti-FGF10 supplementation. As described in the previous study, adding 0.5 ng/ml FGF10 increased ($P<0.05$) cumulus expansion score and co-supplementation with anti-FGF10 (0.1 μg/ml) blocked ($P<0.05$) this effect. In a subsequent study, providing anti-FGF10 in the absence of exogenous FGF10 reduced ($P<0.05$) the mean COC expansion score.

**Effects of FGF10 on Cumulus and Oocyte Gene Expression**

To examine the molecular basis of FGF10 actions during oocyte maturation, expression profiles for candidate genes were completed on cumulus cells and oocytes after 21 h of maturation. Changes in the relative abundance of only a few transcripts were evident in cumulus cells and oocytes after 21 h of maturation (Figure 2-6). Adding 0.5 ng/ml FGF10 decreased ($P<0.05$) concentrations of CTSB and SPRY2 mRNA in cumulus cells (Figure 2-6A) and increased ($P<0.05$) BMP15 mRNA abundance in oocytes (Figure 2-6B).

A second study was completed to describe how FGF10 impacts candidate gene expression after 6 h of maturation. No changes in transcript abundance were detected in the subset of the cumulus-specific transcripts (CTSB, EGFR, FSHR, HAS2, SPRY2) and all of the oocyte-specific transcripts (data not shown).

## Discussion

Embryos generated from IVM and fertilization procedures usually are less able to produce viable offspring than *in vivo*-derived embryos in cattle and humans [25, 38, 250]. The absence of specific thecal-derived products during IVM and development may be one reason for these

reductions in oocyte and embryo competence. This work determined that at least one thecal-and oocyte-derived product, FGF10, improves embryogenesis when provided during *in vitro* oocyte maturation.

The first study described the types of FGFRs expressed in bovine cumulus cells and oocytes as they begin IVM. Transcripts for each of the four genes were readily evident in cumulus cells. Oocytes also contained transcripts for all four receptor classes, although very little FGFR3 mRNA was detected. As described previously, FGF10 reacts primarily with R1b and R2b with high affinity and with several other FGFRs with much lower affinities [247-249]. Transcripts for R1b and R2b were detected in both tissues and R1b mRNA was the predominant FGF10 receptor partner in cumulus cells whereas R2b was more prevalent in oocytes. These differences in expression profiles likely are an important feature to the various FGF10 responses observed in COCs.

Cleavage rates were unaffected in oocytes supplemented with FGF10 during IVM, but subsequent *in vitro* embryo development rates were improved. The most obvious post-fertilization effects included; 1) the proportion of embryos reaching the 8-16 cell stage on day 3, and 2) the proportion of embryos reaching the blastocyst stage on day 7 and advanced blastocysts on day 8 post-IVF. It remains unclear exactly how FGF10 treatment promotes embryo development, but exposure to FGF10 did not affect blastomere numbers at day 8 suggesting that the embryotrophic effects of FGF10 do not require improvements in blastomere numbers on days 7 and 8. Similar outcomes were observed by others [251, 252]. In those studies, bovine oocyte quality affected subsequent blastocyst yields more so than blastocyst quality. Perhaps FGF10 enhances the ooplasm microenvironment by altering concentrations of specific molecules that promote embryo development during the first few cleavage events. EGA occurs at

the 8- to 16-cell stage in bovine embryos [253]. Improvements in embryo development were detectable that this time, and it is quite possible that enhancements in the early developing embryo created a greater proportion of competent 8-16 cell embryos that continued to develop to blastocysts.

Biphasic oocyte responses were evident when FGF10 was added to maturation medium. Observing maximal responses with 0.5 ng/ml indicates that FGF10 likely interacts with high affinity FGFRs (i.e. R1b and/or R2b)to elicit its response (ED50 = 0.1 to 1 ng/ml; [247, 254]). Similar biphasic dose responses to FGF supplementation, and other paracrine-acting factors for that matter, are evident in various cell types [255-259]. This phenomenon could have been caused by receptor down-regulation events associated with ligand overloading [257]. Alternatively, this effect could reflect differential receptor usage that could have prompted secondary signaling systems that interfered with the primary signaling response [255, 260, 261].

Subsequent investigation into how FGF10 improves oocyte competence determined that FGF10 improves several aspects of oocyte maturation. One of the interesting findings was the ability of FGF10 to improve cumulus expansion. The magnitude of FGF10 effects on cumulus expansion scores and the percentage of COCs that were fully expanded after 21 h were not great, likely because expansion rates already were fairly great to begin with, but they did occur when using the same concentration of FGF10 observed to improve subsequent embryo development (0.5 ng/ml). An endogenous source of FGF10 also appears important for cumulus expansion in cattle. In situ hybridization work in bovine follicles found copious amounts of FGF10 mRNA in thecal cells and immunoreactive FGF10 protein throughout the thecal and granulosa layers [228]. Oocytes also contain FGF10 transcripts [228]. The IgG treatments employed for this work likely targeted oocyte-derived FGF10 and any residual thecal-derived FGF10 that remained bound to

extracellular matrix within COCs. The IgG neutralization appeared specific for FGF10. This IgG was used previously to neutralize FGF10 actions on mouse incisor growth [262]. In the present work, providing IgG molecules prevented supplemented FGF10 from stimulating cumulus expansion and subsequent embryo development rates, and providing molar excesses of FGF10 partially overcame the neutralization effects of FGF10 IgG on embryonic development to the blastocyst stage. Also, providing FGF10 IgG in the absence of supplemental FGF10 decreased cumulus expansion. These observations implicate modifications in cumulus cell activity as a potential source for FGF10-induced increases in oocyte competence.

FGF10 also affects several aspects of oocyte maturation. FGF10 improved the percentage of oocytes containing condensed chromatin after 6 h of maturation and oocytes progressing to TI/MII and extruding their first polar body after 21 h. However, the concentrations of FGF10 needed to observe some of these responses were greater than those needed to improve subsequent embryo development. With the exception of the polar body extrusion outcomes, other benefits to oocyte meiotic maturation required 50 ng/ml FGF10. Providing anti-FGF10 did not affect meiotic maturation rates. Also, maturation rates of denuded oocytes were reduced by FGF10 treatment. Meitoic maturation and polar body extrusion occurs, albeit retarded, in the absence of cumulus cells [147, 263]. Therefore, it appears that the rate of oocyte maturation is not a critical component to FGF10-dependent improvements in bovine oocyte competence. Also, cumulus cells appear vital for improvements in oocyte maturation occurring after providing large concentrations of FGF10.

To further understand how FGF10 improves oocyte competence, a series of transcripts identified by others as putative competence markers in cumulus and oocytes were examined. Several cumulus-specific transcripts are linked to oocyte competence. The gene encoding

cathepsin B (CTSB) contained an interesting FGF10-dependent expression pattern. Several cathepsin transcripts, including CTSB and CTSZ, are inversely related to oocyte quality. In one study, cumulus derived from COCs of prepubertal heifers, a model of poor oocyte competence, contained more CTSB mRNA than COCs from mature cows[190]. Oocyte quality and post-fertilization development could be improved by exposing COCs to a membrane-permeable cathepsin inhibitor [190]. In another study, cows with a low rate of antral follicle development contained greater amounts of *CTSB* mRNA than cows with higher numbers of antral follicles [111]. In the present work, *CTSB* mRNA abundance decreased in cumulus from COCs exposed to FGF10.CTSZ, by contrast, exhibited no FGF10-dependent changes in transcript abundance. FGF10 supplementation also reduced cumulus concentrations SPRY2 mRNA. This is not surprising given that SPRY2 is a modulator of FGF signaling [116]. The remaining cumulus competence markers were not impacted by FGF10. Included in this work were the following transcripts: EGFR and FSHR, receptors essential for normal oocyte maturation and cumulus expansion [197, 264]; HAS2, a key player in cumulus expansion [113, 197]; KITL, a component of stem cell survival and oocyte growth [265]; and INHBA, an inhibin subunit [197].

Several oocyte competence factors remained largely unaffected by FGF10 treatment. Included among these were FST[174, 266],GDF9[113],JY1[173]and H2A[264, 267]. FGF10 supplementation did, however, impact BMP15expression. BMP15 is linked with oocyte competence. Supplementation with BMP15 during IVM improves subsequent blastocyst rates in bovine oocytes [266]. Therefore, it is possible that FGF10 improves oocyte competence through BMP15. A more complete understanding of gene expression changes are needed to more completely understand the magnitude of FGF10's effects on the cumulus cell and oocyte during maturation.

It remains unclear what extent using a heterologous recombinant protein had on the outcomes. The human FGF10 protein chosen for this work is 94% identical in amino acid sequence to bovine FGF10. It reacts with approximately the same potency as other FGFs on various tissues, although a subtle reduction in activity was observed when comparing the potency of this protein against other FGFs (FGF1, 2, 7) for their ability to stimulate interferon-tau production in bovine TE [268]. It is presumed that human FGF10 reacts with the same FGFR subtypes as bovine FGF10, although that has not been verified. It remains possible, therefore, that certain aspects of FGF10 activity during oogenesis were not identified by using human FGF10 in bovine COCs.

In summary, work presented herein provides evidence that thecal- and oocyte-derived FGF10 improves oocyte competence. Providing FGF10 to bovine COCs during IVM improved oocyte maturation, cumulus expansion and subsequent embryo development. The mechanisms controlled by FGF10 have not been elucidated. A closer examination of metabolic, transcriptomic and proteomic changes regulated by FGF10 is warranted. However, based on present findings, FGF10 likely acts on cumulus cells in ways that improve their ability to regulate meiosis and provide ooplasm with components of importance for early embryonic survival.

Table 2-1. Primers used for qRT-PCR.

| Transcript | Primer sequence (5' to 3') |
|---|---|
| *Oocyte* | |
| *BMP15* | For: GTCAGCAGCCAAGAGGTAGTG |
| | Rev: CCCGAGGACATACTCCCTTAC |
| *FST* | For: CAGAGCTGCAAGTCCAGTACCA |
| | Rev: CATGTAGAGCTGCCTGGACAGA |
| *GDF9* | For: GGGAAATGTGTTCCTTGCTAATTC |
| | Rev: AGCAGCAAAACCAAAGGAAGAA |
| *H2A* | For: GTCGTGGCAAGCAAGGAG |
| | Rev: GATCTCGGCCGTTAGGTACTC |
| *JY1* | For: TTGGAACTTCCATGGACGACC |
| | Rev: ATTTGCTGGTGATCCCAAGAG |
| *Cumulus* | |
| *CTSB* | For: CGATGCCCGGGAACAGT |
| | Rev: GAGCACAGGATCCCTGATC |
| *CTSZ* | For: GGGAGAAGATGATGGCAGAAAT |
| | Rev: TCTTTTCGGTTGCCATTATGC |
| *EGFR* | For: ACCCTGATCTGGAAGTTTGCA |
| | Rev: TCGAGACCTGGCCCTTCAC |
| *FSHR* | For: TGGTCCTGTTCTACCCCATCA |
| | Rev: GAAGAAATCCCTGCGGAAGTT |
| *HAS2* | For: TAAATGTGGCAGGCGGAAGAAGG |
| | Rev: GTCTTTGTTCAAGTCCCAGCAGCA |
| *INHBA* | For: GCTACCACGCCAACTACTGTGA |
| | Rev: AGAGGGATGAGCCCGATGT |
| *KITL* | For: AATGGGCAGCCGTAGCATT |
| | Rev: TCCAGTAAAAGGCCCCAAAA |
| *SPRY2* | For: CACGTGCTGTCTCTGGATCAG |
| | Rev: GGCCCCTCCGTGTACTCA |
| *FGFRs* | |
| *R1b* | For: ACGTCCTGGTGACGGAGG |
| | Rev: CCGGTGCCATCCATTTGA |
| *R1c* | For: ACTGCTGGAGTTAATACCACCG |
| | Rev: GCAGAGTGATGGGAGAGTCC |
| *R2b* | For: GTGGAAAAGAACGGCAGTAAATA |
| | Rev: GAACTATTTATCCCCGAGTGCTTG |
| *R2c* | For: CACCACGGACAAAGAAATTG |
| | Rev: ATGCAGAGTGAAAGGATATCCC |
| *R3* | For: GCAGCGGCTACAGGTGCTCA |
| | Rev: CAGGCCGCGTCCAGTAAGGG |
| *R4* | For: GCAGACGCTCCTCACCCGAC |
| | Rev: CGAGACTCACGAGGCCAGCG |

Note: The relative standard curve approach (5 serial-dilutions of a follicular/oocyte RNA pool) was used to verify primer efficiencies. The following primers sets were designed previously: *CTSB* and *CTSZ* [190]; *BMP15* [269]; *JY1* and *FSHR* [111]; *H2A* [270]; *HAS2* [197]; *FGFR1b* [271]; *FGFR1c* and *R2c* [272]; *FGFR2b* [273]. Remaining primers were designed using Primer Express Software (version 3.0; Applied Biosystems Inc.).

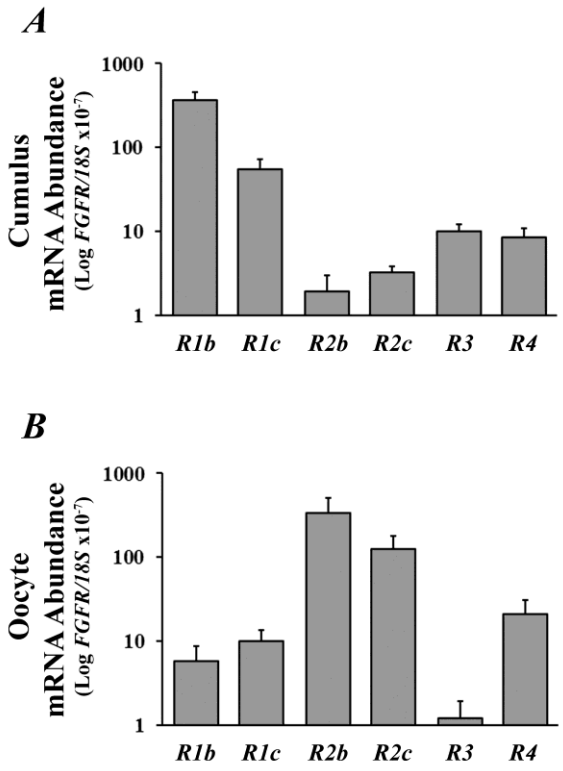


Figure 2-1. Expression profiles of *FGFRs* in bovine cumulus cells and oocytes prior to maturation. Cumulus and oocytes were separated before maturation, tcRNA was extracted and qRT-PCR was completed. Log-transformed ratios of the target mRNA to reference RNA (*18S*) were used for statistical analysis. A): *FGFR* abundance profiles in cumulus cells (each replicate contained cells derived from 20 COCs; 3 replicate studies). B): *FGFR* profiles in denuded oocytes (20 oocytes/replicate; 3 replicate studies). Different superscripts within each transcript represent differences observed due to treatments ($P<0.05$).

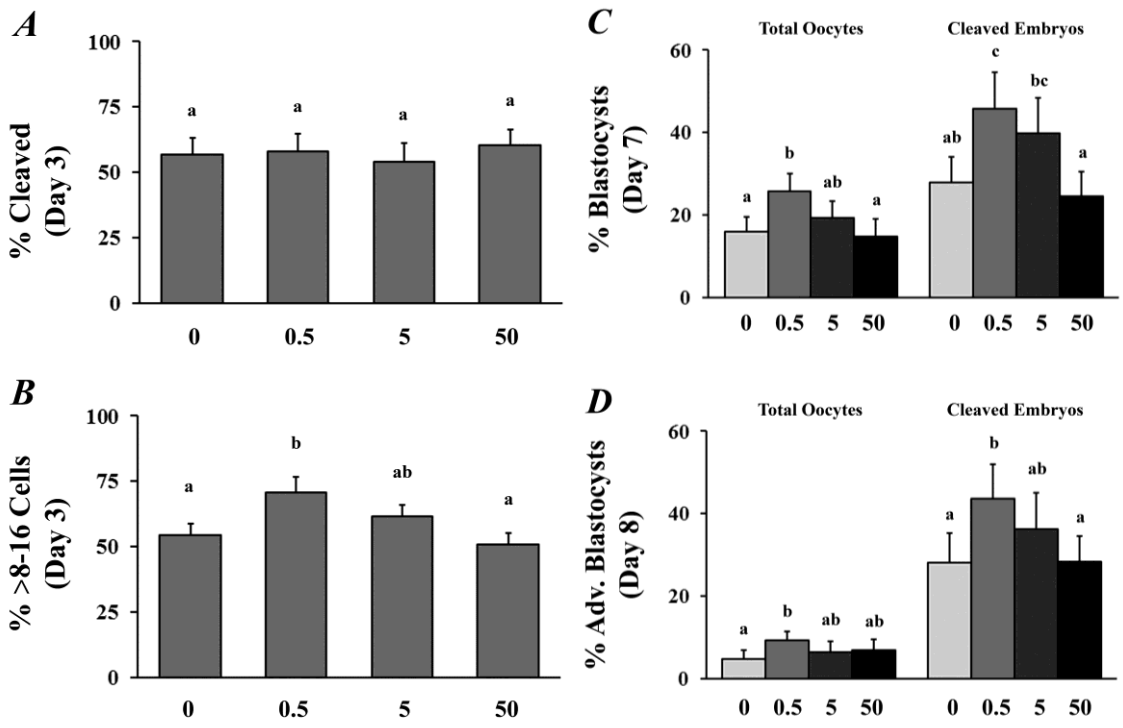


Figure 2-2. *In vitro* development of bovine embryos is enhanced by adding FGF10 to medium during oocyte maturation. COCs were matured for 21-22 h in maturation medium containing 1 mg/ml PVA (no serum) and either 0, 0.5, 5 or 50 ng/ml FGF10, then were fertilized and cultured. Presumptive zygotes were inspected visually using a stereomicroscope to assess embryo development status at specific times. A) The proportion of zygotes that cleaved at 72 h post-IVF. B) The proportion of cleaved zygotes at 72 h post-IVF that contained between 8- and 16-cells. C) The proportion of oocytes (left side) or cleaved embryos (right side) that reached the blastocyst stage by day 7 post-IVF. D) The proportion of oocytes (left side) or cleaved embryos (right side) that reached advanced blastocyst stages on day 8 post-IVF (expansion, hatching, hatched). All values represent means and SEMs from 8 replicate studies, each containing from 20 to 33 zygotes/treatment group. Different superscripts within each panel represent differences observed due to treatments ($P<0.05$).

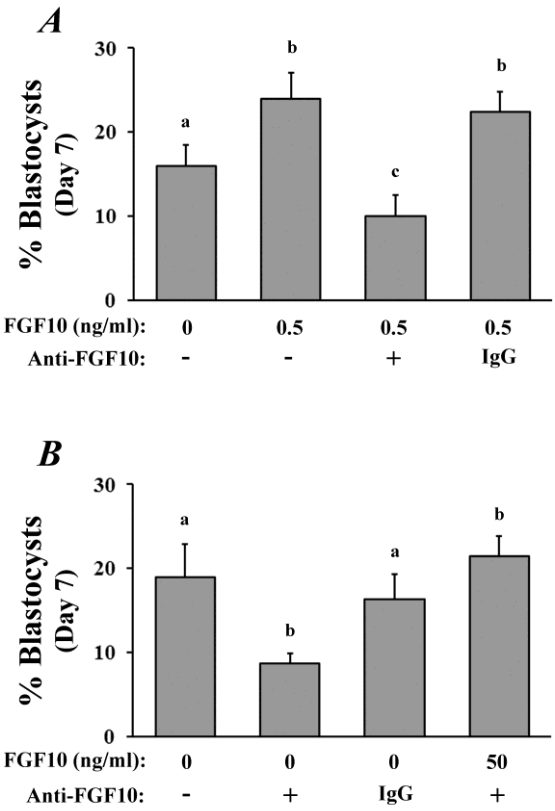


Figure 2-3. Evidence for an endogenous source of FGF10 during oocyte maturation *in vitro*. COCs were cultured in maturation medium in the presence or absence of anti-FGF10. FGF10 was included in some samples to verify the effectiveness of the anti-FGF10 treatment. Control IgG (labeled as IgG) also was included. A) The effects of providing anti-FGF10 (0.1 μg/ml) in the presence or absence of 0.5 ng/ml FGF10 during COC maturation on blastocyst formation on day 7 post-IVF (n=7 replicate studies). B) Limiting the effects of anti-FGF10 by providing excess FGF10 (50 ng/ml) (n=4 replicate studies). Different superscripts within each panel represent differences observed due to treatments ($P<0.05$). Data are presented as the percentage of oocytes that developed to blastocysts on day 7 post-IVF.

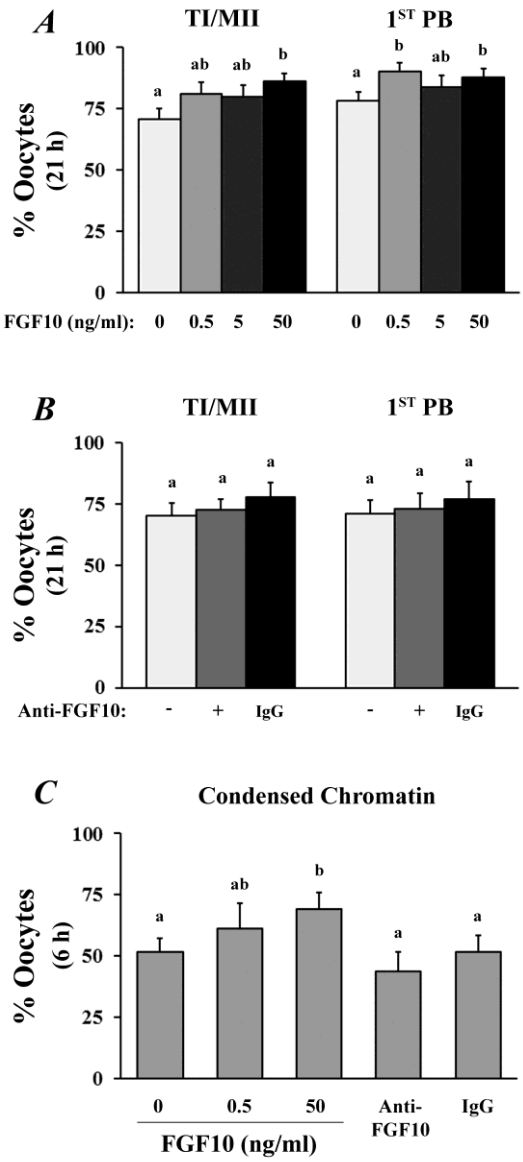


Figure 2-4. FGF10 impacts oocyte progression through meiosis and first polar body extrusion. A, B): After 21-22 h, cumulus was removed and denuded oocytes were processed to determine the proportion of oocytes reaching telophase I (TI), meiosis II (MII; B only) and extruding their first polar body (1st PB). A) The effect of FGF10 supplementation during IVM on oocyte maturation (n=6 replicate studies; 8-10 oocytes/treatment/study). B) The effect of adding anti-FGF10 (or control IgG; each at 0.3 μg/ml) during IVM on oocyte maturation (n=6 replicate studies; 8-10 oocytes/treatment/study). C) After 6 h of maturation, cumulus was removed and denuded oocytes were processed to determine the proportion of oocytes containing condensed chromatin (n=5 replicate studies; 15-20 oocytes/treatment/study). Different superscripts represent differences observed due to treatments ($P<0.05$).

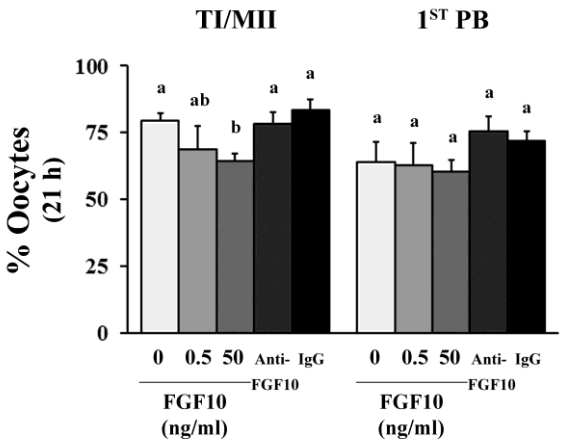


Figure 2-5. FGF10 does not stimulate maturation in denuded oocytes. Cumulus was removed from oocytes before maturation, and denuded oocytes were incubated in maturation medium containing FGF2 (0, 0.5 or 50 ng/ml) or IgG (0.3 μg/ml anti-FGF2 or control IgG) (n=6 replicate studies; 15-20 oocytes/treatment/study). After 21-22 h, the proportion of oocytes reaching TI or MII and undergoing 1st PB extrusion was determined. Different superscripts represent differences observed due to treatments ($P<0.05$).

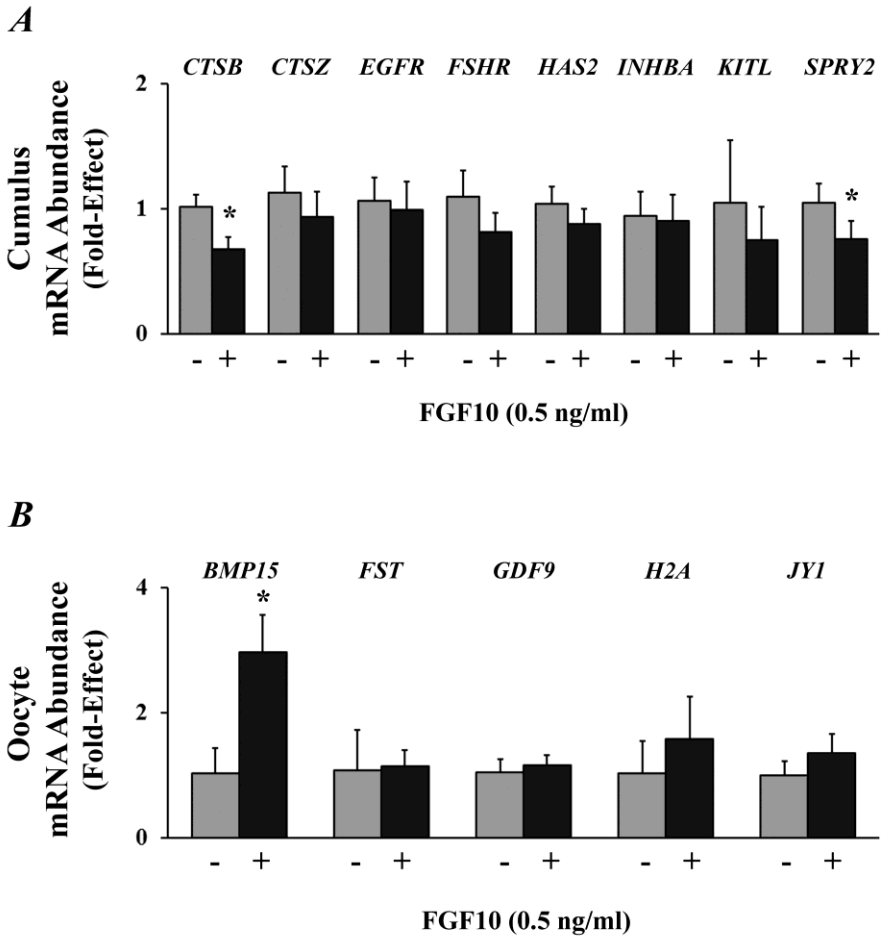


Figure 2-6. The effect of FGF10 supplementation on expression profiles of selective transcripts in cumulus cells and oocytes. COCs were cultured for 21 h in maturation medium containing or lacking 0.5 ng/ml FGF10. Cumulus cells and oocytes were separated, tcRNA was extracted from each and qRT-PCR was completed. A) Expression profiles for cumulus cells (n=5 samples/treatment; each sample contained cumulus cells from 20-25 COCs). B) Expression profiles for oocytes (n=6 pools/treatment; each pool contained 20-25 oocytes). $\Delta C_T$ values were used for statistical analysis, and data are reported based on fold change (± SEM) relative to the control (no FGF10 treatment). Astericks (*) indicates differences observed between treatment groups for a given transcript ($P<0.05$).

# CHAPTER 3
# SUPPLEMENTATION WITH FGF2 PROMOTES MATURATION AND SUBSEQUENT DEVELOPMENTAL COMPETENCE OF CULTURED BOVINE CUMULUS OOCYTE COMPLEXES

A suitable follicular environment is needed to generate an oocyte that can be successfully fertilized and eventually produce a healthy offspring. Several paracrine and autocrine factors within the follicular niche control oocyte maturation and play crucial roles in dictating subsequent embryo competence [25, 98, 238].

One group of paracrine and autocrine factors that have been examined recently for their involvement with folliculogenesis and oogenesis are FGFs. Numerous FGFs exist in mammals, and a majority of these molecules function as local (*i.e.* paracrine, autocrine, intracrine) and endocrine mediators of mitogenesis, morphogenesis and angiogenesis in various cell types [239, 240]. The receptors that associate with FGFs (termed FGFRs) are multigenic, and products for each of the four major *FGFR* genes contain receptor tyrosine kinase activity within their intracellular domains [247, 248]. Numerous alternative splicing events occur within the extracellular region of these receptors to generate a complicated array of ligand-receptor interactions. Various FGFs and FGFRs have been detected in oocytes and follicular somatic cells of cattle [203, 216, 228, 231, 235], pigs [215], mice [141, 213, 230] and women [219, 220]. FGFs are involved in various biological processes during folliculogenesis in rodents, including primordial follicle activation and regulation of granulosa and cumulus cell mitosis, apoptosis and glycolysis [141, 222, 223, 274].

Several activities for FGFs have been described in cattle and other ruminants during follicle development and oogenesis. An FGF of recent interest is FGF10. This molecule is produced by theca cells and oocytes in cattle and its primary receptor partners, FGFR1c and FGFR2b, are produced by granulosa and cumulus cells [203, 228, 235]. Concentrations of

*FGF10* mRNA were greater in healthy, growing bovine follicles than in follicles undergoing atresia [228]. In another study, FGF10 supplementation sustained survival and increased development of cultured preantral caprine follicles [275]. Recently, FGF10 has been implicated as an oocyte competence factor. Supplementing bovine COCs with FGF10 during IVM increased subsequent embryonic development [203].

The concept that selective factors found within the follicular niche can be used to improve *in vitro* production (IVP) of bovine embryos was examined further in this work by describing how FGF2 supplementation during oocyte maturation affects oocyte and embryo development. Immunoreactive FGF2 localizes to theca and granulosa cells in bovine follicles, and the relative abundance of *FGF2* mRNA increases during follicle growth and after GnRH treatment in cattle [272, 276]. Also, mRNA concentrations of *FGF2* increase during bovine IVM [218], and selective *FGF2* single nucleotide polymorphisms (SNPs) are linked with IVF success rate in cattle [11]. Therefore, we proposed that providing FGF2 during IVM would benefit oocyte maturation in ways that subsequently impact embryo development during *in vitro* culture (IVC).

## Materials and Methods

### Reagents and Animal Assurances

Recombinant bovine FGF2 was purchased from R&D Systems (Minneapolis, MN, USA). TCM 199, Hoechst 33342 and primers used for qRT-PCR were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Follicle stimulating hormone (FSH) was purchased from Bioniche Life Sciences (Belleville, Ontario, Canada). mSOF was purchased from Millipore (Cat# BSS-078-D, Billerica, MA, USA). Paraformaldehyde (16% [w/v] ultrapure grade) was purchased from Polysciences Inc. (Warrington, PA, USA). RNase-free DNase was purchased from New England Biolabs (Ipswich, MA, USA). The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit was purchased from Roche Applied Sciences (Indianapolis, IN, USA).

The RNeasy Micro Kit was purchased from Qiagen (Valencia, CA, USA). The High Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems Inc. (Foster City, CA, USA). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

All studies were completed in accordance with and with the approval of the Institutional Animal Care and Use Committee at the University of Florida.

**Bovine IVM, Fertilization and Culture**

Procedures were completed as described previously [38, 124, 203]. Briefly, ovaries were obtained from Central Beef Packing Co. (Center Hill, FL, USA). Cumulus-oocyte complexes (COCs) were collected and groups of 10-12 COCs were cultured in 50 µl drops of OMM (TCM199 containing Earle's salts supplemented with 25 µg/ml bovine FSH (unless otherwise stated), 2 µg/ml estradiol 17-β, 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, 1 mM glutamine, 1 mg/ml PVA. Maturation medium was supplemented with varying concentrations of recombinant bovine FGF2 (0.5 to 50 ng/ml) prepared in TCM199 containing Earle's salts and 1% [w/v] bovine serum albumin (BSA). In one study, IVM was completed using denuded oocytes. For this, COCs were vortexed for 4 min to remove cumulus cells.

After 21 h at 38.5°C in 5% $CO_2$ in humidified air, COCs were transferred to fertilization medium and exposed to Percoll gradient-purified bovine spermatozoa. The same pool of semen from three bulls was used throughout the studies. After 8 to10 h at 38.5°C (5% $CO_2$ in humidified air), cumulus cells were removed by vortexing in 1000 U/ml hyaluronidase and denuded zygotes were placed in groups of 25-30 in 50 µl drops of mSOF [277]. Drops were covered with mineral oil and maintained at 38.5°C in 5% $CO_2$, 5% $O_2$ and 90% $N_2$ for 8 days. At day 3 post-IVF, the proportion of cleaved zygotes and embryos containing ≥8 blastomeres was recorded. The proportion of blastocysts and stage of blastocyst development (early, regular,

expanded, hatching and hatched) was recorded at days 7 and 8 post-IVF. On day 8, blastocysts were fixed with 4% [w/v] paraformaldehyde, permeabilized with 0.1% [v/v] Triton X-100 and stained for 15 min with 1μg/ml Hoechst 33342 [60]. Blastocysts were mounted on slides and number of nuclei per blastocyst was recorded.

**Nuclear Maturation, Cumulus Expansion and Apoptosis Following Oocyte Maturation**

After 21 h of maturation, the degree of cumulus expansion in COCs were scored visually by phase-contrast microscopy on a 1 to 3 scale (1=poor expansion characterized by few morphological changes compared with before maturation; 2=partial expansion characterized by fair expansion but notable clusters lacking expansion; 3=complete or nearly complete expansion) [60, 244]. To assess progression through meiosis during or after IVM, oocytes were denuded by vortexing for 4 min in saline after 6 or 21 h of maturation. Oocytes were fixed with 4% [w/v] paraformaldehyde, permeabilized with 0.1% [v/v] Triton X-100 and stained for 15 min with 1μg/ml Hoechst 33342. Meiotic staging at 21 h was determined with epifluorescence microscopy as described previously [32, 245]. First polar body extrusion was determined by using stereomicroscopy.

In one study, COCs were processed after IVM to determine the percentage of TUNEL-positive cumulus cells using FITC-conjugated dUTP and 1 μg/ml Hoechst 33342 as a counterstain. COCs were mounted on slides and analyzed with epifluorescence microscopy. The proportion of TUNEL-positive cumulus cells was calculated by dividing the number of TUNEL-positive nuclei with total nuclei numbers within each COC.

**Real-time Quantitative (q) RT-PCR**

Repeat pipetting was used to separate cumulus cells and oocytes from each other (n=25 to 30/group) 6 or 21 h after beginning IVM. Denuded oocytes were removed and washed thrice in PBS-PVP. Cumulus cells were transferred to micro-centrifuge tubes, centrifuged at 700 × g for 2

min at room temperature to remove residual solution, snap-frozen in liquid nitrogen and stored at -80°C. Total cellular (tc) RNA was extracted from cumulus cells using the RNeasy Micro Kit. RNA concentrations and integrity of RNA ($A_{260}/A_{280}$ ratio ≥1.8) was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Each tcRNA sample (10 ng/reaction) was incubated in RNase-free DNase and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit. Primers used for this work included *CTSB* [190], *EGFR* and *SPRY2* [60], *FSHR* [111], *HAS2* [197], *BCL2, BAX* and *BAD* [202], and *XIAP* and *FAS* [278]. Primers were used at a concentration of 200 nM and were mixed with RT products and SYBR Green PCR Master Mix. After an initial activation/denaturation step (50°C for 2 min, 95°C for 10 min), 40 cycles of a 2-step amplification protocol (60°C for 1 min, 95°C for 15 sec) were completed using a 7300 Real-Time PCR System. A dissociation curve analysis (60 to 95°C) was used to verify the amplification of a single product. Each sample was run in triplicate and a fourth reaction lacking exposure to reverse transcriptase was included to verify the absence of genomic contamination. Relative amounts of *18S* RNA was used as an internal control for quantifying relative gene expression. No treatment or time-dependent changes in *18S* RNA abundance relative to tcRNA concentration was detected in these studies. The ratio of target to reference RNA was used to determine relative expression ($2^{-CT[target]}/2^{-CT[reference]}$) [60].

**Statistical Analyses**

All analyses were completed with least-squares analysis of variance (LS-ANOVA) using the GLM of the Statistical Analysis System (SAS for Windows, version 9.0; SAS Institute Inc., Cary, NC, USA). All percentage data were arcsine-transformed before analysis. Either orthogonal contracts or pair-wise comparisons (probability of difference analysis [PDIFF]; SAS Institute Inc.) were completed to separate means. Percentage data are presented as non-transformed values and SEMs. COC expansion was analyzed by examining the mean COC

expansion index (calculated for each treatment within each replicate) and by examining the proportion of COCs observed within each of the non-parametric rankings (1, 2 and 3). The ratio of target to reference RNA was used to analyze qRT-PCR data.

## Results

### FGF2 Supplementation During IVM Benefits Subsequent Embryo Development

To describe whether oocytes benefit from FGF2 supplementation during oocyte maturation *in vitro*, COCs were matured in medium containing different concentrations of FGF2 (0.5, 5, 50 ng/ml or carrier only controls). Subsequently, COCs were fertilized and cultured in the absence of supplemental FGF2. Fully defined maturation medium was used (*i.e.* serum was replaced with 1 mg/ml PVA). As shown in Table 3-1, numerical differences that did not approach significance were evident for the percentage of presumptive zygotes that cleaved when COCs were cultured with FGF2. To adjust for these minor differences in cleavage rates, subsequent embryo data were examined based on the number of cleaved embryos.

Supplementation with FGF2 did not affect the percentage of embryos containing ≥8 cells at day 3 post-IVF, but increases (P=0.02) in the percentage of cleaved embryos that became blastocysts at day 7 post-IVF were observed when comparing all the FGF2 treatments with the control (*i.e.* contrasting 0.5, 5 and 50 ng/ml FGF2 versus control). At day 8, FGF2 supplementation did not affect the percentage of total blastocysts, the percentage of advanced blastocysts or blastomere numbers.

### FGF2 Regulates Meiotic Maturation of Bovine Oocytes *In vitro*

A series of studies were completed to understand how FGF2 supplementation may be acting during *in vitro* oocyte maturation. The first set of studies examined oocyte meiotic maturation (Figure 3-1). Supplementation with 0.5, 5 or 50 ng/ml FGF2 increased (P=0.05) the percentage of oocytes with polar bodies after 21 h (Figure 3-1A). Effects of FGF2

supplementation on meiotic maturation also were observed. Pair-wise comparison analysis indicated that exposure to 5 ng/ml increased the percentage of oocytes that developed to metaphase II (MII) when compared with the control (P=0.03) (Figure 3-1B). However, exposure to 0.5 or 50 ng/ml FGF2 did not affect meiotic maturation.

Follow-up studies were completed to determine if these positive effects of FGF2 supplementation on meiotic maturation required cumulus cells and FSH supplementation. In one study, oocytes were denuded by rapid vortexing and cultured in medium containing 0 or 5 ng/ml FGF2. Supplementation with FGF2 did not affect the percentage of oocytes extruding their first polar body (71 ± 8 vs. 56 ± 8% for 0 and 5 ng/ml FGF, respectively) or achieving the MII stage (78 ± 6 vs. 57 ± 9% for 0 and 5 ng/ml FGF, respectively). In a second study, cumulus cells were not removed but COCs were matured in medium lacking FSH. In these cultures, supplementation with FGF2 did not affect the percentage of oocytes extruding their first polar body or reaching MII after 21 h (40 ± 3 vs. 35 ± 5 for polar body extrusion and 33 ± 5 vs. 32 ± 5 for meiosis II for 0 and 5 ng/ml FGF2, respectively).

**FGF2 Increases Expansion and Reduces Apoptosis of Cumulus Cells**

The effects on FGF2 on cumulus cell activity and gene expression were examined to describe how beneficial effects of FGF2 may be achieved during COC maturation. Cumulus expansion is a good predictor of oocyte competence for fertilization and subsequent embryo development [147, 190, 279]. FGF2 supplementation during COC maturation had some minor influences on cumulus expansion (Figure 3-2). When compared with the control, supplementation with 0.5 ng/ml FGF2 increased (P=0.04) the percentage of COCs that were fully expanded (*i.e.* score=3). Supplementation with 5 or 50 ng/ml FGF2 did not affect expansion scores.

A separate study determined that FGF2 supplementation also may act on the cumulus to limit cumulus cell apoptosis (Figure 3-3). COCs were cultured with 0, 0.5 or 5 ng/ml FGF2 for 21 and the percentage of TUNEL-positive cumulus nuclei were then determined. Supplementation with either concentration of FGF2 reduced (P=0.03) the percentage of TUNEL-positive cumulus cells when compared with the non-treated control. To follow up on this observation, the influence of FGF2 supplementation on expression profiles of several apoptosis-related genes was examined after 6 and 21 h of maturation. Supplementation with 5 ng/ml FGF2 did not affect the relative abundance of *BCL2*, *XIAP*, *BAX*, *BAD* and *FAS* in cumulus cells at either time point (data not shown).

A final study was completed to determine if FGF2 supplementation altered the expression of selective cumulus-expressed transcripts associated with oocyte competence [113, 116, 190, 197, 264]. For this, COCs were cultured in 0 or 5 ng/ml FGF2 for 6 or 21 h, COCs were vortexed to liberate cumulus cells, and RNA was extracted from cumulus cells. Subsequent qRT-PCR analysis did not detect FGF2-dependent differences in the relative abundance of *CTSB*, *SPRY2*, *EGFR*, *FSHR* and *HAS2* mRNA after 6 or 21 h of culture (data not shown).

## Discussion

Bovine IVM/IVF/IVC procedures generate embryos that usually are inferior at producing viable offspring than *in vivo*-derived embryos. Pregnancy rates at day 28-32 usually are reduced and subsequent pregnancy losses are increased following transfer of IVP-embryos to recipients at day 7 post-estrus when compared with inseminated cows [38, 280]. This work tested whether addition of selective biological agents during IVM would improve subsequent embryogenesis. Recent work from this laboratory determined that FGF10 supplementation during IVM increased subsequent embryo development *in vitro* [60]. This FGF is produced by thecal cells and oocytes in antral follicles, and concentrations of FGF10 are positively related to health status of the

follicle [228, 235]. Our interest in examining FGF2 as a second oocyte competence factor stemmed from descriptions of FGF2 being expressed within the follicle, with notable expression within theca, granulosa and cumulus cells [272, 276] and by observing transcripts for several *FGFR* isotypes that bind FGF2 in cumulus cells and oocytes [60].

The consequences of providing supplemental FGF2 during IVM on subsequent embryo development were very specific. Treatment of COCs with FGF2 did not impact cleavage rates, early embryo development or overall development of blastocysts, but increased the onset of blastocyst formation at day 7. Cell numbers at day 8 post-IVF were not different in embryos derived from FGF2-treated COCs, indicating that FGF2 did not mediate initial blastocyst formation by altering blastomere division rates. Small increases in advanced blastocyst rates at day 8 were observed in embryos from FGF2-treated COCs (4 vs. 9 to 11%; see Table 3-1). Similar increases in blastocyst rates that are not accompanied by changes in blastomere numbers also were observed after supplementing FGF10 to COCs [60]. Observing increases in the onset of blastocyst development are significant because more pregnancies are maintained to term from early forming blastocysts versus blastocysts forming later following IVF [281, 282].

Several FGF2-dependent alterations in oocyte maturation were observed in this work. Its ability to promote first polar body extrusion was observed at all of the FGF2 concentrations tested (0.5 to 50 ng/ml) whereas benefits on progression of oocytes to metaphase II was only observed in COCs provided 5 ng/ml FGF2. The involvement of FSH in these FGF2-dependent effects was examined by removing this supplement from the culture system. As also shown by others, rates of oocyte maturation were reduced in the absence of FSH [283]. Treatment with FGF2 did not affect oocyte maturation in absence of FSH. This could indicate that FSH controls FGF-dependent events during maturation. A definitive role for FSH in controlling *FGFR*

expression during folliculogenesis has not been described but FSH increases *FGFR1* mRNA abundance in cultured rat sertoli cells [284] and GnRH treatment, which induces LH and FSH surges, increases *FGFR1* mRNA abundance in bovine follicular cells [272]. Both of the major splice variant forms of FGFR1 (R1b and R1c) exhibit high affinity interactions with FGF2 [247].

A second culture model used to examine the effects of FGF2 during IVM involved removing cumulus cells before maturation. Granulosa cells and presumably cumulus cells produce FGF2 during bovine oocyte maturation *in vivo* [272, 276] and removal of cumulus cells allowed for describing if any direct effects of FGF2 on oocytes exist. No FGF2 responses were detected in denuded oocyte cultures.

Several parameters were examined to describe how FGF2 acts indirectly via cumulus cells to mediate oocyte competence. Cumulus cells exchange signaling molecules, energy substrate and others with oocyte by communicating through gap junctions [98]. Transcripts identified by others as putative cumulus markers of oocyte competence were examined in an effort to understand how FGF2 regulates oocyte competence. Cathepsin B (*CTSB)* mRNA and protein abundance is inversely related to oocyte quality in cattle [190]. Other cumulus-specific transcripts are important for normal oocyte maturation and cumulus expansion (*EGFR*, *FSHR*, *HAS2*, *SPRY2*) [113, 197, 264]. FGF2 supplementation did not affect the abundance of any of these transcripts. The lack of detectable changes in these transcripts after FGF2 treatment indicates that FGF2 must act through alternative pathways to impact oocyte competence.

It also was interesting to observe that FGF2 limits cumulus cell apoptosis. Cumulus cells undergo spontaneous apoptosis during IVM [285]. The mechanism controlling this activity has not been identified. The expression of several candidate markers of cell survival was not affected by FGF2 treatment.

In summary, this work shows for the first time that supplementing FGF2 during IVM stimulates various aspects of oocyte maturation and cumulus activity. This treatment also benefits subsequent developmental competence, and specifically promotes the onset of blastocyst formation in cultured bovine embryos. The specific mechanisms controlled by FGF2 during oocyte maturation that confers competence to form blastocysts has not been described, and future examinations into the actions of FGF2 should include further investigation into how this factor affects cumulus cell activity and components of the ooplasm after maturation.

Table 3-1. The effect of supplementing FGF2 during COC maturation on subsequent *in vitro* development.

| Parameter[1] | FGF2 (ng/ml) | | | | P Value[2] |
|---|---|---|---|---|---|
| | 0 | 0.5 | 5 | 50 | |
| Day 3: % Cleaved Zygotes[3] | 82 ± 2 | 78 ± 1 | 77 ± 4 | 73 ± 3 | 0.14 |
| Day 3: % ≥ 8-cell Embryos[4] | 59 ± 9 | 65 ± 6 | 58 ± 5 | 68 ± 2 | 0.59 |
| Day 7: % Blastocysts[4] | 16 ± 3 | 30 ± 2 | 26 ± 2 | 29 ± 7 | 0.02 |
| Day 8: % Blastocysts[4] | 38 ± 8 | 46 ± 6 | 40 ± 3 | 38 ± 8 | 0.69 |
| Day 8: % Advanced Blastocysts[4] | 4 ± 2 | 13 ± 3 | 11 ± 2 | 9 ± 5 | 0.22 |
| Blastomere Number (Day 8) | 124 ± 8 | 121 ± 8 | 129 ± 10 | 129 ± 9 | 0.96 |

[1]For embryo development data, n=4 replicate studies; 26 to 35 COCs/replicate. For cell number assessment, n=26 to 34 blastocysts examined over 4 replicate studies.
[2]Represents the comparison (via Orthogonal contrast analysis) of controls (no FGF2) versus oocytes cultured in 0.5, 5 or 50 ng/ml FGF2.
[3]Based on the percentage of total oocytes.
[4]Based on the percentage of cleaved embryos.

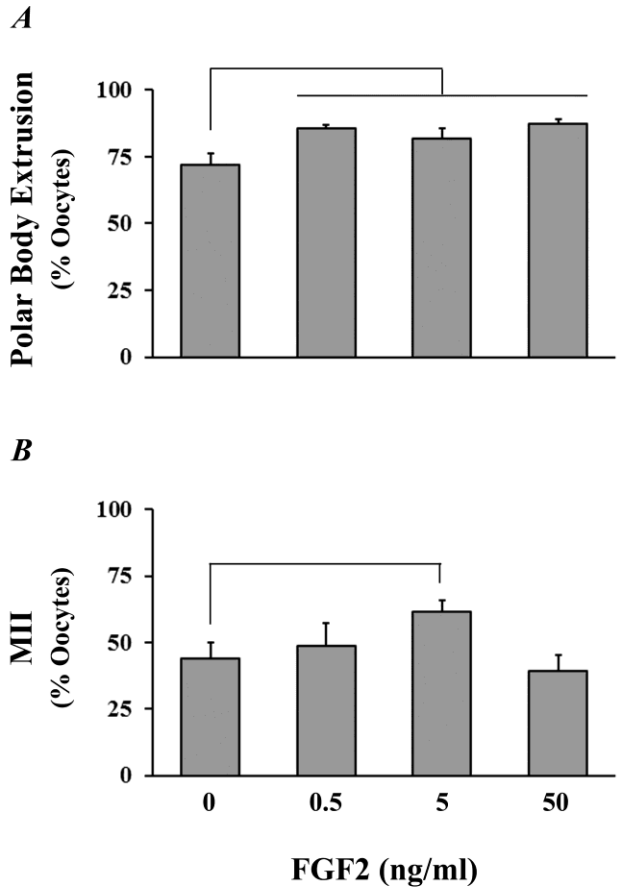


Figure 3-1. The effect of FGF2 supplementation during IVM on oocyte maturation. COCs (n=10-22/replicate; 7 replicate studies) were cultured in maturation medium containing FSH and 0, 0.5, 5 or 50 ng/ml FSH. After 21 h, cumulus cells were removed by vortexing and the percentage of oocytes that extruded their first polar body (A) and achieved MII (B) was determined. A) A significant difference between controls and all FGF2 treatment groups was detected by using Orthogonal contrasts (P=0.05). B) Percentage of oocytes that reached MII differed between the control and oocytes exposed to 5 ng/ml FGF2. The other FGF2 treatments did not differ from the control.

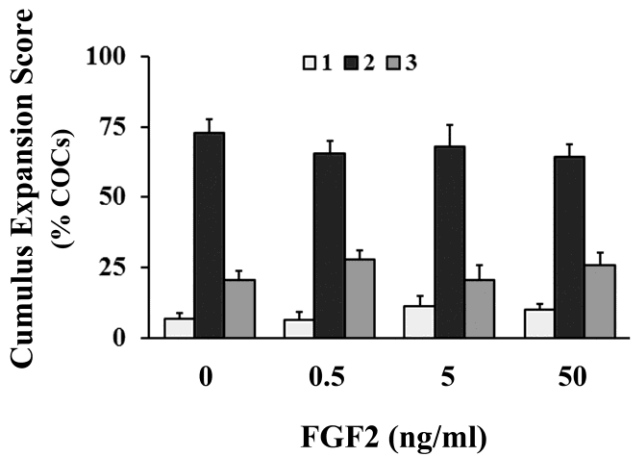


Figure 3-2. The effect of FGF2 supplementation during IVM on cumulus expansion. COCs (n=10-22/replicate; 6 replicate studies) were cultured in maturation medium containing FSH and 0, 0.5, 5 or 50 ng/ml FSH. After 21 h, cumulus expansion scores were assigned to each COC (rank from 1 to 3 based on no or little expansion, moderate expansion, or complete expansion) and the percentage of COCs in each category were compared between treatments. There was an increase (P=0.04) in the percentage of fully-expanded cumulus (score 3) in COCs incubated win medium containing 0.5 ng/ml FGF2 when compared with controls. The other FGF2 treatments did not differ from controls in their relative portion of cumulus expansion.

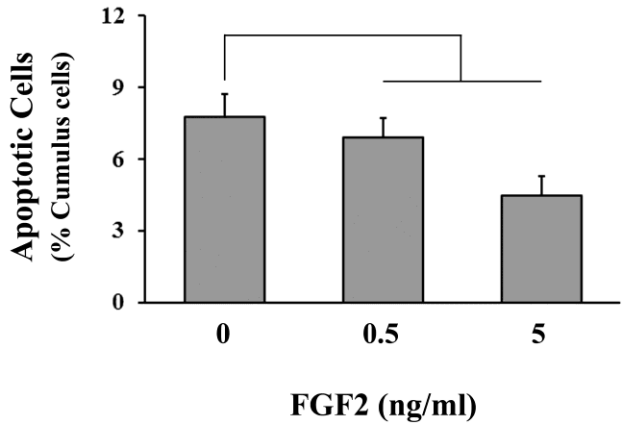


Figure 3-3. The effect of FGF2 supplementation during IVM on cumulus cell apoptosis. COCs (n=25-35/replicate; 4 replicate studies) were cultured in maturation medium containing FSH and 0, 0.5, 5 or 50 ng/ml FSH. After 21 h, COCs were fixed and processed to determine the percentage of TUNEL-positive cumulus cells in each COC. A significant difference between controls and both FGF2 treatment groups was detected by using Orthogonal contrasts (P=0.03).

# CHAPTER 4
# DISRUPTION OF FGF RECEPTOR SIGNALING IN CUMULUS-OOCYTE COMPLEXES REDUCES MEIOTIC MATURATION AND SUBSEQUENT DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES

FGFs control various aspects of embryonic, fetal and adult physiology. They are best known as mediators of cell proliferation and differentiation, organogenesis, angiogenesis and tumorigenesis [211, 239]. Most FGFs act as paracrine factors in various tissues although some FGFs serve as endocrine factors and control vitamin D metabolism, kidney function and bone mineralization while others function primarily as intracrine mediators of cell survival [286, 287].

Multiple FGF transcripts are expressed in bovine oocytes (*FGF8/10/17*), theca cells (*FGF2/7/10/18*) and granulosa cells (*FGF2, 8, 18*) [228, 231, 233, 276]. Several FGF receptor variants (*FGFRs*) are expressed in bovine theca, granulosa and cumulus cells and in oocytes [228, 231, 276, 288]. Four genes encode these tyrosine kinase receptors mammals (*FGFR1/2/3/4*), and alternative splicing in three of these receptors (*FGFR1/2/3*) generate numerous receptor subtypes [211, 247]. One splicing event occurs within the third extracellular immunoglobulin-like domain of *FGFR1/2/3*, and this generates receptor subtypes known as *IIIb*/*IIIc* or *b/c* variants that bind FGFs with various affinities. For example, the FGFR2b subtype (also known as keratinocyte growth factor receptor) reacts with the FGF7 subfamily of ligands (FGF3, 7, 10, 22) whereas the FGFR2c subtype interacts with other FGFs (*e.g.* FGF2, 4, 6, 9) [211, 247].

There is mounting evidence that locally produced FGFs are important for folliculogenesis. In mice, oocyte-derived FGF8 acts cooperatively with BMP15, another oocyte-derived factor, to stimulate glycolysis in cumulus cells [141]. Theca- and oocyte-derived FGF2 regulates LH receptor expression in granulosa cells and primordial follicle development [223, 224]. Follicle development and estrogen production also are increased in human ovarian tissue cultures

containing FGF2 [221]. In cattle, the steroidogenic capacity of granulosa cells is regulated by FGF7, 10 and 18, all of which are produced primarily by theca cells [228, 232, 235, 276].

Recently this laboratory found that supplementing FGFs during *in vitro* oocyte maturation (IVM) increase the overall success of *in vitro* bovine embryo production [60, 289]. Since theca cells are not utilized during IVM of cumulus-oocyte complexes (COCs), we proposed that supplementing theca-derived FGFs would improve the ability of the oocytes to mature, be fertilized and develop into viable embryos. Supplementing FGF10 during IVM increased bovine oocyte competence [60]. Specifically, FGF10 supplementation during IVM did not greatly affect oocyte maturation and fertilization success but increased embryo development rates to the blastocyst stage. Similar findings were described when FGF2 was supplemented to bovine COCs during IVM [289].

Endogenous sources of FGFs (*i.e.* FGFs derived from cumulus cells or oocytes) also appear important for oocyte competence during *in vitro* embryo production. Neutralizing FGF10 activity during IVM by IgG capture did not affect oocyte maturation or fertilization but reduced subsequent embryo development [60]. The present work was completed to better understand the functional importance of FGFs during the final stages of oocyte maturation in cattle. Specific objectives were to describe whether gonadotropins affect the expression of specific *FGFR* isotypes in cumulus cells and oocytes during IVM and to examine whether blocking FGFR activity with pharmacological inhibitors during IVM affects oocyte maturation, fertilization and embryo development.

## Materials and Methods

### Reagents and Animal Assurance

TCM 199, Hoechst 33342 and primers used in qRT-PCR were purchased from Invitrogen Corp. (Carlsbad, CA, USA). FSH was purchased from Bioniche Life Sciences (Belleville,

Ontario, Canada). mSOF was purchased from Millipore (Billerica, MA, USA). The pharmacological inhibitors of FGFRs were purchased from Symansis, Shanghai, China (SU5402) and EMD Chemicals, Gibbstown, NJ (PD173074). Paraformaldehyde (16% [w/v] ultrapure grade) was purchased from Polysciences Inc. (Warrington, PA, USA). RNase-free DNase was purchased from New England Biolabs (Ipswich, MA, USA). The RNeasy Micro Kit was purchased from Qiagen (Valencia, CA, USA) and the PicoPure$^{TM}$ RNA Isolation Kit was purchased from MDS Analytical Technologies (Sunnyvale, CA, USA). The High Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems Inc. (Foster City, CA, USA). RIPA buffer, Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail were purchased from Thermo Scientific (Pittsburgh, PA USA). AlexaFluor 594 was purchased from Molecular Probes Inc. (Eugene, OR, USA). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

All experiments were carried out in accordance with the approval of the institutional animal care and use committee at the University of Florida.

**IVM, Fertilization and Culture**

Oocyte maturation, fertilization and embryo culture *in vitro* were completed as described previously [38, 124, 203]. Briefly, ovaries were obtained from a local slaughterhouse (Central Packing, Center Hill, FL, USA). COCs were collected and pools of 10-12 COCs were placed in 50 µl drops of OMM (TCM199 containing Earle's salts, 25 µg/ml bovine FSH [unless stated otherwise], 2 µg/ml estradiol 17-β, 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, 1 mM glutamine, 1 mg/ml PVA). Pharmacological inhibitors of FGFR kinases (SU5402, PD 173074) were prepared in DMSO and supplemented to OMM immediately before use (0.25% and 0.005% DMSO final dilution, respectively). In one study, denuded oocytes were generated

before maturation by vortexing COCs 4 min to remove cumulus cells. Maturation was completed for 21 h at 38.5°C in an atmosphere of 5% $CO_2$ in humidified air.

After maturation, COCs were placed in fertilization medium and incubated with Percoll gradient-purified bovine spermatozoa for 8 to10 h at 38.5°C (5% $CO_2$ in humidified air) [60]. A pool of semen from three bulls was used for all studies. After fertilization, cumulus cells were removed by vortexing in 1000 U/ml hyaluronidase and putative zygotes were cultured in groups of 25-30 in 50 µl drops of mSOF (25 µg/ml gentamicin sulfate, 0.4 mM sodium pyruvate, 2.77 mM myo-inositol, 0.5 mM sodium citrate, 1 mM alanyl glutamine, 5.3 mM sodium lactate syrup, 10 µl/ml non-essential amino acids, 20 µl/ml essential amino acids and 4 mg/ml fatty acid-free BSA) [277]. Drops were covered with mineral oil and maintained at 38.5°C in 5% $CO_2$, 5% $O_2$ and 90% $N_2$ for 8 days.

**Nuclear Maturation Determination**

To assess progression through meiosis during or after IVM, oocytes were denuded by vortexing for 4 min in saline after 21 h of maturation. Oocytes were fixed with 4% [w/v] paraformaldehyde, permeabilized with 0.1% [v/v] Triton X-100 and stained for 15 min with 1µg/ml Hoechst 33342. Meiotic staging at 21 h was determined with epifluorescence microscopy as described previously [32, 245]. First polar body extrusion was determined under stereomicroscopy.

**Quantitative, Real-Time RT-PCR Analysis of Cumulus Cells and Oocytes**

Cumulus cells and oocytes (n=20/group) were separated from each other either immediately after COC isolation and prior to maturation (0 h) or at 6 or 21 h after IVM by vortexing for 4 min. Denuded oocytes were washed thrice in PBS-PVP, snap-frozen in liquid nitrogen and stored at -80ºC until use. Cumulus cells were centrifuged (700 × g for 2 min) to remove residual solution and were snap-frozen in liquid nitrogen and stored at -80°C until use.

Total RNA was isolated from cumulus cells using the RNeasy Micro Kit and from oocytes using the PicoPure$^{TM}$ RNA Isolation Kit. RNA concentrations and integrity ($A_{260}/A_{280}$ ratio $\geq$1.8) were determined by using the Nano Drop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA (10 ng/reaction for cumulus samples; entire sample for oocytes) was treated with RNase-free DNase and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit. The SYBR® Green detection system and a 7300 Real-Time PCR System (Applied Biosystems Inc.) was used to provide relative quantities of specific transcripts in cumulus and oocyte transcripts. Primers (200 nM) were described previously [60]. After an initial activation/denaturation step (50°C for 2 min, 95°C for 10 min), 40 cycles of a 2-step amplification protocol (60°C for 1 min, 95°C for 15 sec) were completed. A dissociation curve analysis (60 to 95°C) was used to verify the amplification of a single product. Each sample was run in triplicate and a fourth reaction lacking exposure to reverse transcriptase was included to verify the absence of genomic contamination. Relative amounts of *18S* RNA was used as an internal control.

**Statistical analyses**

All data were analyzed by analysis of variance (ANOVA) using the GLM of the Statistical Analysis System (SAS for Windows, version 9.0; SAS Institute Inc., Cary, NC, USA). Percentage data were arcsine-transformed before analysis. Differences in individual means were separated further by completing pair-wise comparisons (probability of difference analysis [PDIFF]; SAS Institute Inc.). Percentage data were graphed using non-transformed values and SEMs. The qRT-PCR data were analyzed after determining the ratio of target to reference RNA ($2^{-CT[target]}/2^{-CT[reference]}$) [60]. Data are presented as fold-changes over 0 h controls by using the comparative threshold cycle ($C_T$) approach, where the average $\Delta C_T$ value for each transcript was determined (target $C_T$– *18S*$C_T$) and used to calculate the fold-change ($2^{-\Delta\Delta CT}$) [246].

## Results

### FGFR mRNA Abundance in COCs

Recent work described the relative abundance of various *FGFR* transcripts in bovine cumulus cells and oocytes before COC culture [60]. Cumulus cells contained high amounts of both *FGFR1* spliced variant mRNAs (*R1b/R1c*) and moderate amounts of the spliced *FGFR2* mRNA variants (*R2b/R2c*) whereas oocytes contained large amounts of *R2b/R2c* and moderate amounts of *R1b/R1c.* Since notable increases in bovine granulosa/cumulus *FGFR* mRNA abundances occur around the time of ovulation [272], we proposed that changes in *FGFR1* and *FGFR2* mRNA abundances would occur in bovine cumulus cells and oocytes during IVM.

Cumulus cells were separated from oocytes by vortexing either before COC culture or after 6 or 21 h of culture and RNA was extracted from groups of cumulus cells and oocytes. Samples collected before culture served as reference controls for initial transcript abundance of *R1b*, *R1c*, *R2b* and *R2c*. Supplementation with FSH increased ($P<0.05$) the abundance of each *FGFR* isotype after 6 h of culture (Figure 4-1). This beneficial effect was observed when cumulus cells from FSH-treated COCs were compared with non-FSH-treated samples and cumulus cells collected before culture. The FSH effect was transient for three of the four transcripts. Abundance of *R1b*, *R1c* and *R2b* mRNA were not different between FSH-treated and untreated samples after 21 h of culture whereas *R2c* mRNA was greater ($P<0.05$) in cumulus cells exposed to FSH than those not exposed to this gonadotropin.

Changes in the relative abundance of *FGFRs* also were detected over time in culture. The *R2c* transcript contained the most dynamic changes (Figure 4-1D), with increased ($P<0.001$) *R2c* mRNA abundance after 6 h of culture in the absence of FSH (14 ± 5 fold change versus 0 h control) and a further increase ($P<0.001$) after 6 h in cultures containing FSH (71 ± 15 fold change versus 0 h control). The relative abundance of *R2c* mRNA in cumulus cells was less after

21 h of culture than at 6 h ($P<0.05$) but mRNA abundance at 21 h remained greater ($P<0.001$) than that for cumulus cells collected before culture (12 ± 4 fold change versus 0 h control) and FSH supplementation increased ($P<0.01$) *R2c* mRNA abundance at this time point (35 ± 6 fold change versus 0 h control).

Additional studies were completed to further characterize the *FGFR1* and *FGFR2* expression profiles in cumulus cells. In one study, a lower concentration of FSH was examined for its ability to mediate *FGFR* mRNA abundance in cumulus cells. FSH (1 µg/ml) was added to the medium and COCs were cultured for 6 h before cumulus cell removal. Increases ($P<0.05$) in *R1b*, *R1c*, *R2b* and *R2c* transcripts were observed when compared with cumulus cells from COCs lacking FSH exposure (2.1 ± 0.3, 2.0 ± 0.3, 42.7 ± 13.9 and 21.0 ± 4.0 fold increase for each respective variant). In another study, COCs were cultured in medium lacking FSH but containing 3 µg/ml LH. After 6 h, the relative abundance of cumulus *R1b*, *R1c*, *R2b* and *R2c* transcripts did not differ between LH-treated and non-treated COCs (data not shown).

Supplementation with FSH did not affect mRNA abundance of *R1b*, *R1c*, *R2b* and *R2c* in oocytes (data not shown). No changes in abundance of *R1b*, *R1c* and *R2b* transcripts were detected in oocytes after 6 or 21 h of culture, and *R2c* mRNA abundance decreased ($P<0.05$) after 21 h of culture in oocytes derived from FSH-supplemented and non-supplemented COCs (0.55 ± 08 fold change versus 0 h control).

**FGFR mRNA Abundance is not Dependent on MAPK3/1**

FSH-dependent activation of mitogen activated kinase pathways, and specifically ERK1/2-dependent systems (termed MAPK3/1), are required for resumption of oocyte meiosis and cumulus cell activation in mice [140, 290]. A study was completed to determine if FSH utilizes MAPK3/1 to stimulate *FGFR* mRNA abundance in bovine cumulus cells (Figure 4-2). Addition of U0126, an inhibitor of MAPK3/1 activation, to COCs during culture did not affect

FSH-induced and non-FSH-induced concentrations of *R1b*, *R1c*, *R2b* and *R2c* mRNA in cumulus cells after 6 h (Figure 4-2). The effectiveness of the inhibitor was verified by observing that it could block the FSH-dependent increase in *SPRY2* mRNA, a MAPK3/1-responsive feedback inhibitor of FGF signaling [249] (Figure 4-2E).

**Interrupting FGFR Signaling During Oocyte Maturation Compromises Embryo Development**

To describe the significance of FGFR signals during COC maturation, sequential studies were completed with two pharmacological inhibitors of FGFR kinase activity (Figure 4-3 and 4-4). Both inhibitors (SU5402 and PD173074) were added at the beginning of IVM and COCs were washed after maturation to remove inhibitors before fertilization. Fertilization and subsequent embryo culture occurred in media lacking inhibitors. A range of concentrations were examined for each inhibitor, and the largest concentration of each inhibitor prevented FGF2-dependent gene expression in bovine trophoblast cells (25 μM SU5402 and 1 μM PD173074) [277, 291]. No exogenous FGFs were provided during IVM/IVF/IVC.

The percentage of cleaved embryos at day 3 post-IVF was not affected by SU5402 treatment during oocyte maturation (Figure 4-3A). However, exposure to 25 μM SU5402 during maturation reduced ($P<0.007$) the percentage of cleaved embryos containing ≥8 cells at day 3 (Figure 3A). Also, the percentage of embryos that reached the blastocyst stage at day 7 and 8 post-IVF were decreased ($P<0.01$) in COCs exposed to 25 μM SU5402 (Figure 3B). Exposure to lower concentrations of SU5402 did not affect embryo development potential at days 3, 7 or 8.

Exposure to PD173074 during oocyte maturation did not affect the percentage of cleaved embryos at day 3 post-IVF, but exposure to 1 μM PD173074 decreased ($P=0.01$) the percentage of embryos containing ≥8 cells (Figure 4-4A). Exposure to 100 nM PD173074 tended ($P=0.09$) to decrease the percentage of ≥8 cells embryos whereas exposure to 10 nM of the inhibitor did

not affect this outcome. At day 7 post-IVF, exposure to ≥10 nM PD173074 decreased ($P<0.05$) the percentage of cleaved embryos that were blastocysts (Figure 4-4B). At day 8, blastocyst formation was reduced when oocytes were exposed to 1 μM PD173074 ($P=0.007$), tended to be reduced after exposure to 100 nM of the inhibitor ($P=0.07$) and was not affected by exposure to 10 nM of inhibitor.

**FGFR Signaling and Oocyte Meiotic Maturation**

The ramifications of blocking FGFR activity during COC culture were examined by surveying how exposure to the FGFR inhibitors affected the meiotic maturation of oocytes (Figure 4-5). Incubation with 25 μM SU5402 decreased ($P<0.05$) the percentage of oocytes that extruded the first polar body and achieved metaphase II (MII) whereas incubation with 1 μM PD173074 did not affect polar body extrusion and MII rates. To clarify how FSH supplementation impacts the ability of the FGFR inhibitors to mediate oocyte maturation, a study was completed using maturation medium that lacked FSH (Figure 4-5B). Fewer oocytes extruded their first polar body and achieved MII when FSH was not added to maturation medium, and SU5402 exposure further decreased ($P<0.05$) both parameters whereas exposure to PD173074 did not affect oocyte maturation.

## Discussion

This work provides evidence that dynamic changes in cumulus *FGFR* expression occurs during *in vitro* oocyte maturation and inhibiting FGFR kinase activity during IVM negatively affects bovine embryo development. To our knowledge this is the first report describing that oocyte competence is dependent on endogenous FGF signaling during the final stages of oocyte maturation preceding fertilization.

Bovine follicles and oocytes contain several *FGFR* isotypes [228, 272, 276], and this laboratory determined that *FGFR1b/c* were highly abundant in cumulus cells and *FGFR2b/c*

were highly abundant in denuded oocytes [60]. Each of these *FGFRs* responded to FSH in cumulus cells, and profound increases in mRNA abundances were found after 6 h of COC maturation when using FSH concentrations commonly used for IVM culture (25 μg/ml) or lower amounts of FSH (1 μg/ml). The *FGFR2b/c* isotypes were very responsive to FSH treatment. The relative abundances of the *R2b/c* transcripts also were increased during the first 6 h of COC maturation in the absence of FSH, albeit at reduced levels. Most of the current findings are consistent with previous observations. FSH increased *R2b* and *R3c* mRNA abundance in cultured bovine granulosa cells [228, 231]. Also, induction of a gonadotropin surge in cattle induced a transient increase in *R1c* mRNA concentrations in follicular cells (theca and granulosa) [272]. However, no changes in *R2c* mRNA abundance were noted in that study. Collectively, previous and current findings suggest that COC maturation in the presence of FSH enhances cumulus cell responsiveness to FGFs that bind to FGFR1 and FGFR2 receptor subtypes (*e.g.* FGF7 and 10 for R2b; FGF2 for R2c). However, not all of the *FGFR* expression profiles described during IVM necessarily exist in follicular cells during peri-ovulatory follicle development *in vivo*.

Each of the *FGFR* transcripts was greater in abundance in cumulus cells after 6 h of COC maturation than either before culture and after 21 h of maturation. This biphasic response to COC culture and FSH supplementation was not examined further, but similar findings were observed in follicles from cattle after GnRH challenge [272]. In that study, greatest concentrations of *FGFR1c* mRNA were detected 4 h after GnRH treatment than before or ≥10 h after GnRH treatment. Other dynamic changes in granulosa cells that may drive this *FGFR* expression pattern include changes in steroid and prostaglandin production [272]. Alternatively, *FGFR* expression and activity are tightly regulated by several intracellular feedback inhibitors

[211, 239] and changes in this activity also may explain the temporal expression patterns of these receptors.

No increases in cumulus *FGFR* mRNA levels were evident after COC exposure to LH. In mice, LH induces the production of EGF-like molecules in granulosa cells, and these factors ultimately regulate the resumption of meiosis and ovulation [114]. Also, no FSH-dependent or independent increases in *FGFR* mRNA abundance were evident in oocytes at 6 or 21 h. These observations implicate FSH-dependent activation of cumulus cells as the predominant modifier of *FGFR* transcript abundance in COCs during IVM.

A follow-up study determined that the FSH-dependent increases in cumulus cell *FGFR* mRNA levels were not altered after blocking MAPK3/1 activation. MAPK3/1 signaling is essential in granulosa cells for fertility in female mice [139, 140, 290]. Current evidence indicates that cAMP-dependent liberation of catalytic PKA from its regulatory subunits increases MAPK3/1 phosphorylation status by mechanisms that are not fully understood but appear to involve reducing the activities of phosphotyrosine phosphatases that serve as feedback inhibitors for MAPKs [158, 290].

Two sequential studies utilized two pan-FGFR kinase inhibitors to examine the importance of FGFR activity during IVM on subsequent embryo development. Both inhibitors are competitive inhibitors of ATP binding within tyrosine kinase regions, and both inhibitors contain high affinity for FGFR kinases. Each inhibitor was used at concentrations effective at blocking FGFR activation in other tissues [292-295], including bovine trophoblast cells [277, 291]. No differences in cleavage rates were detected at day 3 post-IVF when COCs were cultured with either inhibitor. However, decreases in the percentage of ≥8 cell embryos at day 3 and blastocysts at days 7 and 8 were detected with either SU5402 or PD173074. Complementary

outcomes were observed by supplementing COCs with FGF10 during IVM [60]. Supplementing FGF10 during bovine IVM did not affect cleavage rates but increased the proportion of 8 cell embryos at day 3 and blastocysts at day 7 post-IVF.

It is important to note that the studies with FGFR inhibitors were completed without providing exogenous FGFs. The maturation medium was fully defined and devoid of serum, and the only sources of FGFs were derived from oocytes or cumulus cells. Bovine oocytes and cumulus cells produce several FGFs [228, 231, 233, 276]. Also, theca-derived FGFs probably also contribute to FGF activity even after COCs are removed from their follicles because several FGFs have high affinity for transmembrane and extracellular heparin sulfate proteoglycans and will be sequestered within the cumulus layers [211, 239]. The necessity for these endogenous FGFs during COC maturation is consistent with previous findings examining the need for endogenous FGF10 during IVM [60]. In that work, antibody capture of FGF10 during IVM reduced subsequent embryo development.

A final series of studies were completed to examine whether inhibiting FGFR kinase activity affects bovine oocyte maturation. SU5402 inhibited the percentage of first polar body extrusion and progression to MII after maturation but PD173074 did not affect either outcome. These effects were noted in the presence and absence of supplemental FSH. One reason for these disparate outcomes is that these inhibitors could act through non-FGFR targets. These inhibitors are not exclusive for FGFRs, and perhaps SU5402 reacted with other tyrosine kinase receptors (*e.g.* EGFR) to mediate oocyte meiosis differently than PD173074. Alternatively, perhaps greater amounts of PD173074 were needed to detected effects with this inhibitor. In a previous study, greater concentrations of FGF10 were needed to improve meiotic maturation of oocytes than were needed to improve subsequent embryo development rates [60]. These outcomes may

reflect differential usage of FGFR subtypes for controlling meiotic maturation and oocyte competence. Regardless of the reason for these disparate outcomes, a firm conclusion about the role of FGFs in controlling oocyte meiotic maturation cannot be made at the present time.

In summary, this work shows that *FGFR* expression profiles in cumulus cells are regulated by FSH in the final stages of oocyte maturation and blocking FGFR activity during IVM impacts subsequent embryo development. We speculate that FGFs are improving oocyte competence during IVM by acting on cumulus cells to affect the accumulation of maternal transcripts, proteins and metabolites deposited in oocytes prior to ovulation so that oocytes are able to survive until EGA. The specific transcripts that serve as downstream regulators of this activity have yet to be identified. Understanding the underlying mechanisms mediated by FGFs during COC culture may open new avenues of research aimed at improving the oocyte's ability to form a viable embryo and ultimately produce a viable offspring.

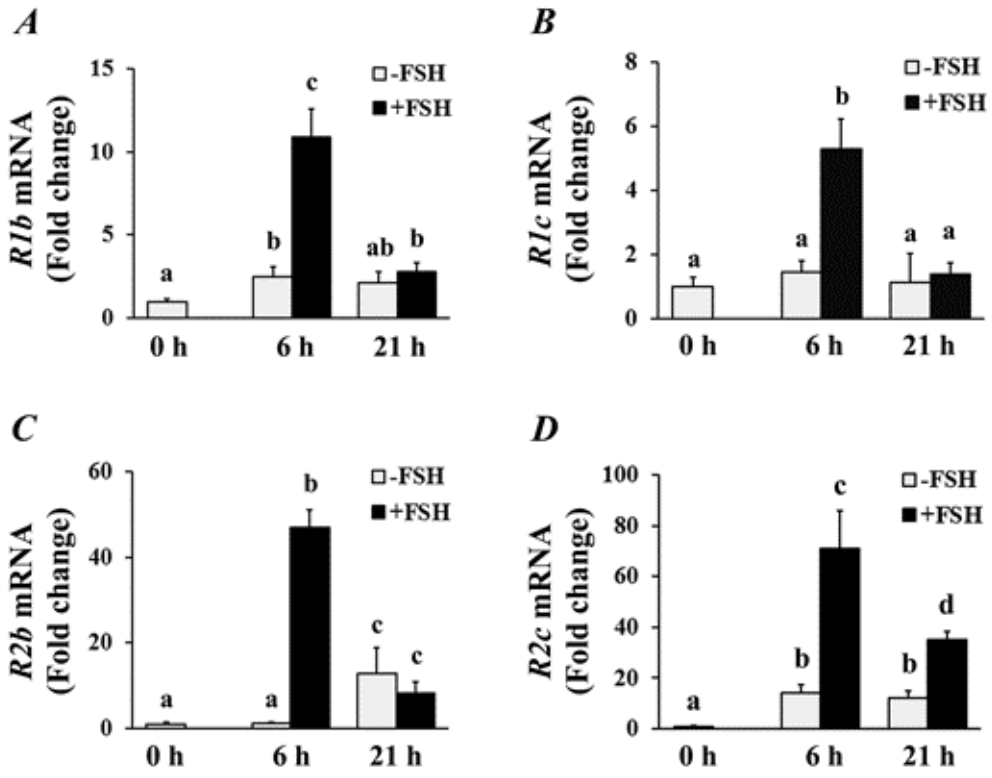


Figure 4-1. Changes in transcript abundance for *FGFR1* and *R2* splice variants in cumulus cells from COCs cultured in the presence or absence of FSH. Bovine COCs were collected at cumulus cells were separated from oocytes by vortexing either before culture (0 h) or 6 or 21 h after beginning IVM. Medium either contained or lacked 25 µg/ml FSH in COCs cultured for 6 or 21 h. Abundance of transcripts for *R1b*, *R1c*, *R2b* and *R2c* (A through D, respectively) was determined by qRT-PCR. Values are expressed as fold change relative to mRNA abundance of each transcript at 0 h. Differences superscripts within each panel indicate differences ($P<0.05$; n=15 COCs/time point/replicate, 3 replicate studies).

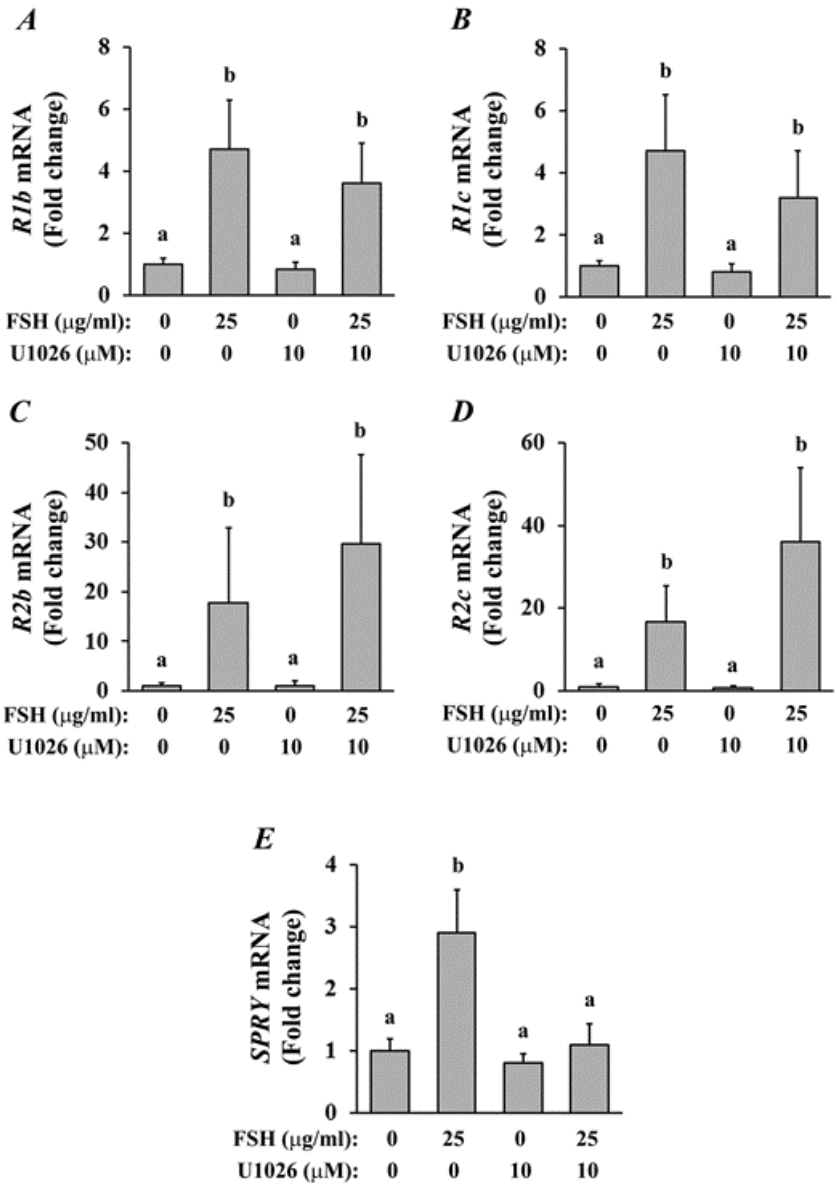


Figure 4-2. The FSH-dependent increase in FGFR mRNA abundance is not MAPK-dependent. COCs were treated with or without 25 μg/ml FSH and with or with 10 μM U0126, a MAPK3/1 inhibitor. After 6 h of maturation, cumulus cells were separated from oocytes by vortexing and qRT-PCR was used to examine abundance of transcripts for *R1b*, *R1c*, *R2b* and *R2c* (A through D, respectively) and *SPRY2* (E). Data are expressed as fold changes relative to the samples from COCs not treated with FSH or U0126. Differences superscripts within each panel indicate differences ($P<0.05$; n=15 COCs/replicate; 4 replicate studies).

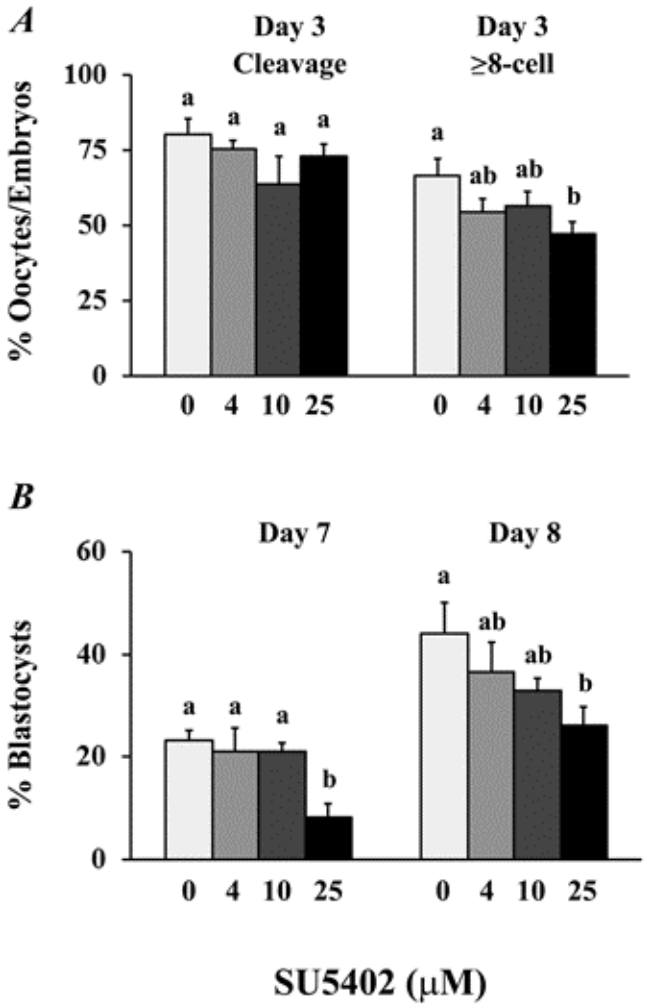


Figure 4-3. Disruption of FGFR signaling during IVM with SU5402 compromises subsequent embryo development *in vitro*. COCs were matured in medium containing 25 μg/ml FSH and SU5402 (4 to 25 μM). Controls contained carrier only (DMSO). After maturation, COCs were washed to remove inhibitors, oocytes were fertilized and presumptive zygotes were cultured. A) After 3 days, the percentage of cleaved embryos and the cleaved embryos that contained ≥8 cells were determined. B) At days 7 and 8 post-IVF, the percentage of cleaved embryos that were blastocysts was determined. Different superscripts in each graph denote differences ($P<0.05$; n=30 COCs/treatment/replicate; 6 replicate studies).

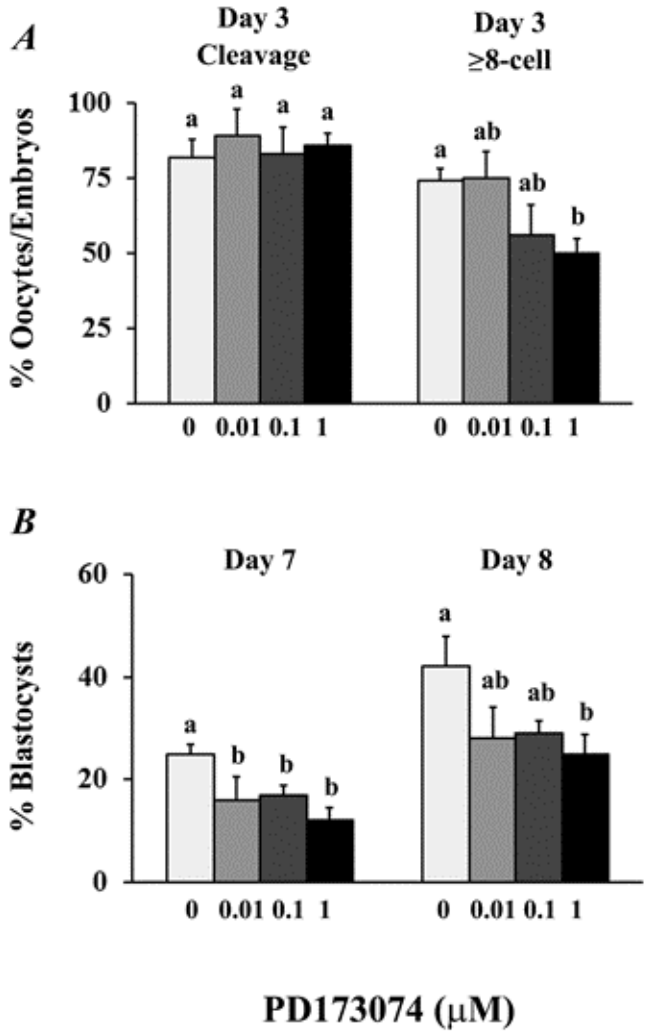


Figure 4-4. Disruption of FGFR signaling during IVM with PD173074 compromises subsequent embryo development *in vitro*. COCs were matured in medium containing 25 μg/ml FSH and PD173074 (10 nM to 1 μM). Controls contained carrier only (DMSO). After maturation, COCs were washed to remove inhibitors, oocytes were fertilized *in vitro*, and presumptive zygotes were cultured. A) After 3 days, the percentage of cleaved embryos and the cleaved embryos that contained ≥8 cells were determined. B) At days 7 and 8 post-IVF, the percentage of cleaved embryos that were blastocysts was determined. Different superscripts in each graph denote differences ($P<0.05$; n=30 COCs/treatment/replicate; 7 replicate studies).

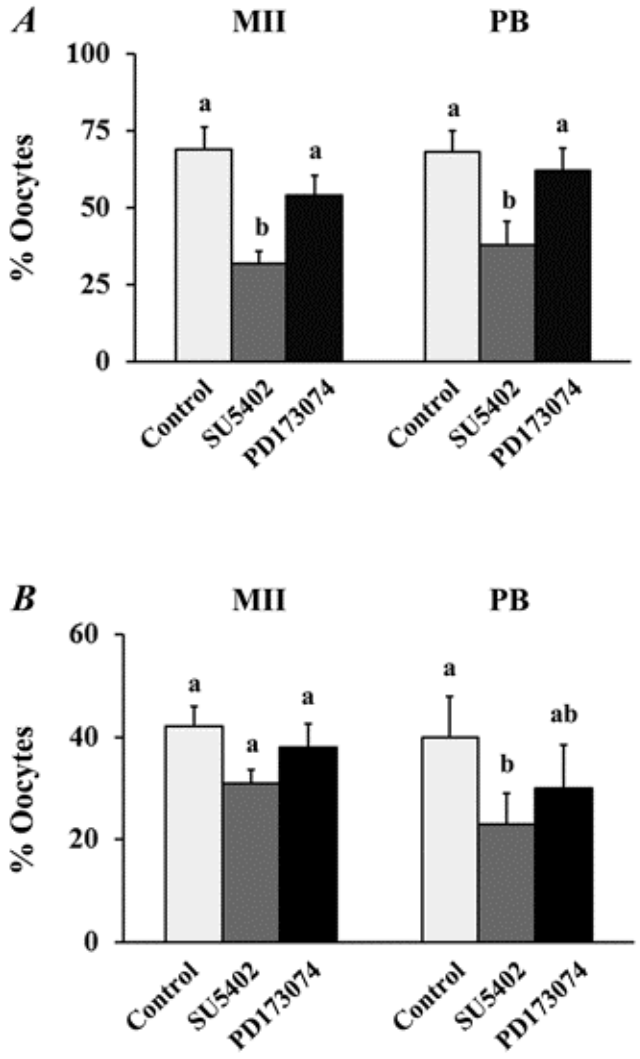


Figure 4-5. Meiotic maturation of oocytes is delayed by disruption of FGFR signaling during IVM. A) and B) COC were cultured in medium containing 25 (A) or 0 μg/ml FSH (B) and either 25 μM SU5402, 1 μM PD173074, or carrier only (DMSO). After 21 h, cumulus cells were separated from oocytes and the percentage of oocytes with extruded polar bodies and percentage of oocytes that matured to the metaphase II (MII) stage was determined (n=10 COCs/treatment/replicate; 4 replicate studies).

# CHAPTER 5
# SUPPLEMENTING OOCYTE MATURATION MEDIUM WITH FGFS IMPROVES *IN VITRO* PRODUCTION OF BOVINE EMBRYOS

Problems associated with oocyte quality contributes to infertility in cattle, humans and other mammals [27, 98]. The competence of oocytes to become fertilized and develop conceptuses that will maintain viable pregnancies to term are reduced when oocytes are matured *in vitro*. In cattle, fewer blastocysts develop from oocytes matured *in vitro* than oocytes matured *in vivo* [251], and fewer pregnancies result from transferring *in vitro* produced (IVP) embryos to cattle than using *in vivo* generated embryos or inseminating cows [30, 38, 296]. The premise of work presented herein is that embryo transfer success rates may be improved in cattle by optimizing oocyte cultures in ways that increase the proficiency of oocyte maturation, fertilization and/or early embryo development.

The oocyte grows within a follicular niche and acquires developmental competence through a series of events controlled by various endocrine, paracrine and autocrine factors (reviewed by [25]). Supplementation of OMM with molecules found in the follicular niche improves oocyte maturation rate and subsequent embryo production efficiency. EGF supplementation during IVM improves bovine oocyte maturation, cumulus expansion, fertilization rates and eventual development of blastocysts [147]. Similarly, supplementing vascular endothelial growth factor (VEGF) to bovine COCs during IVM increases subsequent fertilization and embryo development rates [297, 298]. Supplementing BMP15 or GDF9 individually or together during IVM does not affect fertilization rate of bovine oocytes but increases *in vitro* blastocyst development [266].

Several FGFs have been implicated as mediators of folliculogenesis and oogenesis in cattle [231, 234]. One FGF that has received recent attention as a putative regulator of follicle and oocyte development is FGF10. This factor is produced by theca cells and the oocyte and its

primary receptor partner is produced by granulosa/cumulus cells [60, 228]. Another FGF of interest is FGF2. Expression of this factor is up-regulated in theca and granulosa cells during the final stages of folliculogenesis preceding ovulation [216, 217] and in cumulus cells during IVM [218]. Also, certain single nucleotide polymorphisms (SNPs) for FGF2 associate with reduced IVF and embryo development potential [11]. Recently this laboratory determined that both these FGFs appear to be important for oocyte maturation. One recent study found that endogenous sources of FGF10 are required for optimal bovine cumulus oocyte complex (COC) maturation *in vitro*, and supplementing FGF10 during bovine IVM increases subsequent IVP embryo development (Chapter 2) [60]. Another study determined that FGF2 supplementation during IVM increases subsequent bovine embryo development (Chapter 3).

Previous work describing FGF2 and FGF10 actions when supplemented during IVM were completed in serum-free conditions. Most IVP embryo production systems include serum in OMM to achieve maximal embryo production. To explore the potential inclusion of FGFs during IVM as a scheme for improving oocyte competence, studies were completed to determine if FGF2 and FGF10 supplementation to OMM containing serum improves oocyte maturation and subsequent *in vitro* embryo development.

## Materials and Methods

### Reagents

Recombinant bovine FGF2 was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human FGF10, TCM 199, Hoechst 33342 and primers used in qRT-PCR were purchased from Invitrogen Corp. (Carlsbad, CA, USA). FSH was purchased from Bioniche Life Sciences (Belleville, Ontario, Canada). mSOF was purchased from Millipore (Billerica, MA, USA). Paraformaldehyde (16% [w/v] ultrapure grade) was purchased from Polysciences Inc. (Warrington, PA, USA). Mouse anti-Cdx2 was purchased from Biogenex (San Ramon, CA) and

FITC-labeled goat anti-mouse IgG was purchased from AbCam (Cambridge, MA). RNase-free DNase was purchased from New England Biolabs (Ipswich, MA, USA). The Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit was purchased from Roche Applied Sciences (Indianapolis, IN, USA). The RNeasy Micro Kit was purchased from Qiagen (Valencia, CA, USA) and the PicoPure$^{TM}$ RNA Isolation Kit was purchased from MDS Analytical Technologies (Sunnyvale, CA, USA). The High Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems Inc. (Foster City, CA, USA). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

All studies were completed in accordance and with the approval of the institutional animal care and use committee at the University of Florida.

**COCs Collection and Oocyte IVM**

Ovaries were collected from Central Beef Packing Co. (Center Hill, FL, USA). They were then washed with 0.9% [w/v] sodium chloride supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin. COCs were harvested and groups of 10-12 COCs were matured in 50 µl drops of OMM (TCM199 containing Earle's salts, 10% BSS, 25 µg/ml bovine FSH, 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, 1 mM glutamine). Varying concentrations of FGF2 or FGF10 were added to OMM immediately before beginning COC maturation. Both proteins were prepared in TCM199 containing 1% [w/v] BSA and stored in aliquots at -20ºC until use. COCs were cultured for 21 h at 38.5°C in 5% $CO_2$ in humidified air.

**IVF and IVC**

The IVF and IVC procedures were carried out as described previously [203]. Briefly, COCs were incubated with Percoll gradient-purified bovine spermatozoa in fertilization medium supplemented with PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine

in 0.9% [w/v] NaCl). A pool of semen from three bulls was used each time. After 8 h at 38.5°C (5% $CO_2$ in humidified air), cumulus cells were separated from oocytes by vortexing in 1000 U/ml hyaluronidase. Putative zygotes were cultured in groups of 25-30 in 50 µl drops of mSOF; Millipore, Billerica, MA, USA) containing 25 µg/ml gentamicin sulfate, 0.4 mM sodium pyruvate, 2.77 mM myo-inositol, 0.5 mM sodium citrate, 1 mM alanyl glutamine, 5.3 mM sodium lactate syrup, 10 µl/ml non-essential amino acids, 20 µl/ml essential amino acids and 4 mg/ml fatty acid-free BSA. Drops were covered with mineral oil and maintained at 38.5°C in 5% $CO_2$, 5% $O_2$ and 90% $N_2$ for 8 days.

**Nuclear Maturation and Cumulus Expansion**

After maturation, the degree of cumulus expansion in COCs was scored visually by phase-contrast microscopy on a 1 to 3 scale (1=poor expansion characterized by few morphological changes compared with before maturation; 2=partial expansion characterized by fair expansion but notable clusters lacking expansion; 3=complete or nearly complete expansion) [60, 244]. To assess meiotic stage after maturation, oocytes were denuded by vortexing for 4 min in saline. Oocytes were fixed with 4% [w/v] paraformaldehyde, permeabilized with0.1% [v/v] Triton X-100 and stained for 15 min with 1µg/ml Hoechst 33342. Meiotic staging was determined with epifluorescence microscopy as described previously [32, 245].

**Differential Staining in Blastocysts**

Blastocysts obtained at day 8 post-IVF were incubated with 4% [w/v] paraformaldehyde for 30 min. After three washes, they were incubated with 0.5% [v/v] Triton X-100and 1 mg/ml PVP in PBS for 20min. Blastocysts then were blocked with 5% BSA for 1 h before incubation with mouse anti-Cdx2 IgG at 4ºC overnight and FITC-labeled goat anti-mouse IgG at room temperature for 1 h. Blastocysts were counterstained with1 µg/ml Hoechst 33342 for 15 min and were placed into glycerol drops on microscope slides. Total cell numbers (all Hoechst stained

nuclei), TE (FITC-stained nuclei) and ICM cells (total minus TE) were determined by using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) with FITC and DAPI filter. Digital images were acquired using the AxioVision software and a high-resolution black and white AxioCam MRm digital camera (Zeiss).

**Real-time Quantitative (q) RT-PCR**

At the end of the maturation period, cumulus cells and oocytes were separated by repeated pipetting (n=25 to 30/group). Denuded oocytes were removed and washed thrice in PBS-PVP. Cumulus cells were transferred to microcentrifuge tubes and centrifuged at 700 × g for 2 min at room temperature to remove residual solution. Both oocytes and cumulus cells were snap-frozen in liquid nitrogen and stored at -80°C. Total cellular (tc) RNA was extracted from cumulus cells using the RNeasy Micro Kit and from oocytes using the PicoPure™ RNA Isolation Kit. RNA concentrations and integrity ($A_{260}/A_{280}$ ratio ≥1.8) of cumulus samples were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Limited amounts of RNA prevented determination of RNA quality in the oocyte samples. Each tcRNA sample (10 ng/reaction for cumulus samples; entire RNA sample for oocytes) was incubated in RNase-free DNase and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit. Primers used for this work were reported previously [60, 111, 190, 197, 270]. Primers were used at a concentration of 200 nM and were mixed with RT products and SYBR Green PCR Master Mix. After an initial activation/denaturation step (50°C for 2 min, 95°C for 10 min), 40 cycles of a 2-step amplification protocol (60°C for 1 min, 95°C for 15 sec) were completed using a 7300 Real-Time PCR System. A dissociation curve analysis (60 to 95°C) was used to verify the amplification of a single product. Each sample was run in triplicate and a fourth reaction lacking exposure to reverse transcriptase was included to verify the absence of genomic contamination. Relative amounts of *18S* RNA was used as an internal control for quantifying

relative gene expression. The ratio of target to reference RNA was used to determine relative expression ($2^{-CT[target]}/2^{-CT[reference]}$) [60].

**Statistical Analyses**

All analyses were carried out with least-squares analysis of variance (LS-ANOVA) with the GLM of the Statistical Analysis System (SAS for Windows, version 9.0; SAS Institute Inc., Cary, NC, USA). Statistical analyses used arcsine-transformed percentage data generated from each replicate (experimental unit = average percentage within each replicate). Differences in individual means were separated further by completing pair-wise comparisons (probability of difference analysis [PDIFF]; SAS Institute Inc.). Percentage data were graphed using non-transformed values and SEMs.

## Results

**FGF10 Promotes Bovine Oocyte Developmental Competence *In vitro***

Previous work by this laboratory determined that supplementing FGF10 to serum-free OMM benefits oocyte and embryo development in culture [60]. This study determined if similar effects were possible when OMM contained serum. COCs were collected and cultured in OMM containing 10% BSS and different concentrations of FGF10 (0.5, 5 or 50 ng/ml or carrier only control). After maturation, COCs were fertilized and cultured in the absence of supplemental FGF10. As outlined in Table 3-1, supplementation with FGF10 did not affect the percentage of cleaved zygotes present at day 3 post-IVF. Providing 50 ng/ml FGF10 increased (P=0.04) the percentage of cleaved embryos that were ≥ 8-cells at day 3 when compared with control. Supplementing lower concentrations of FGF10 did not affect percentage of ≥8 cell embryos.

Development to the blastocyst stage also was influenced by FGF10 supplementation. Specifically, supplementation with 50 ng/ml FGF10 but not lower FGF10 concentrations increased (P=0.03) the percentage of blastocysts observed at day 7 and 8 post-IVF. Also,

exposure to 0.5, 5 or 50 ng/ml FGF10 increased ($P<0.05$) the percentage of advanced blastocysts and the percentage of expanded blastocysts at day 7 post-IVF whereas none of these FGF10 treatments benefited advanced blastocyst rates at day 8.

An additional study determined that supplementation with 50 ng/ml FGF10 did not affect total cell number, the number of TE and ICM, and the ICM to TE ratio when compared with controls at day 8 post-IVF (Table 5-2). Immunoreactivity to Cdx2 anti-sera was used to distinguish TE from non-TE cells. Additional studies examined how FGF10 supplementation affects oocyte and cumulus maturation status *in vitro*. Incubation with 50 ng/ml FGF10 during maturation did not influence the percentage of oocytes achieving metaphase II (89 ± 5 vs. 79 ± 8 for 0 vs. 50 ng/ml FGF10, respectively) and did not alter cumulus expansion (data not shown).

**FGF10 Treatment During IVM Does Not Affect the Expression of Selective Transcripts Implicated in Cumulus and Oocyte Competence**

Numerous cumulus- and oocyte-specific genes have been implicated as mediators of COC maturation and oocyte competence. Selective transcripts were examined to determine if FGF10 regulated their abundance during COC maturation [111, 113, 173, 190, 197, 264, 266, 267, 299, 300]. No changes in the relative abundance of oocyte-specific transcripts (*BMP15, FST, GDF9, HIST2H2AC, JY1*) or cumulus-specific transcripts (*CTSB*, CTSZ, *EGFR, FSHR, HAS2, INHBA, KITLG, SPRY2)* were detected after 21 h of maturation in OMM containing BSS and either 0 or 50 ng/ml FGF10 (data not shown).

**FGF2 Promotes Bovine Oocyte Developmental Competence *In vitro***

A study was completed to determine whether supplementation of serum-containing OMM with FGF2 improves subsequent *in vitro* embryo development (Table 5-3). Supplementing with 0.5, 5 or 50 ng/ml FGF2 did not affect the percentage of cleaved embryos and embryos that contained ≥8 cells at day 3. Supplementation of 0.5 ng/ml FGF2 increased ($P=0.05$) the

percentage of blastocysts at day 7 but not at day 8. None of the other FGF2 treatments affected blastocyst formation. FGF2 supplementation did not affect the percentage of advanced blastocysts at day 8.

## Discussion

The proficiency of IVP systems to generate high quality, transferable bovine embryos is a major limitation to the widespread incorporation of IVP embryos in commercial settings. Pregnancy rates and pregnancy retention to term usually are lower following transfer of IVP-derived embryos than for inseminated cows and cows receiving embryos from multiple ovulation strategies [30, 38]. Also, a prevalence of extended gestation lengths, excessive birth weights and skewed gender ratios still exists in pregnancies derived from IVP embryos [301, 302]. Improvements in IVM technologies may provide opportunities to improve the overall percentage of transferable IVP embryos and, more importantly, improve the competence of these embryos to maintain pregnancies to term and yield a viable offspring.

This work and previous observations [60] show that FGF10 supplementation during IVM improves blastocyst yields in bovine IVP embryo production systems. Regardless of whether OMM contained or lacked serum, supplementation with FGF10 did not affect cleavage rates but increased the percentage of ≥8-cell embryos at day 3, blastocysts and advanced blastocysts. However, differences in FGF10-mediated effects during oocyte maturation were observed dependent on whether OMM contained or lacked serum. Neither oocyte progression to metaphase II nor cumulus expansion scores were affected by FGF10 supplementation in the presence of serum. Also, none of the cumulus- and oocyte-specific transcripts chosen for analysis based on previous reports of their involvement with oocyte quality and competence were altered by FGF10 in the presence of serum. In previous work, FGF10 supplemented to serum-free OMM stimulated oocyte maturation and cumulus expansion [60]. It also altered the

abundance for certain cumulus (*CTSB*, *SPRY2*) and oocyte (*BMP15*) transcripts. Such disparities indicate that FGF10-dependent improvements in embryo development rate do not require changes in rates of oocyte meiotic maturation, cumulus expansion or selective gene expression.

Supplementation with FGF10 during IVM did not affect total blastomere numbers and the number of TE and ICM cells at day 8 post-IVF. Previous work testing FGF10 effects in OMM without serum also failed to detect effects on blastomere numbers in blastocysts [60]. These observations are consistent with the contention that quality of the oocytes dictates blastocyst yield more so than the quality of the blastocyst [251].

The mechanisms used by FGF10 to manipulate COC maturation in ways that ultimately improve embryo development remain speculative. Observing increases in embryo development as early as the 8-cell stage suggest that perhaps FGF10 improves the developmental potential of IVP embryos by optimizing early embryo development. EGA occurs at the 8- to 16-cell stage in bovine embryos, and prior to this time the zygote must rely on maternally-derived mRNA, proteins and other molecules for regulating development [253]. It is quite possible that FGF10 impacts some aspect of the maternal control over early embryo development and this activity improves the proportion and timing of embryo development *in vitro*.

FGF10 exerted similar outcomes when supplemented to maturation medium containing or lacking serum supplementation, but there was a marked difference in the effective concentration ranges for FGF10 under these conditions. Removing serum from maturation medium reduced the effective dose for FGF10 (0.5 ng/ml without serum). In the presence of serum, 50 ng/ml FGF10 was needed in most cases to detect differences from controls. This outcome likely indicates that an inhibitory factor is present in the BSS. Heparin and heparan sulfate are detectable in serum and plasma [303], and several FGFs, including FGF10, have a great affinity for these molecules

[304]. Under normal situations heparin and heparan sulfate are crucial co-factors for FGF binding to receptors, but excesses in these molecules can limit FGF actions [304]. Alternatively, other FGF inhibitory factors may exist in serum. The identity of these molecules was not pursued in the work.

The effects of supplementing FGF2 during IVM were less pronounced than for FGF10 supplementation. An increase in blastocyst development was detected at day 7 post-IVF when supplementing 0.5 ng/ml FGF2 during COC maturation. Greater amounts of FGF2 did not affect blastocyst formation. Also, FGF2 did not alter cleavage rates, the percentage of ≥8-cell embryos at day 3 and advanced blastocyst formation. Previous work examining FGF2 supplementation during IVM in serum-free conditions found no improvements in fertilization and early embryo development rates but detected greater blastocyst development rates when provided at various concentrations (0.5 to 50 ng/ml) (Chapter 3). It is reasonable to speculate that limited effects observed with FGF2 supplementation resulted from the presence of an inhibitory molecule in serum.

In conclusion, providing FGF10 during IVM of bovine COCs increase blastocyst production. No special modifications to OMM are needed to detect FGF10-dependent effects on embryo development, but the FGF10 dosage must be increased in OMM containing serum for this treatment to be beneficial. FGF2 supplementation during IVM did not greatly impact subsequent embryo development in this work, indicating that this FGF is not as effective as FGF10 to increasing oocyte quality and early embryo development.

Table 5-1. Effects of FGF10 supplementation during IVM on subsequent *in vitro* embryo development.

| Parameter[1] | FGF10 (ng/ml) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 5 | 50 |
| 30 h: % Cleaved Zygotes[2] | $27 \pm 7^{a}$ | $24 \pm 6^{a}$ | $26 \pm 6^{a}$ | $29 \pm 6^{a}$ |
| Day 3: % Cleaved Zygotes[2] | $71 \pm 4^{a}$ | $77 \pm 3^{a}$ | $74 \pm 4^{a}$ | $72 \pm 3^{a}$ |
| Day 3: % ≥ 8-cell Embryos[3] | $54 \pm 12^{a}$ | $58 \pm 12^{ab}$ | $57 \pm 12^{ab}$ | $61 \pm 13^{b}$ |
| Day 7: % Blastocysts[3] | $37 \pm 3^{a}$ | $40 \pm 4^{ab}$ | $39 \pm 2^{ab}$ | $49 \pm 4^{b}$ |
| Day 7: % Advanced Blastocysts[3] | $15 \pm 4^{a}$ | $25 \pm 3^{b}$ | $28 \pm 4^{b}$ | $30 \pm 4^{b}$ |
| Day 8: % Blastocysts[3] | $51 \pm 3^{a}$ | $54 \pm 4^{ab}$ | $51 \pm 4^{a}$ | $59 \pm 3^{b}$ |
| Day 8: % Advanced Blastocysts[3] | $38 \pm 4^{ab}$ | $42 \pm 3^{ab}$ | $36 \pm 3^{a}$ | $46 \pm 4^{b}$ |

[1]For embryo development data, n=6 replicate studies; 26 to 35 COCs/replicate.
[2]Based on the percentage of total oocytes.
[3]Based on the percentage of cleaved embryos.
Different superscripts within each row (*i.e.* parameter) represents differences.

Table 5-2. The effect of FGF10 supplementation during IVM on inner cell mass and TE cell numbers at day 8 post-IVF.

| Cell Number[1] | FGF10 (ng/ml) | |
|---|---|---|
| | 0 | 50 |
| Total | 131 ± 6 | 126 ± 5 |
| Trophectoderm | 108 ± 6 | 104 ± 4 |
| Inner Cell Mass | 23 ± 2 | 22 ± 1 |
| Ratio Inner Cell Mass/Trophectoderm | 0.23 ± 0.01 | 0.23 ± 0.01 |

[1]n=60-61 blastocysts examined over 6 replicate studies.

Table 5-3. Effect of FGF2 supplementation during IVM on subsequent *in vitro* embryo development.

| Parameter[1] | FGF2 (ng/ml) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 5 | 50 |
| Day 3: % Cleaved Zygotes[2] | $61 \pm 8^{a}$ | $64 \pm 10^{a}$ | $58 \pm 9^{a}$ | $64 \pm 7^{a}$ |
| Day 3: % ≥ 8-cell Embryos[3] | $54 \pm 4^{a}$ | $67 \pm 5^{a}$ | $65 \pm 8^{a}$ | $58 \pm 4^{a}$ |
| Day 7: % Blastocysts[3] | $28 \pm 5^{a}$ | $41 \pm 3^{b}$ | $34 \pm 8^{ab}$ | $35 \pm 11^{ab}$ |
| Day 8: % Blastocysts[3] | $36 \pm 10^{a}$ | $51 \pm 3^{a}$ | $49 \pm 10^{a}$ | $49 \pm 10^{a}$ |
| Day 8: % Advanced Blastocysts[3] | $10 \pm 4^{a}$ | $10 \pm 2^{a}$ | $15 \pm 8^{a}$ | $11 \pm 3^{a}$ |

[1]For embryo development data, n=3 replicate studies; 26 to 35 COCs/replicate.
[2]Based on the percentage of total oocytes.
[3]Based on the percentage of cleaved embryos.
Different superscripts within each row (*i.e.* parameter) represents differences.

# CHAPTER 6
# GENERAL DISCUSSION

The ability of oocytes to resume meiosis, become fertilized and generate viable pregnancies, also called oocyte competence, is controlled during folliculogenesis by various endocrine, paracrine and autocrine factors. Having a better understanding of the acquisition of competence for cultured oocytes utilized for reproductive technologies may provide new strategies for improving *in vitro* reproductive technologies in humans and agricultural animals.

This dissertation focused on describing how specific paracrine factors affect the developmental potential of bovine embryos generated by IVM/IVF/IVC procedures. These procedures are of special concern because pregnancy rates following embryo transfer are often reduced for cows receiving IVP embryos when compared with inseminated cows [38, 280]. Recent studies implicate FGFs as important regulators of folliculogenesis. Thus, a series of studies were designed to define the role of FGFs in the oocyte maturation and subsequent embryo development.

The aim of the first and second project was to determine if FGF2 and FGF10 can improve bovine oocyte competence after their supplementation to OMM. Exposing cumulus-oocyte complexes to FGF10 during IVM increased the percentage of embryos at the 8-16 cell stage on day 3 and blastocysts on day 7 post-IVF. Exposure to FGF2 increased blastocyst rates but did not affect the proportion of embryos at the 8-16 cell stage. This result is interesting as EGA takes place at the 8- to 16-cell stage in bovine embryos [253]. Perhaps improvements in blastocyst formation by FGF10 are mediated by improving early embryo competence (i.e. before the 8-cell stage) whereas the beneficial effects of FGF2 act through later events of embryogenesis.

However, in both studies, there was no effect of FGF2 and FGF10 on either the total cell number per blastocyst or the proportion of ICM out of total cell number. These observation are

consistent with previous studies [251, 252]. In those studies, bovine oocyte quality affected subsequent blastocyst yields more so than blastocyst quality. It is likely that FGF2/10 improves the ooplasm microenvironment by changing specific molecules that enhance embryo development during the first few rounds of cleavage.

To better understand the effects of FGF2 and 10, oocyte maturation and cumulus expansion were examined. Both FGF2 and FGF10 increased the percentage of oocytes reaching metaphase II in intact COCs. However, these effects were compromised when using oocytes removed from cumulus before culture. This indicates that the enhancement in the meiotic maturation by FGF2 and 10 is mediated by cumulus cells.

Cumulus expansion was improved by FGF10 or FGF2 and FGF2 reduced the proportion of apoptotic cumulus cells after culture. We examined if FGF2 and 10 affected the expression of genes related to cumulus expansion and apoptosis. None of the genes examined were influenced by either FGF. Further studies are needed to define how these FGFs impact the cumulus cell transcriptome and also to understand how post-transcriptional regulation of mRNAs may be influenced by FGFs. Based on available evidence, it appears that the beneficial effects of FGF 2 and 10 on oocyte competence was attributed, at least in part, to the improved meiotic maturation or cumulus expansion and survival.

Given the FGF10 used in this work is human recombinant, the specificity of FGF10-dependent effects on oocyte maturation and cumulus expansion were evaluated. The use of anti-FGF10 IgG prevented the FGF10-dependent effects on maturation and cumulus expansion compared with the non-immune IgG control. Also, exposure to individual supplementation of anti-FGF10 IgG also inhibited meiotic maturation and cumulus expansion. Thus, both exogenous and endogenous FGF10 are beneficial for the COC maturation *in vitro*.

The involvement of FSH in FGF2 and 10-dependent effects was also evaluated by removing this supplement from the culture system. Previous studies show that rates of oocyte maturation were reduced in the absence of FSH [283]. Treatment with FGF2 and 10 did not affect oocyte maturation in absence of FSH. This implicates that FSH controls FGF-dependent events during maturation..

The expression pattern of FGFRs and the role of FSH were described. After 6 h of culture, R1b, R1c, R2b and R2c mRNA abundance were increased in cumulus cells but not in oocytes when COCs were cultured with FSH. However, this effect was compromised after 21 h of culture. The similar phenomena have been seen in follicles from cattle after GnRH treatement [272]. Specifically, increased FGFR1c mRNA abundance was detected 4 h after GnRH challenge than before or ≥10 h after GnRH treatment. This change may be driven by dynamic changes in steroid and prostaglandin production [272]. Alternatively, FGFR expression and activity are tightly regulated by several intracellular feedback inhibitors [211, 239] and such activity could explain the temporal expression patterns of these receptors.

A follow-up study showed that the FSH-dependent increases in cumulus cell *FGFR* mRNA levels were not mediated by MAPK3/1 signaling, which is required in granulosa cells for fertility in female mice [139, 140, 290]. However, the increase of sprouty2 mRNA in response to FSH is mediated by MAPK3/1.

Another important aspect of this work was to examine whether FGF2 and 10 improved oocyte competence by mediating the actions of other cumulus and oocyte competence factors. . FGF10 influenced the expression of CTSB and SPRY2 in cumulus cells and BMP15 in oocytes. Nonetheless, none of these genes were affected by FGF2. Similar findings have been observed in previous studies. For example, CTSB mRNA abundance has been found negatively associated

with the oocyte competence in cattle. Exposure to BMP15 during IVM improves blastocyst formation. These other studies and ours implicate FGF10 maybe one upstream molecules of those oocyte competence factors.

The first two projects were using "gain of function" approach and revealed that both FGFs of interest are beneficial for oocyte developmental potential. This prompted us to decipher what about the roles of other FGFs during oocyte maturation. Also, we discovered that FGF10 present within COCs were functionally significant. Preventing its activity by antibody capture limited bovine oocyte competence.

A "loss of function" approach was used to determine if preventing FGF10 and potentially other FGFs from acting on COCs during IVM affects oocyte maturation, fertilization and subsequent embryo development. After maturation, COCs were washed and fertilized and cultured in the absence of inhibitors. Addition of either inhibitor decreased the percentage of ≥8-cell embryos and blastocyst formation. Collectively, the gain of function and loss of function studies indicated that FGF signaling is very important for induction of oocyte maturation and cumulus expansion and thus critical for the subsequent embryo development.

Several implications may be possible from this work. First and foremost, we speculate if supplementing FGF2 or FGF10 to maturation medium during *in vitro* culture of bovine COCs might result in embryos that are better suited for transfer into recipient cows. One set of studies determined that supplementing FGF2 or 10 to OMM containing serum, the oocyte medium formulation commonly used for IVP, improves blastocyst development rates. FGF10 appeared to be better suited for improving embryo development than FGF2.

The predominat endpoint used in most laboratories for evaluating the developmental potential of the IVP embryo is blastocyst formation, which is also used in this work. However,

the full potential has to be examined through embryo transfer. Thus, further work is needed to determine whether FGF2/10's beneficial effect last in the long term.

The second potential application from this work is selection of the high yielding cows based on the genetic sequence of FGF2/10. Several recent studies have reported SNPs of *FGF2* or *STAT5* in cows are associated with embryos survival [11, 305]. In addition, BMP15 homozygous mutation in sheep reduces ovulation rate while heterozygous mutation increases this rate [104]. Perhaps, the selection of cows based on FGF2/10 sequence will improve the reproductive efficiency.

Our work may have relevance to human IVF clinics. The use of IVM in human clinics is limited due to the low efficiency of IVM. The reproductive physiology and embryo developmental pattern as well as ART in women is similar with those in the cow (both are single-ovulating species) than the mouse [306, 307]. Thus, the outcomes in this work implicates FGF2/10 are conceivably to improve oocyte competence in women.

To conclude, this work indicates that improvements with *in vitro* embryo production efficiency in cattle can be made by supplementing COCs with FGF10 and FGF2 to a lesser extent (Figure 6-1). This work also provides a novel insight into the importance of FGFRs and locally-derived FGF2 and 10 during oocyte maturation in cattle. Their subsequent impact on *in vitro* embryo development implicates them as noteworthy oocyte competence factors.

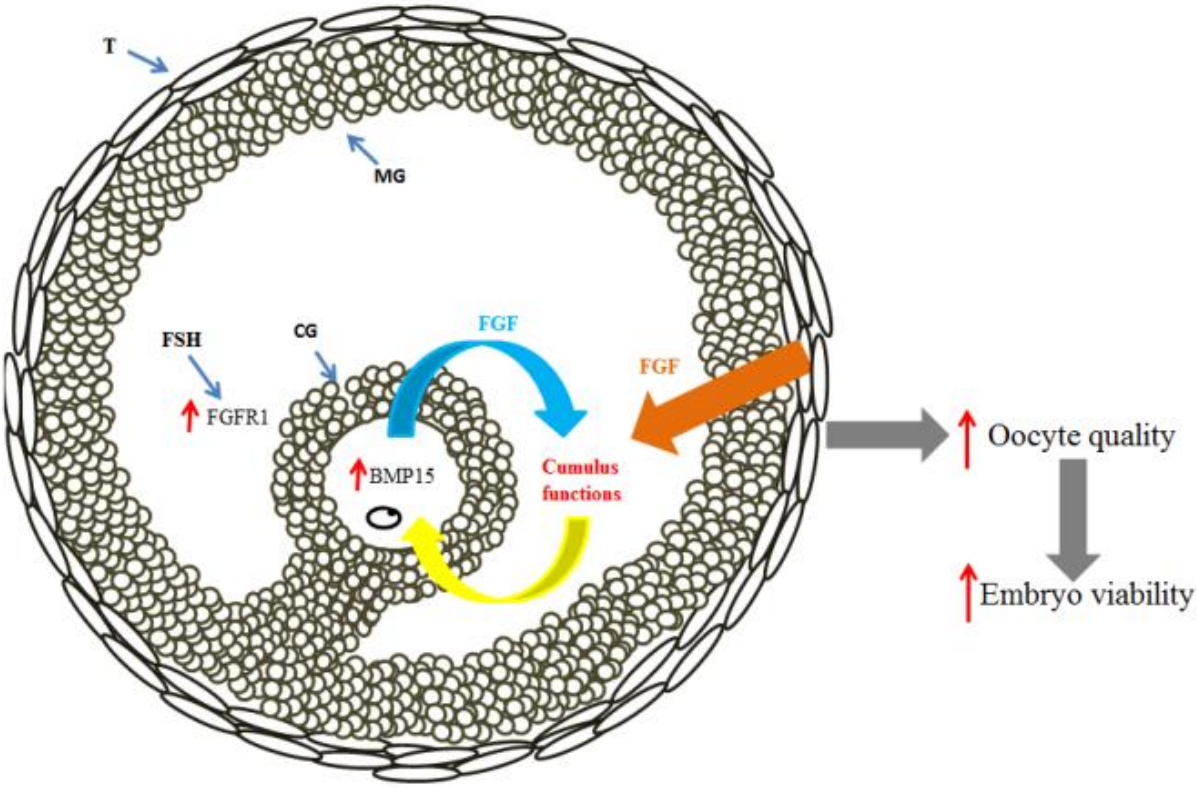


Figure 6-1. Summary of the FGF signaling requirement for the oocyte maturation and developmental competence. Paracrine factors FGFs (i.e. FGF2/10) secreted from either the oocyte or follicular somatic cells (theca cells and granulosa cells) act on the cumulus cells to affect cumulus expansion and change specific molecules (i.e. *CTSB*, *Sprouty 2*) or potentially other functions. The remodeling of the cumulus cells by FGFs may influences the oocyte microenvironment by communicating through the gap junctions between the oocyte and the cumulus cells. For example, one important oocyte signal BMP15 is up-regulated by FGF10. Also, FGF appears activates ERK1/2, the essential components for mammal fertility, by phosphorylation. By these changes, the oocyte quality is improved and thus the future embryos are viable. The FGF signaling is under the control of FSH. For example, FGFR1 mRNA is increased by FSH during oocyte maturation. T: theca cells; CG: cumulus granulosa cells; MG: mural granulosa cells.

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# BIOGRAPHICAL SKETCH

Kun Zhang was born in 1982 in Guo Yang County, Anhui Province in China. As the youngest of three sons in the family, Kun graduated from No.1 Middle School of Bo Zhou with honors in 2000. He then came to Beijing to attend China Agricultural University (CAU) for undergraduate study and earned a Bachelor of Agriculture degree in Animal Sciences in summer of 2004. Following graduation, he continued at CAU for graduate study in September of 2004. His research was focused on optimization of pig cloning systems under the supervision of Dr. Ning Li. In the summer of 2007, Kun moved to Florida and began his doctoral study in the Animal Molecular and Cellular Biology Graduate Program. From August, 2007 to August, 2008, his project focused on characterizing the role of DNA methylation in development of bovine cloned embryos under the supervision of Dr. Karen Moore. Afterwards, he switched to work with Dr. Alan Ealy to study the role of FGF signaling in oocyte maturation and embryo development. After completing his doctoral studies, Kun will move to the University of Massachusetts at Amherst to continue his research on reproductive biology. Kun is married to Shaohua Wang, who is a molecular biologist.