ABSTRACT

HOCKNEY, JESSICA EILEEN. Candidate mRNAs Regulating Meiotic Resumption in Bovine Cumulus-Oocyte-Complexes. (Under the direction of Charlotte Elizabeth Farin.)

In bovine oocytes, the resumption of meiosis is characterized by the breakdown of the germinal vesicle (GVBD). When cumulus-oocyte complexes (COCs) are cultured in-vitro in the presence of gonadotropins, GVBD is characterized by an initial inhibitory phase, which is followed by an acceleration in the rate of GVBD. An initial transcriptional event is required for gonadotropin-induced in-vitro maturation. The objectives of this thesis were: 1) to define the time course required for transcriptional initiation in bovine cumulus oocyte complexes (COCs); 2) to determine the pattern of expression for Nr4A1 and Egr1 mRNAs in bovine COCs; and 3) to reduce the expression of Nr4A1 mRNA expression to determine its effect on oocyte maturation.

Bovine COCs were cultured in the presence follicle stimulating hormone (FSH) alone or FSH with the transcriptional inhibitor, 5,6-dichloro-1-B-D-ribofuranosylbenzamidazole (DRB), in order to refine the time course required for transcription initiation and to determine the pattern of expression for Nr4A1 and Egr1 mRNAs. All experiments contained a control group of COCs that were cultured for the entire duration in the presence of DRB. By adding DRB at 0, 30, 60, 90, 120, 150, or 180 minutes after the initiation of culture, it was determined that gene transcription required for GVBD occurs between 0 and 60 minutes after the start of culture. Analysis of COCs cultured for 0, 30, 60, 90 or 180 minutes demonstrated

that Nr4A1 mRNA levels increased significantly ($P<0.05$) at 30 minutes after the start of culture, which is consistent with the time of transcription initiation required for GVBD. In contrast, Egr1 mRNA levels did not significantly differ throughout the culture period.

Small interfering RNAs (siRNAs) designed from the sequence for Nr4A1 were used to reduce Nr4A1 mRNA expression and determine the effects of Nr4A1 mRNAs on GVBD in bovine COCs. Expression of Nr4A1 mRNA decreased in abundance in treatment groups containing siRNAs specific to Nr4A1 (siNr4A1) with the greatest decrease in expression occurring in the 25nM and 50nM siNr4A1 treatments. As expected, fewer COCs underwent GVBD when cultured in the presence of DRB at 9 and 20 hours as compared to COCs cultured in FSH alone. Additionally, no significant differences were observed between the FSH and non-specific siRNA (siNS) treatment groups in the proportion of COCs undergoing GVBD at either 9 or 20 hours of culture. Fewer COCs cultured in the presence of 50nM siNr4A1 underwent GVBD by 9 hours of culture as compared to those cultured in FSH alone. The percentage of COCs that underwent GVBD did not differ between the siNr4A1 and FSH control treatments at 20 hours. The expression of Nr4A1 mRNA at 30 minutes after the start of culture did not differ with FSH, siNr4A1, or siNS treatments.

In summary, gene transcription required for GVBD in bovine COCs occurs within 0 to 60 minutes of culture. Nr4A1 mRNAs are present in bovine COCs and these mRNA levels increase significantly after 30 minutes of culture. Furthermore, Egr1 mRNAs are present in bovine COCs, but Egr1 mRNA levels do not change

throughout culture. Bovine COCs cultured with siNr4A1 showed a significant decrease in the percentage of oocytes undergoing GVBD after 9 hours of culture. In conclusion, it appears that Nr4A1 plays an active role in GVBD in bovine COCs.

Candidate mRNAs Regulating Meiotic Resumption in Bovine Cumulus-Oocyte-Complexes

by

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REVIEW OF LITERATURE

OOCYTE DEVELOPMENT AND MATURATION

OOGENESIS AND FOLLICULOGENESIS

Background

In mammalian systems, female germ cell development begins during gastrulation, as primordial germ cells, formed from the embryonic ectoderm (Drummond, 2005), migrate from the yolk sac to the urogenital ridge (Knobil and Neil, 1998). The germ cells, or oogonia, are clustered into nests of cells undergoing incomplete cytokinesis, with daughter cells connected by intercellular bridges (Pepling, 2006). Oogonia stain positive for alkaline phosphatase, allowing their migratory path to be traced (Chiquoine, 1954; Ginsburg et al., 1990). Following migration, the cells undergo a brief period of mitosis while expanding their population. The oogonia then enter meiosis and become primary oocytes (Downs, 1990), with one flat layer of flattened, squamous epithelial cells surrounding the primary oocyte, forming a primordial follicle (Jamnongijt and Hammes, 2005) just before or shortly after birth (Drummond, 2005).

Prophase I

Primary oocytes enter the first stage of meiosis, prophase I, which is categorized into five transitory stages, which include preleptotene, leptotene, zygotene, pachytene, and, finally, diplotene where the oocyte enters into dictyate

arrest. Dictyate arrest is easily identified by the presence of decondensed chromatin and the germinal vesicle (GV; Martus, 1994), and is referred to as the "diffuse diplotene" stage. When the oocyte is activated, the GV undergoes germinal vesicle breakdown (GVBD) at the resumption of meiosis (Shultz et al., 1983). For many years it was believed that all mammals were born with a finite number of oocytes that were held in dictyate arrest until puberty, with small groups of follicles maturing during each cycle (Mandl and Zuckerman, 1951). However, recent evidence suggests that murine oocytes might undergo atresia at a steady rate and then regenerate from germline stem cells throughout the reproductive life of the animal (Johnson et al., 2004).

Primary Follicular Development

Once the primary oocytes are arrested in the diplotene stage of meiosis, the primordial follicles are recruited by unknown factors to begin the transformation of primordial follicle to primary follicle, with the transformation of follicular granulosa cells from a squamous to cuboidal shape (Drummond, 2005). During this transition, mitochondria, smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER) increase as the oocyte's new energy requirements increase (Fair et al., 1997). While the factors responsible for transformation remain unclear, many members of the transforming growth factor-ß (TGF-ß) superfamily have been found in the ovary during this critical developmental period and may represent potential

candidates for key regulators of the primordial to primary follicular transition (Drummond et al., 2003; Drummond 2005). Furthermore, TGF-ß is thought to stimulate preantral follicle growth when cultured in the presence of FSH (Drummond, 2005). Additional candidates for this role include anti-mullerian hormone (AMH), growth and differentiation factor-9 (GDF9), bone morphogenetic proteins 4 and 7 (BMP4,-7; Drummond, 2005).

Preantral Follicular Development

As granulosa cells proliferate in primary follicles, layers form and a multilayered preantral follicle (secondary follicle) develops. During this phase, cortical granules are formed in the oocyte cytoplasm (Fair et al., 1997), the zona pellucida begins to form, and oocyte RNA synthesis is detected for the first time (Fair, 2003). Thecal cells are also recruited to the follicles during this phase, allowing follicular response to lutenizing hormone (LH). In response to FSH, the preantral follicle forms a fluid filled cavity (antrum) and is now an antral, or tertiary, follicle (Drummond, 2005). Early tertiary follicles have at least one active nucleolus and are fully capable of transcription (Fair, 2003). At this stage, AMH is known to inhibit granulosa cell proliferation (diClemente et al., 1994), aromatase activity, and LH receptor expression (Kim et al., 1992; Seifer et al., 1993). In addition, AMH appears to affect both preantral and small antral follicles (Baarends et al., 1995) by decreasing the responsiveness of these follicles to FSH, while assisting in selection of

the Graffian follicle and subsequent atresia of remaining follicles (Durlinger et al., 2001; Drummond 2005). Other factors possibly playing a role in antrum formation include activin A, which has been shown to stimulate rat antral formation in-vitro (Zhao et al., 2001).

Importance of Cumulus-Oocyte Interactions

In order to understand the mechanisms by which oocytes undergo meiosis it is vital to understand interactions between the oocyte and the surrounding cumulus granulosa cells, collectively referred to as the cumulus-oocyte-complex (COC). Cumulus granulosa cells are closely connected to the oocyte via gap junctions, which are made of proteins from the connexin family, most specifically connexin 43 (Cx43; Jamnongjit and Hammes, 2006). These gap junctions are necessary for cumulus cell expansion during follicular growth and for subsequent oocyte development and maturation (Jamnongjit and Hammes, 2006) as they allow cell-to-cell communication and are necessary for the transportation of nutrients and metabolic precursors to the oocyte (Herlands and Schultz, 1984). Notably, oocytes lacking intact cumulus cells are incapable of maintaining meiotic arrest when cultured in vitro. During meiotic maturation COCs undergo cumulus cell expansion where gap junctions are severed and oocytes undergo subsequent maturation (Eppig, 1994). It is hypothesized that these gap junctions are important for the transfer of factors inhibiting meiosis, such as cAMP, from the cumulus cells to the oocyte (Downs and Hunzicker-Dunn, 1995;

Jamnongjit and Hammes, 2006). When these gap junctions are broken, the oocyte no longer receives inhibitory factors and undergoes maturation (Downs and Hunzicker-Dunn, 1995; Jamnongjit and Hammes, 2006).

Follicular Size and Oocyte Developmental Competence

Oocytes in primordial follicles are incapable of resuming meiosis and subsequent embryo development. However, as follicular size increases, meiotic and developmental competence of the oocyte also increases (Fair et al., 1995). In bovine oocytes, full meiotic competence is not reached until the follicle reaches the antral stage and is approximately 1.5-2mm in diameter. These oocytes, typically greater than 100μm in diameter, first gain the ability to undergo germinal vesicle breakdown (GVBD). Then they acquire the ability to reach the metaphase I (MI) stage and finally the metaphase II (MII) stage where they remain arrested until subsequent fertilization (Fair et al., 1995). Furthermore, bovine oocytes cannot undergo fertilization and development until developmental competence is acquired, typically when follicles are greater then 2mm in diameter (Fair et al., 1995).

COCs must be removed from follicles between 2mm and 10mm in order to be meiotically and developmentally competent (Rodriguez and Farin, 2004). When COCs originate from follicles less than 2mm, they are unable to reach MII, whereas COCs from follicles larger then 10mm may be atretic, having already started meiosis prior to culture (Fair et al., 1995). COCs must have at least 4 complete layers of

cumulus cells in order for proper communication to occur between the oocyte and cumulus cells through gap junctions. This communication is vital to oocyte maturation and oocytes lacking complete cumulus layers will not reach full meiotic and developmental competence (Eppig et al., 2002).

Effects of Gonadotropins

After puberty, meiosis resumes in oocytes of preovulatory follicles following the gonadotropin surge of lutenizing hormone (LH) and follicle-stimulating hormone (FSH; Masui and Clarke, 1979). The oocyte proceeds into meiosis, extrudes the first polar body, and is arrested again at the MII stage until fertilization (Knobil and Neil, 1998). While oocytes resume meiosis in the presence of LH, it should be noted that there are no LH receptors present on either oocytes or their surrounding cumulus-granulosa cells. In fact, LH receptors are only expressed on the thecal and mural granulosa cells of the preovulatory follicle. Thus for LH to be the hormone responsible for oocyte maturation and cumulus expansion, some unknown paracrine factor(s) must be released by these thecal and granulosal cells in response to LH to trigger oocyte maturation and cumulus cell expansion events (Peng et al., 1991; Jamnongijt and Hammes, 2005). In recent years members of the epidermal growth factor (EGF) family have been suggested to be some of these previously unknown paracrine regulators (Downs et al., 1988; Park et al., 2004). Some of these growth factors include amphiregulin and epiregulin, which are thought to simulate oocyte

maturation and ovulation (Downs et al., 1988; Park et al., 2004). In this proposed model, LH promotes both steroid production and the release of these growth factors from thecal and/or mural granulosa cells, with the growth factors acting in a paracrine manner to cause cumulus cell expansion, breaking apart the cumulus-oocyte gap junctions, and causing oocyte maturation (Park et al., 2004; Jamnongijt and Hammes, 2005).

CUMULUS CELL-OOCYTE INTERACTIONS

Development of Gap Junctions

In order for folliculogenesis to proceed and oocyte developmental competence to be acquired, growth and development of both the somatic and germ cells must occur in a synchronized manner (Gilchrist et al., 2004). In the primordial follicle, the oocyte and squamous granulosal cells are connected by adherent-like junctions, with oolema projections penetrating adjacent granulosa cells (Fair et al., 1997). At this stage of development, gap junctions are present between adjacent granulosa cells, but are not yet connecting the oocyte and granulosa cells (Fair et al., 1997).

During follicular growth, the antrum of the follicle is formed and the granulosa cells develop into two different subtypes with distinct anatomical and functional differences, the mural granulosa cells and the cumulus granulosa cells (Gilchrist et al., 2004). Mural granulosa cells line the follicular wall around the antrum of the follicle, and form an epithelial layer with a basal lamina (Gilchrist et

al., 2004; Khamsi and Roberge, 2001). Cumulus granulosa cells surround the oocyte proper and are held in place by "trans-zonal cytoplasmic processes" which penetrate through the zona pellucida and parts of the oocyte membrane, creating cumulus-oocyte complexes (Gilchrist et al., 2004). Gap junctions occur on the ends of these cytoplasmic processes and also between the cumulus granulosa cells themselves (Gilchrist et al., 2004). The appearance of gap junctions occurs simultaneously with antrum formation, coinciding with the ability of the oocyte to undergo meiosis (Fair et al., 1997; Gilchrist et al., 2004).

Gap Junctions

Gap junctions are made of connexons, with each connexon composed of six protein subunits called connexins (Kidder and Mhawi, 2002). Each connexin is made of four transmembrane domains, two extracellular loops, and one intracellular loop, with N- and C-termini located in the cytoplasm (Kidder and Mhawi, 2002). Gap junctions between the oocyte and the surrounding cumulus cells allow for the transfer of relatively small molecules, less then 1 kDa in size (Bruzzone et al., 1996), between the oocyte and the cumulus cells without resorting to receptor-mediated endocytosis (Gilchrist et al., 2004). Such molecules are primarily associated with metabolism and include nucleotides, glucose and other metabolites (Eppig 1991; Gilchrist et al., 2004). In addition, amino acids, ions, second messengers such as cAMP, and other small regulatory molecules are also transferred via gap junctions (Eppig 1991;

Gilchrist et al., 2004). Gap junctional communication is known to play an important role in oocyte development and subsequent fertility (Gilchrist et al., 2004). Furthermore, gap junctional communication is thought to be involved in broadcasting endocrine messages from the cumulus cells directly to the oocyte (Gilchrist et al., 2004). In experiments designed to decrease Conexin 43 (Cx43) in vitro, the primary gap junctional protein in COCs, a recombinant adenovirus expressing the antisense code for Cx43 resulted a two-fold decrease in coupling between cumulus cells and the oocyte and an overall fifty percent decrease in oocyte maturation (Vozzi et al, 2001). This experiment demonstrates that gap junctional communication between oocytes and the surrounding cumulus cells is vital for oocyte maturation (Vozzi et al, 2001).

Oocyte and Cumulus Cell Interactions

For decades the oocyte was thought to play a relatively passive role in the regulation of folliculogenesis and its subsequent maturation, but in the past few years it has become abundantly clear that the oocyte is, itself, an important regulator of folliculogenesis, primarily through the use of bidirectional paracrine signaling (Gilchrist et al., 2004). Communication from the oocyte to the cumulus cells and vice versa is thought to be necessary for granulosa cell development (Gilchrist et al., 2004). This communication is vital in that folliculogenesis will not occur in the absence of paracrine signaling between the oocyte and surrounding cumulus cells (Gilchrist et al., 2004).

Oocyte Secreted Factors

Several paracrine factors secreted by the oocyte that are thought to play a role in the resumption of meiosis are part of the TGF-ß superfamily (Gilchrist et al., 2004). In particular, growth differentiation factor-9 (GDF9), GDF9-B, bone morphogenetic protein-15 (BMP15), and possibly various activins are of interest for regulating meiotic resumption (Gilchrist et al., 2004). Inactivation of GDF9 and GDF9B by introduction of inactivating mutagens in ovine and murine models has shown that these oocyte-secreted factor(s) promote DNA synthesis and subsequent cell proliferation of both mural and cumulus granulosa cells (Gilchrist et al., 2004). Most interestingly, these oocyte-derived mitogenic factor(s) enhance and promote granulosa cell proliferation by stimulating synthesis of insulin-like growth factor-1 (IGF1) and androgens (Gilchrist et al., 2004; Hussein et al., 2006).

Growth Differentiation Factor-9 (GDF9)

GDF9 appears to induce the expression of genes that are associated with oocyte maturation and acquisition of oocyte developmental competence by maintaining a "highly specialized microenvironment" in the cumulus cells surrounding the oocyte (Hussein et al., 2006). GDF9 has been shown to play a crucial role in cumulus cell expansion in the mouse (Buccione et al., 1990; Dragovic et al., 2005), as well as in upregulation of expression of hylauronic acid synthase 2 (HAS2; Elvin et al., 1999) and cyclooxygenase 2 (COX2; Su et al., 2004). Both

HAS2 and COX2 are required for cumulus cell expansion and subsequent maturation. In addition, GDF9 appears to stimulate gene expression in cumulus cells while regulating cumulus cell uptake of amino acids and substrates, as well as transport into the oocyte (Hussein et al., 2006; Sugiwura and Eppig, 2005; Su et al., 2004). In the mouse, deletion of GDF9 causes an arrest of primary follicle development (Su et al., 2004). This is due to a lack of cuboidal granulosal cell proliferation (Su et al., 2004). GDF9 has also been found to promote granulosa cell growth while repressing the expression of the LH receptor in the cumulus cells (Su et al., 2004).

Bone Morphogenetic Protein-15 (BMP15)

Oocyte-secreted factors help maintain cumulus cells in a non-lutenized state by promoting growth and reducing steroid production (Li et al., 2000) as well as by preventing cumulus cell apoptosis (Hussein et al. 2005, 2006). BMP-15 performs this function by maintaining the level of anti-apoptotic factors (Hussein et al. 2005, 2006). Deletion of the BMP15 gene in mice has a negative impact on fertility, in part by reducing the number of oocytes successfully undergoing fertilization (Otsuka et al., 2000, 2001; Su et al., 2004). Recombinant BMP15 has also been found to promote the growth of rat granulosa cells in culture while suppressing FSH receptor number (Otsuka et al., 2000, 2001; Su et al., 2004). Mice lacking the GDF9 and BMP15 genes ovulated oocytes that lacked solid cumulus cell-oocyte connections and had lower rates of fertilization (Su et al., 2004). Thus, it appears that the BMP15 and

GDF9 act in a synergistic manner to affect COC development (Su et al., 2004). Working together, BMP15 and GDF9 alter cumulus cell phenotype, allowing these cells to respond to LH, albeit indirectly, and induce the process of cumulus cell expansion (Su et al., 2004).

Insulin-Like Growth Factor System (IGF System)

The insulin-like growth factor (IGF) system has long been recognized to play a role in follicular development in mammalian species, primarily affecting cell proliferation and steroidogenesis (Nuttinck et al., 2004). The ligands IGF-1 and -2, their receptors, and the IGF-binding proteins have been identified and expression patterns verified during bovine folliculogenesis (Nuttinck et al., 2004). The IGF1 receptor (IGFR1) has been identified in both the granulosa and thecal cells (Nuttinck et al., 2004). Neither the expression of IGF1 nor IGFR1 is affected by the stage of follicular development (Armstrong et al., 2000), but IGF binding proteins (IGFBPs) can bind to IGF (Jones and Clemmons 1995; Nuttinck et al., 2004) and can prevent IGF from binding to IGFR1, activating the signaling pathway of IGFR1 (Jones and Clemmons 1995) or simply slowing IGF clearance (Jones and Clemmons 1995; Nuttinck et al., 2004). Paracrine factors secreted by the oocyte do affect IGF-induced steroidogenesis (Nuttinck et al., 2004) and the addition of IGF1 to in vitro culture medium further acts as a promoter for meiosis (Nuttinck et al., 2004; Sakaguchi et al., 2002). Cell-specific expression patterns have been determined and demonstrate that

IGFR1 and IGFBP2 mRNAs are located in the oocyte, while IGF1 transcripts are isolated in the cumulus cells (Nuttinck et al., 2004). These data suggest that the IGF system is activated via a paracrine control system between the oocyte and surrounding cumulus cells. Furthermore, IGF1 combined with epidermal growth factor (EGF) appears to play a role in cumulus expansion, oxidative metabolism, and oocyte maturation (Nuttinck et al., 2004; Roeger et al,, 1998; Sakaguchi et al., 2002).

Epidermal Growth Factor (EGF) and Epidermal Growth Factor-Like Factor

In vivo, the LH surge mid-cycle triggers oocyte maturation and subsequent ovulation, yet the cumulus cells of rodents contain very few if any LH receptors. Messenger RNAs for the EGF-like factors amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) have been found to be upregulated by LH in mouse and rat preovulatory follicles, indicating a potential role for these factors as communicators between the LH surge and the oocyte itself, triggering cumulus cell expansion and oocyte maturation (Park, et al., 2004; Shimada et al., 2006). Conti et al. (2006) have proposed a possible paracrine mechanism in which LH induces AREG, EREG, and BTC release in granulosa cells of preovulatory follicles. These ligands then activate EGF receptors in the cumulus cells, leading to the production of mRNAs for haylauron synthase-2 (HAS2), prostaglandin synthase-2 (PTGS2) (also known as cyclooxygenase-2 (COX2)) and tumor necrosis factor α-induced protein-6 (TNFAIP6) (Ochsner al., 2003), all of which are essential for cumulus cell expansion

(Conti et al., 2006; Hsieh and Conti, 2005; Shimada et al., 2006). TNFAIP6 binds to the hyluronan backbone and interacts with the α-chain to stabilize the cellular matrix (Fulop et al., 2004; Shimada et al., 2006). Interestingly, knockout mice for PTGS2 or PTGS2 receptor (PTGSR2) also have decreased levels of TNFAIP6 mRNA and protein (Ochsner al., 2003). Cumulus cell expansion has also been shown to be decreased with COCs were cultured with the progesterone receptor (PR) agonist RU486, even in the presence of gonadotropins (Shimada et al., 2004, 2006).

OOCYTE MATURATION

After puberty, meiosis resumes in oocytes of preovulatory follicles following a gonadotropin surge, which consists of surges of follicle-stimulating hormone (FSH) and lutenizing hormone (LH) (Masui and Clarke, 1979). The dictyate oocyte undergoes GVBD and proceeds to metaphase II, extrudes the first polar body, and is arrested again at the MII stage until fertilization (Knobil and Neil, 1998).

Molecular Mechanisms Responsible for Oocyte Maturation

High cAMP levels within the oocyte keep PKA in an active state, thereby phosphorylating unknown proteins responsible for the maintenance of the GV (Conti et al., 2002; Richard 2007). The mechanism by which cAMP levels remain high in the oocyte are unclear, but one hypothesis suggests that cAMP diffuses through the gap junctions from the supportive granulosa cells to the oocyte proper (Conti et al.,

2002) and that this pool of cAMP is controlled by phosphodiesterase 3A (PDE3A) (Shitsukawa et al., 2001; Conti et al., 2002). High levels of cAMP prevent maturation-promoting factor (MPF) activation via phosphorylating $p34^{cdc2}$ and repressing the synthesis of cyclin B1 (Dekel, 2005). It is thought that GVBD is dependent upon activation of signaling pathways, possibly utilizing PDE3A, and is responsible for a reduction in cAMP levels or blocking the inhibitory effects of cAMP (Conti et al., 2002).

When cAMP levels drop or become blocked, PKA is inactivated, allowing the oocyte to undergo GVBD (Conti et al., 2002). This drop in cAMP levels and subsequent inactivation of PKA is thought to allow for MPF activation, possibly acting as an upstream regulator of the mitogen-activating protein kinase/Mos (MAPK/Mos) pathway, thus stimulating Mos translation and activation of MAPK (Dekel, 2005). MPF is then inactivated at the MI stage in order complete the first meiotic division with the extrusion of the first polar body (Dekel, 2005). It is thought that the decrease in the cAMP levels and subsequent inactivation of PKA results in a reactivation of MPF, again activating MAPK and Mos translation (Dekel, 2005). Consequently, the activated MPF regulates MAPK/Mos pathway and subsequent Mos translation, and the oocytes arrest at the MII stage until fertilization (Maller et al., 2002; Dekel, 2005).

Regulation of the Meiotic Cell Cycle

Initiation and regulation of the meiotic cell cycle is known to be a complex and involved mechanism that is not clearly defined. Several factors have been identified as playing an active role in this process including but not limited to transcriptional events, subsequent protein synthesis and phosphorylation events (Sirard et al., 1998).

Factors Known to Regulate Oocyte Maturation

mRNA Transcription

While it was originally thought that mRNA and subsequent protein synthesis was not needed for murine oocytes to undergo GVBD (Jagiello, 1969; Stern et al., 1972; Golbus and Stein, 1976; Crozet and Szollosi, 1980), other mammalian systems were found to depend on protein synthesis at or before the resumption of meiosis (Ekholm and Mangnusson, 1979; Osborn and Moor, 1983; Moor and Crosby, 1986). It should be noted that more recent literature suggests the murine system does, in fact, need mRNA synthesis prior to the resumption of meiosis in order to continue meiotic maturation (Rodriguez et al., 2002).

Gene transcription is vital for the resumption of meiosis and subsequent GVBD, and occurs within the first hour to hour and a half of culture for bovine oocytes (Farin and Yang, 1994; Kastrop et al., 1991; Meineke and Meinecke-Tillmann, 1993; Osborn and Moore, 1983; Sirard et al., 1998). Oocytes removed

from follicles prior to ovulation can be prevented from undergoing meiotic maturation and subsequent GVBD by being cultured in the presence of chemicals inhibiting the function of RNA polymerase II (Sirard et al., 1998). Compounds that act to inhibit GVBD at the level of RNA transcription initiation, include α-amanitin (Osborn and Moor, 1983; Moor and Crosby, 1986; Hunter and Moor, 1987; Kastrop et al., 1991; Meinecke and Meinecke-Tillman, 1993; Rodriguez et al., 2002) and the adenosine analog, 5,6-dichloro-1-B-D-ribofuranosylbenzamidazole (DRB), which prevents the formation of a stable transcription initiation complex (Farin and Yang, 1994; Rodriguez et al., 2002). It should be noted that these inhibitors are only effective when used on intact COCs, as the cumulus cells are required for control of meiotic maturation (Tsafriri et al., 1982; Eppig et al., 1983; Schultz et al., 1983; Downs et al., 1988; Rodriguez et al., 2002).

Protein Synthesis

Based on experiments using reversible protein synthesis inhibitors such as cycloheximide, bovine oocytes require an initial synthesis of proteins at the resumption of meiosis in order for the oocyte to undergo GVBD (Sirard et al., 1998). In addition, transferring cytoplasm from oocytes that have already resumed meiosis into oocytes in the early GV stage will cause the arrested oocyte to resume meiosis, even when cultured with cycloheximide (Simon et al., 1989; Sirard et al., 1998). Current data suggests that this protein synthesis is in response to a rise in MPF

(Simon et al., 1989; Sirard et al., 1998; Tatemoto and Horiuchi, 1995). Specifically, cyclin B, part of the MPF molecule, is thought to play a key role in the resumption of meiosis due to its association with cdc32 and subsequent phosphorylation and dephosphorylation events (Sirard et al., 1998).

Kinases

Phosphorylation events are critical during meiosis (Sirard et al., 1998), and play a role in both GVBD and chromatin condensation (Motlik et al., 1990; Sirard et al., 1998; Dekel 2005). The use of the kinase inhibitor, 6-dimethylaminopurine (6-DMAP), a puromycin analog, inhibited meiosis in a dose-dependent manner in a variety of species across two phyla (Sirard et al., 1998). It is currently hypothesized that MPF is not activated in the presence of 6-DMAP, preventing phosphorylation events and the subsequent activation of cyclin B (Sirard et al., 1998; Han and Conti, 2006). It should be noted that when cultured in the presence of 6-DMAP, bovine COCs are arrested during the first 4 hours of culture and remain sensitive to 6-DMAP, but then undergo meiosis even in the presence of the phosphorylation inhibitor (Fulka et al., 1991; Sirard et al., 1998).

Protein Kinase A

Protein kinase A (PKA) is a kinase that is activated downstream of adenylate cyclase (AC) conversion of ATP to cAMP. cAMP, in turn, binds to the regulatory

unit of PKA (Sirard et al., 1998; Dekel 2005). This pathway is governed by a G-protein coupled gateway, and is activated when the proper agonist binds to the receptor, causing the G-protein to disassociate and activate AC (Rodriguez and Farin, 2004; Sirard et al., 1998). In order to prevent intracellular levels of cAMP from increasing too rapidly, phosphodiesterase-3A (PDE3A) converts cAMP to 5'-AMP, which is normally inactive in this system (Rodriguez and Farin, 2004; Sirard et al., 1998; Conti et al., 2002). It should be noted that PKA is a tetramer with two regulatory subunits and two catalytic subunits (Sirard et al., 1998). Furthermore, the two different PKA isozymes, PKA type I and PKA type II, differ in their regulatory unit structure and properties (Sirard et al., 1998). PKA type II is located in the cumulus cells and is responsible for the resumption of meiosis while PKA type I is located both within the oocyte proper and in the cumulus cells (Rodriguez and Farin, 2004; Sirard et al., 1998). Activation of PKA type I results in meiotic arrest (Rodriguez, 2002) and is responsible for maintaining meiotic arrest (Rodriguez and Farin, 2004; Sirard et al., 1998). High levels of cAMP within the oocyte help to maintain meiotic arrest and a decrease in cAMP levels occurs directly before the resumption of meiosis (Sirard et al., 1998; Dekel 2005).

Phosphatases

The phosphatase inhibitor okadaic acid (OA) acts by inhibiting phosphatase 1 and 2A, and has been shown to increase general protein phosphorylation by inhibiting

these two phosphatases (Lévesque and Sirard, 1996; Sirard et al., 1998). This treatment resulted in the acceleration of meiotic resumption and subsequent GVBD in cattle (Lévesque and Sirard, 1996; Sirard et al., 1998). Furthermore, OA treatment reduced the effects of the kinase inhibitor, 6-DMAP, and the protein synthesis inhibitor cyclohexamide (Sirard et al., 1998). These observations demonstrate that an OA treatment increases meiotic resumption by increasing protein synthesis and protein phosphorylation (Sirard et al., 1998). The tyrosine phosphatase inhibitor, vanadate, inhibits the activation of MPF and consequently prevents the resumption of meiosis (Sirard et al., 1998; Solomon et al., 1990). In addition, vanadate also damages microtubule function in the oocyte (Aquino et al., 1995; Sirard et al., 1998).

Follicular Fluid Components

Several factors have been identified in follicular fluid that play important roles in oocyte maturation. Follicular fluid (FF) contains proteins, glycoproteins, and steroids (Sirard et al., 1998). Based on biochemical analyses, differences in bovine FF (BFF) have been demonstrated to occur during different phases of follicular development (Sirard et al., 1998). Műllerian inhibiting substance (MIS) is one of several factors present in follicular fluid that prevents meiotic resumption (Sirard et al., 1998). Another of these factors is an unidentified, 8,000 dalton protein that has been shown to block progesterone-induced maturation (Sirard et al., 1998). A soluble protein kinase acting in a cAMP-dependent manner has also been identified (Sirard et

al., 1998). In addition, a relatively high level of linoleic acid, which inhibits resumption of meiosis in naked bovine oocytes, is present in BFF (Sirard et al., 1998). It was originally suggested that MIS and the unidentified proteins originate from the thecal or granulosal cells of the follicular wall, but subsequent studies have proposed that these factors originate solely in the granulosa cells and are transferred to the oocyte via gap junctions that connect the oocyte to the surrounding cumulus cells (Sirard et al., 1998).

Mechanism of Intrafollicular Arrest

When located in the inhibitory follicular environment, the oocyte is arrested in the GV state. A follicular signal binds to a G-protein coupled receptor, causing it to disassociate, activating adenylate cyclase (AC) and consequently converting ATP to cAMP (Sirard et al., 1998). These elevated cAMP levels activate PKA type I, located in the oocyte cytoplasm, and therefore maintain meiotic arrest (Rodriguez and Farin, 2004; see figure 1).

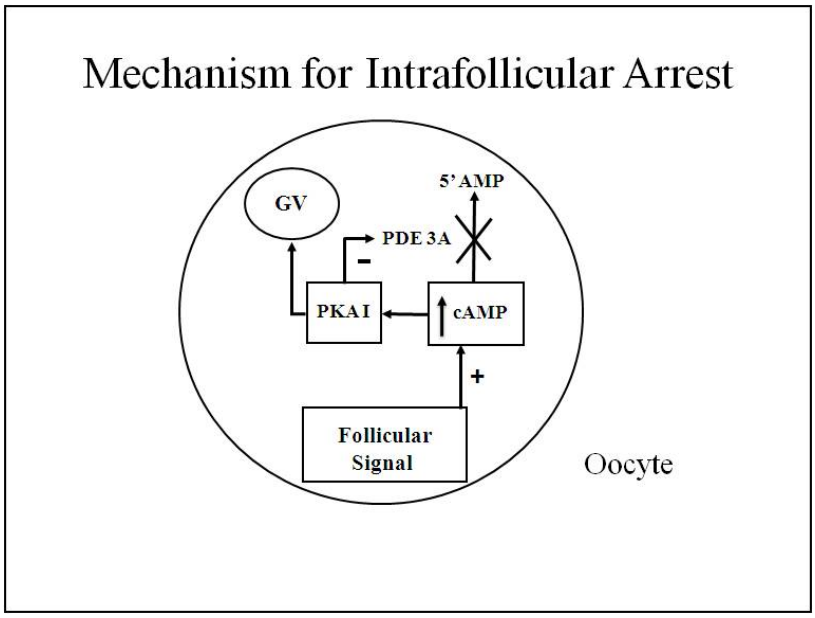

Figure 1. Mechanism for intrafollicular arrest. High levels of cAMP maintained by a follicular signal activate PKA type I, which in turn inactivates PDE3A, and subsequently maintains the GV. Figure taken from Rodriguez and Farin, 2004.

In Vivo vs. In Vitro Maturation

Resumption of meiosis in a GV-stage oocyte occurs in vivo due to an increase in the gonadotropin levels of lutenizing hormone (LH) and follicle stimulating hormone (FSH; Jamnongijt and Hammes, 2005). It is thought that the follicular cells produce various inhibitory factors that prevent the oocyte from resuming meiosis while still within the follicle proper (Sirard et al., 1998). In vitro, resumption of meiosis occurs following removal of the oocyte from the follicle (Pincus and Enzmann, 1935; Edwards, 1965). When the follicle is punctured during collection,

the oocyte is removed from the inhibitory environment and consequently resumes meiosis (Sirard et al., 1998).

Spontaneous vs. Gonadotropin-Induced In Vitro Maturation

When cultured in vitro, the oocyte can spontaneously resume meiosis in the absence of gonadotropins (Farin and Yang, 1994). Additionally, oocytes can resume meiosis when cultured in the presence gonadotropins, although maturation occurs in an altered manner. When gonadotropins are included in culture medium, GVBD is characterized by an initial inhibitory phase followed by acceleration in the rate of GVBD (Rodriguez and Farin, 2004). This gonadotropin-induced maturation requires an initial transcriptional event mediated by unidentified mRNA products. When cultured in the presence of gonadotropins, GVBD occurs after COCs have been in culture for approximately 6-8 hours (Farin and Yang, 1994). Metaphase I occurs around 12-15 hours in culture whereas MII occurs at approximately 18-24 hours of culture (Sirard et al., 1989).

Spontaneous In Vitro Maturation

Spontaneous in vitro maturation is thought to occur simply as a consequence of the COC being removed from the inhibitory follicular environment, resulting in removal of the inhibitory follicular signal and a subsequent drop in cAMP levels (Rodriguez and Farin, 2004). Spontaneous maturation can be prevented by including

PDE inhibitors, cAMP analogs, AC activators, or invasive AC directly to the culture medium (Rodriguez and Farin, 2004), and therefore spontaneous maturation is believed to be simply an artifact of in vitro culture (Downs, 1995; Rodriguez and Farin, 2004; see figure 2).

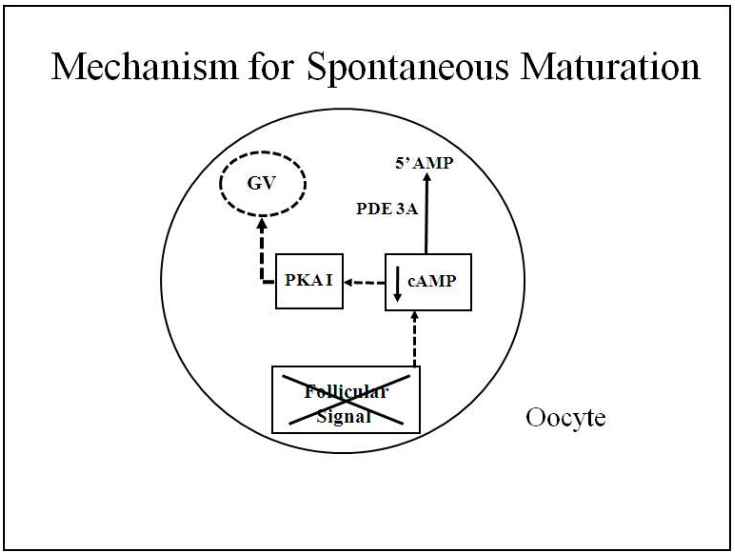

Figure 2. Mechanism for spontaneous maturation. When the oocyte is removed from the follicular environment, the inhibitory follicular signal is removed and subsequently cAMP levels drop. When cAMP levels decrease, PKA type I is inactivated and GVBD occurs. PDE3A may also be activated, further decreasing cAMP levels. Figure taken from Rodriguez and Farin, 2004.

Gonadotropin-Induced In Vitro Maturation

Inhibitory Phase of Gonadotropin-Induced In Vitro Maturation

When COCs are cultured in the presence of gonadotropins, an initial inhibitory period is present in which the COCs maintain their arrest in the GV state (Eppig et al., 1983; Farin and Yang, 1994). It is hypothesized that this phenomenon is due to the maintenance of high cAMP levels in response to FSH binding to receptors on the cumulus cells surrounding the oocyte (Downs, 1990; Rodriguez and Farin, 2004). FSH binds to receptors on the cumulus cells, which results in an initial activation of PKA type I in the cumulus cell (Rodriguez and Farin, 2004). This results in an unknown signal that enters the oocyte via gap junctions, which results in the maintenance of high levels of cAMP within the oocyte (Rodriguez and Farin, 2004). These high levels of cAMP, in turn, continue to activate PKA type I within the oocyte, and maintain the GV state (Rodriguez and Farin, 2004). This temporary period of FSH-induced meiotic arrest lasts for approximately six hours in bovine COCs (Farin and Yang, 1994; Rodriguez et al., 2002; see figure 3).

It should be noted that when transcriptional inhibitors such as DRB or α-amanitin are included in the culture medium before the onset of the initial transcriptional event, the inhibitory phase of gonadotropin-induced in vitro maturation is prolonged (Rodriguez and Farin, 2004). As long as these inhibitors remain in the medium, oocytes will not enter the stimulatory phase of gonadotropin-induced in vitro maturation and will not undergo GVBD (Rodriguez and Farin, 2004).

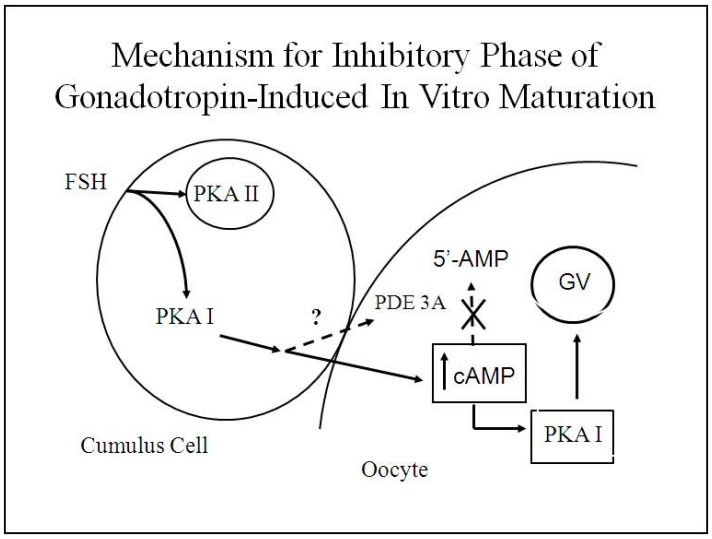

Figure 3. Mechanism for inhibitory phase of gonadotropin-induced in vitro maturation. FSH binds to the cumulus cell membrane, increasing cAMP levels within the cumulus cell, and subsequently activating PKA types I and II. Activation of PKA type I within the cumulus cell maintains high cAMP levels within the oocyte, which in turn activates PKA type I within the oocyte, maintaining the oocyte in the GV stage. Figure taken from Rodriguez and Farin, 2004.

Stimulatory Phase of Gonadotropin-Induced In Vitro Maturation

Following the initial inhibitory phase of gonadotropin-induced maturation, there is a notable acceleration in the rate of GVBD compared to that of spontaneous maturation (Farin and Yang, 1994, Rodriguez and Farin, 2004). This stimulatory phase is dependent upon a transcriptional event occurring within one hour from the

start of culture in bovine, porcine and ovine systems (Osborn and Moore, 1983; Kastrop et al., 1991; Meineke and Meinecke-Tillmann, 1993; Farin and Yang, 1994). Transcriptional inhibitors, such as DRB or α-amanitin, must be added to the culture within this same time frame to stop mRNA synthesis and cause meiotic arrest. Since the kinetics of maturation in mice is rapid, this transcriptional event occurs in murine systems within the first 20 to 30 minutes of culture (Rodriguez et al., 2002).

Binding of FSH to cumulus cell receptors activates PKA type II, initiating gene transcription and subsequent protein formation (Rodriguez and Farin, 2004). These proteins either enter the oocyte directly via gap junctions or, more likely, establish a signal communication from the cumulus cells to the oocyte through the gap junctions and are thought to stimulate PDE3A, which converts the relatively high levels of cAMP into the inactive 5'-AMP, therefore overriding the initial meiotic arrest caused by PKA type I during the inhibitory phase (Rodriguez and Farin, 2004). The decrease in cAMP levels causes an inactivation of PKA type I within the oocyte, and this inactivation allows the oocyte to undergo GVBD (Rodriguez and Farin, 2004; see figure 4).

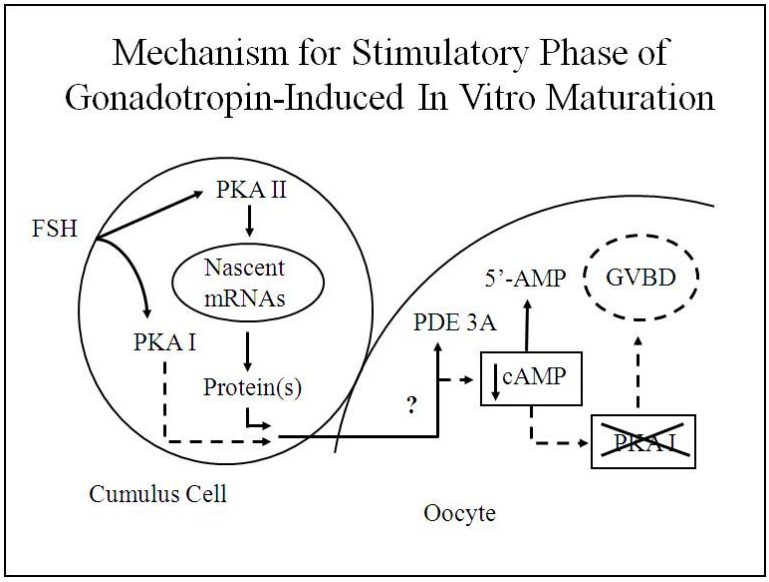

Figure 4. Mechanism for stimulatory phase of gonadotropin-induced in vitro maturation. When FSH binds to it's receptor on the cumulus cell, PKA type II is activated, which initiates gene transcription and subsequent translation. The proteins produced then enter the oocyte and are thought to activate PDE3A, decreasing cAMP levels, consequently inactivating PKA type I within the oocyte, and therefore inducing GVBD. Figure taken from Rodriguez and Farin, 2004.

GENOMIC METHODOLOGIES FOR STUDYING OOCYTE MATURATION

METHODS FOR ANALYSIS OF TRANSCRIPTOME EXPRESSION

In the past twenty years, technology has advanced to a level that allows for large scale genome analysis. These techniques provide large quantities of information regarding potential candidate genes involved in various biological mechanisms. While several types of genomic analyses are now available, microarray and serial analysis of gene expression (SAGE) remain the most promising for the identification of transcripts actively playing a role in oocyte GVBD.

Microarray

Developed in the early 1990's, microarray technology allows one to monitor the expression of many genes simultaneously (Schena et al., 1995; Tavera-Mendoza et al., 2006). DNA microarrays consist of a glass slide that contains thousands of DNA sequences to be identified and expression patterns determined (Tavera-Mendoza et al., 2006). These sequences are fixed to the slide in a grid, with each portion of the grid representing a particular portion of a gene (Tavera-Mendoza et al., 2006). RNA samples are then converted to cDNA, labeled with various fluorescent dyes, and then hybridized to the slide (Tavera-Mendoza et al., 2006). Comparisons of fluorescently labeled probe sets are used to determine the identity and abundance of various sequences (Tavera-Mendoza et al., 2006). cDNA microarrays use probes that

are amplified by RT-PCR, and are fixed on slides by UV cross-linking the thymidine residues of the cDNA and the amine groups on the slide (Cheung et al., 1999; Tavera-Mendoza et al., 2006). In contrast, oligonucleotide microarrays use 25mer oligonucleotides that are complementary to the desired gene and are formed in silicone (Lipshutz et al., 1999; Tavera-Mendoza et al., 2006). Oligonucleotide microarrays are the most commonly at this time, as they allow for detection of RNA from a single cell or small pools of cells or embryos (Brambrink et al., 2002; Niemann et al., 2007).

Serial Analysis of Gene Expression (SAGE)

Like microarray technology, serial analysis of gene expression (SAGE) was also developed in the mid 1990's (Tavera-Mendoza et al., 2006). In SAGE, short cDNA sequences, called tags, are generated by cleavage of cDNA with class II restriction enzymes while leaving 4 base pair overhangs (Tavera-Mendoza et al., 2006). These tags are then concatemerized into longer strands for sequencing and the number of times a tag appears is counted, determining the relative abundance of the tag (Tavera-Mendoza et al., 2006; Tuteja and Tuteja, 2004).

Comparison of Microarray and SAGE

Like all technologies, both microarrays and SAGE each have inherent advantages and drawbacks. Both of these technologies allow for a large scale

analysis of the desired transcriptome while identifying previously unknown transcripts that can be cross-referenced with identified or predicted sequences (Tavera-Mendoza et al., 2006). Microarrays are generally considered to be technically easier to perform then generation of SAGE libraries and make a relatively "quick" snapshot of genes (or mRNAs) involved in a particular process (Tavera-Mendoza et al., 2006). Drawbacks of microarray technology include limiting numbers of transcripts on a single chip and the inability of the array to detect multiple copies of the same transcript (Tavera-Mendoza et al., 2006). Therefore quantification is inherently difficult when using microarrays. Unfortunately, SAGE cannot identify transcripts that do not have class II restriction sites, as tags will not be generated, and some "orphan" tags will be generated in all libraries (Tavera-Mendoza et al., 2006). Therefore, only tags that are noted several times in various libraries are viable candidates. Notably, SAGE does not require previous knowledge of the mRNA sequences to be identified, can recognize a tag several times in a library, and therefore is a promising technology for identification of novel mRNA sequences (Tavera-Mendoza et al., 2006). For this reason, SAGE was used to generate two libraries used to compare mRNAs present in murine oocytes arrested at the GV stage to those mRNAs present in COCs that underwent GVBD (Rodriguez et al., 2006).

SAGE Library Generation for mRNAs present in Gonadotropin-Induced In Vitro GVBD

In order to identify candidate mRNAs responsible for the resumption of meiosis, SAGE libraries were created to compare gene expression profiles from murine cumulus oocyte complexes (COCs) cultured in the presence of FSH to those from transcriptionally arrested COCs cultured with FSH plus DRB (Rodriguez et al., 2006). SAGE tags were compared and assigned identities from databases such as the NCBI SAGE Map. SAGE tag counts were compared between the FSH-induced and DRB-arrested COC treatment groups. From these data, a list of candidate mRNAs involved in murine cumulus-oocyte maturation were identified (Rodriguez et al., 2006).

Four of these potential candidate mRNAs are of particular interest as they play active roles in biochemical pathways associated with meiotic maturation and subsequent oocyte developmental competence. Nuclear Receptor Subfamily 4, Member 1 (Nr4A1), Early Growth Response 1 (Egr1), Dual Specificity Phosphatase 1 (Dusp1), and Immediate Early Response 3 (Ier3) were all demonstrated to have between a 5- and 40-fold decreased expression when murine COCs were cultured in the presence of the transcriptional inhibitor, DRB, compared to untreated control COCs (Rodriguez et al., 2006). These four candidate mRNAs affect cellular systems recognized as important in cumulus-oocyte function. Nr4A1, also called Nur77, is a parathyroid hormone-induced gene and is thought play a role in activation of the

cAMP-protein kinase A pathway (Kovalovsky et al., 2002; Maira et al., 2003; Pirih et al., 2005). Egr1 is an extremely important mRNA in oocytes, having been identified bovine granulosa cells and found to have a profound effect on oocyte developmental competence (Roberts et al., 2001). Furthermore, Egr1 is thought to be induced by FSH and play an active role in follicular growth and ovulation while closing off gap junctions during oocyte maturation (Fritz et al., 2002; Russell et al., 2003). Dusp1 is thought to be involved in the down-regulation of MAPK activity and possibly play a role in spindle formation during meiosis (Gordo et al., 2001). Ier3 is thought to cause rapid cell progression in high doses, while having the opposite effect when removed from culture (Wu, 2003).

SMALL INTERFERING RNAs (siRNAs)

RNA Interference (RNAi)

RNA interference (RNAi) is a new approach for studies of mRNA function and is based on the silencing of mRNAs through the use of double-stranded RNA (dsRNA; McManus and Sharp, 2002). Discovered in 1998 with the injection of dsRNA into *C. elegans*, RNAi results in selective mRNA degradation (Fire et al., 1998) and uses small portions of dsRNA to block the expression of a target mRNA homologous to the dsRNA (McManus and Sharp, 2002; Sledz et al., 2003). This allows for identification of specific mRNA function (McManus and Sharp, 2002; Sledz et al., 2003). Small interfering RNAs (siRNAs), 21-23 nucleotide (nt) dsRNAs

with 2nt 3'-end overhangs, determine RNAi specificity, acting as an intermediate of RNAi in mRNA silencing (McManus and Sharp, 2002). Therefore, specific siRNAs can be synthesized and used to silence a target mRNA (McManus and Sharp, 2002; Sledz et al., 2003). This technology allows one to decrease specific mRNA expression in cells or tissues of a variety of species without having to prepare knockout model animals.

Formation and Function of siRNAs

siRNA formation in vivo is thought to occur in a two step manner. First, the dsRNA is cleaved by the enzyme dicer (Agrawal et al., 2003). Through the use of ATP as an energy source, the dsRNA is broken down into 21-23 nt dsRNAs containing uridine residues with 2nt 3'-hydroxyl-containing overhangs (Agrawal et al., 2003). These 21-23nt sequences then undergo a phosphorylation reaction with an unidentified kinase which adds a phosphate group to the 5'-ends of the dsRNA fragments, which are now termed siRNAs (Argawal et al., 2003; see figure 5).

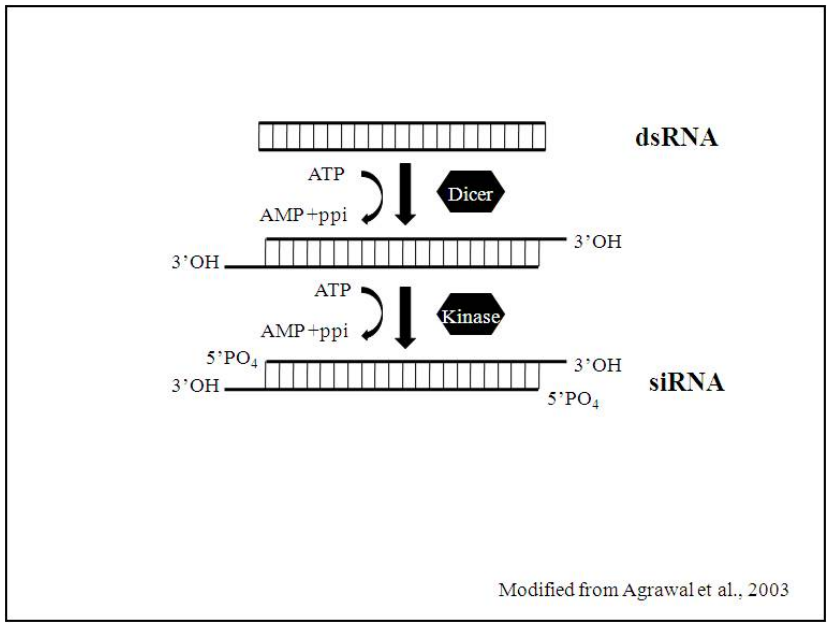

Figure 5. In vivo siRNA formation. Double stranded RNA is cleaved by dicer, and subsequent 21-23nt dsRNAs are phosphorylated by a kinase, adding a phosphate group to the 5- ends and forming siRNAs.

During the second phase, the newly formed siRNAs then bind to an RNAi-specific protein complex, forming an RNAi-Induced Silencing Complex (RISC; Argawal et al., 2003). It is hypothesized that the RISC becomes activated in the presence of ATP, and a helicase in the RISC complex unwinds the siRNA, exposing the siRNA and allowing the antisense siRNAs to pair with the mRNA stand (Argawal; et al., 2003). After the antisense siRNA and the sense mRNA pair, the RISC complex then cuts the mRNA, and therefore breaks it into smaller portions

which cannot be read and used in protein synthesis (Argawal et al., 2003; see figure 6).

Part of the RISC complex is a nuclease responsible for the cleavage of the mRNA strand. This nuclease, or mRNA-cleaving RNA protein complex, is also referred to as a small interfering ribonucleoprotein particle (siRNP; Argawal et al., 2003). It is thought that siRNPs differ from Dicer, and cleave the mRNA 11 to 12nt from the 5'-end of the antisense siRNA (Argawal et al., 2003; see figure 6).

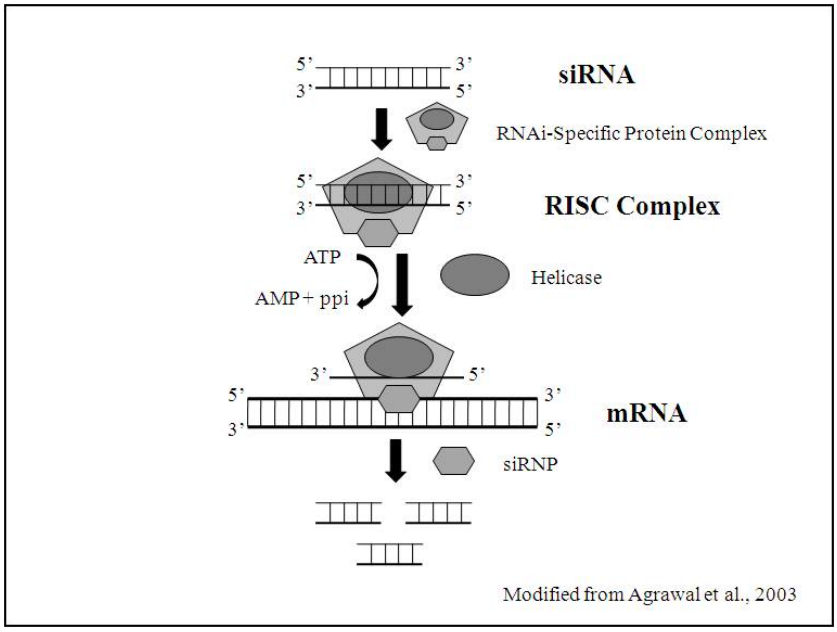

Figure 6. In vivo siRNA function. The siRNA binds to the RNAi-specific protein complex, forming a RISC complex. A helicase within the RISC complex unwinds the siRNA, exposing the antisense siRNA strand and allowing it to bind to the sense mRNA strand. After the antisense siRNA strand and the sense mRNA strand bind, the siRNP within the RISC complex cuts the mRNA into short regions that cannot be translated.

When performing in vitro siRNA formation, a sequence 50 to 100nt downstream of the start codon is typically selected from a portion of amplified cDNA, avoiding 5'- and 3'- untranslated regions (Kumar and Clarke, 2007). Standard siRNAs are 21 to 23 nucleotides in length, have uridine residues in the 3'-overhang, and a 5'-hydroxyl group (Kumar and Clarke, 2007). siRNAs have been produced in vitro using a U6-expression vector or by chemical means, both of which have proved to be effective in degrading specific mRNAs for gene inhibition (Yu et al., 2002).

siRNA usage in Biological Systems to Study mRNA Function

As one of the most promising technological processes recently developed, RNAi and siRNA usage is a current "hot topic." In addition to many research uses, it is thought that RNAi will play a role in developing many future technological applications, including treatment of cancer and genetic diseases (Argawal et al., 2003). RNAi has been used to elucidate biological pathways in various mammalian species, including but not limited to the human, monkey and mouse (Paradis et al., 2005). Examples of this include identification of genes involved in signaling cascades, embryonic development, and basic cellular processes (Argawal et al., 2003; Clemens et al., 2000). While RNAi will likely never replace the use of knockouts, it is a promising technology for instances in which knockout animals are not feasible (Argawal et al., 2003). Furthermore, RNAi can be used in conjunction with

sequencing, allowing for relatively rapid analysis of gene function (Argawal et al., 2003). Several laboratories have had success using dsRNAs or siRNAs (Paradis et al., 2005). Examples of studies using dsRNAs or siRNAs in conjunction with oocyte or embryo culture include the introduction of cyclin B1 dsRNA into oocytes to reduce cyclin B1 mRNA levels (Paradis et al., 2005), identification of MAPK roles in GVBD (Yu et al., 2007), and identifying Cdc42 function in meiotic resumption (Cui et al., 2007).

One of the first studies to utilize RNAi for determination of gene function in mammalian oocytes was performed by Svoboda et al. in 2000. In this study, dsRNAs for Mos and tissue plasminogen activator (tPA) were developed and successfully used to inactivate MAPK and tPA respectively (Svoboda et al., 2000).

In one study, Paradis et al. injected cyclin B1 dsRNA into oocytes, determining that the dsRNA did, in fact, knock down cyclin B1 expression without affecting cyclin B2 (Paradis et al., 2005). Furthermore, the injection of green florescent protein dsRNA had no effect on cyclin B1 mRNA levels or on the ability of the oocyte to mature properly (Paradis et al., 2005). This paper demonstrated that RNAi technology is a useful instrument for studying gene function in oocytes with no discernible side effects (Paradis et al., 2005).

Common concerns among those considering using RNAi as a tool for identifying gene function in mammalian cells is the potential non-specific effects or the potential induction of an interferon response, which turns off all protein synthesis

or causes random mRNA degradation within the cell (Stein et al., 2005). Stein et al. (2005) found no evidence of non-specific effects associated with the introduction of long dsRNAs into murine oocytes and further determined that no genes involved in the interferon response were active in the oocytes, indicating that RNAi technology may in fact be an extremely valuable tool for determining gene function in oocytes (Stein et al., 2005). It should be noted that this experiment was performed on oocytes themselves, not COCs, and that the granulosa cells surrounding the oocyte may be problematic if an interferon response was induced.

From these and other studies, it appears that RNAi is a very viable tool for assessing mRNA function in mammalian oocytes, allowing for determination of specific gene function with minimal to no side effects. As a novel technology, new applications for RNAi are being rapidly developed, and these techniques will have vast potential uses for identifying biological pathways and mRNA function.

STATEMENT OF THE PROBLEM

Resumption of meiosis is characterized by germinal vesicle breakdown (GVBD) in bovine COCs. Studies have indicated that GVBD in vitro occurs in a two-step manner with an initial inhibitory phase followed by a stimulatory phase (Rodriguez and Farin, 2004). Gonadotropin-induced in vitro maturation in bovine COCs is dependent upon an initial transcriptional event occurring within one to one and a half hours after the start of culture (Farin and Yang, 1994; Rodriguez and Farin, 2004).

To date, the mRNA products responsible for the initiation of the initial transcriptional event have not yet been identified (Rodriguez et al., 2006). A list of candidate mRNAs thought to play a role in the resumption of meiosis in murine COCs was generated by comparison of SAGE libraries showing that the candidates Nuclear Receptor Subfamily 4, Member 1 (Nr4A1) and Early Growth Response 1 (Egr1) had significantly decreased expression when COCs were arrested in the GV stage (Rodriguez et al., 2006). Therefore, the objectives of this study were to determine if Nr4A1 and Egr1 mRNAs are present in bovine COCs, determine their pattern of expression, and determine their effects on oocyte maturation. The function of these candidate mRNAs were assessed using a siRNA approach.

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CANDIDATE mRNAs REGULATING MEIOTIC RESUMPTION IN BOVINE CUMULUS-OOCYTE COMPLEXES

ABSTRACT

In bovine oocytes, the resumption of meiosis is characterized by the breakdown of the germinal vesicle (GVBD). When cumulus-oocyte complexes (COCs) are cultured in-vitro in the presence of gonadotropins, GVBD is characterized by an initial inhibitory phase, which is followed by an acceleration in the rate of GVBD. An initial transcriptional event is required for gonadotropin-induced in-vitro maturation. The objectives of this study were: 1) to define the time course required for transcriptional initiation in bovine COCs; 2) to determine the pattern of expression for Nr4A1 and Egr1 mRNAs in bovine COCs; and 3) to reduce Nr4A1 mRNA expression using a siRNA approach to determine its effects on oocyte maturation.

Bovine COCs were cultured in the presence of follicle stimulating hormone (FSH) alone or FSH with the transcriptional inhibitor, 5,6-dichloro-1-B-D-ribofuranosylbenzamidazole (DRB). Small interfering RNAs (siRNAs) designed from the sequence for Nr4A1 were used to reduce Nr4A1 mRNA expression and determine the effects of Nr4A1 mRNAs on GVBD in bovine COCs.

Gene transcription required for GVBD in bovine COCs occurred within 0 to 60 minutes of culture. In addition, Nr4A1 mRNAs were present in bovine COCs and their mRNA levels increased significantly after 30 minutes of culture. Egr1 mRNAs were also present in bovine COCs, but Egr1 mRNA levels did not change throughout

culture. Bovine COCs cultured with siNr4A1 showed a significant decrease in the percentage of oocytes undergoing GVBD after 9 hours of culture. In conclusion, it appears that Nr4A1 plays an active role in GVBD in bovine COCs.

INTRODUCTION

In bovine oocytes, the resumption of meiosis is distinguished by the breakdown of the germinal vesicle (GVBD). In vivo, due to an increase in gonadotropin levels, the dictyate oocyte undergoes GVBD, proceeds to metaphase II as it extrudes the first polar body and is arrested again at the MII stage until fertilization (Knobil and Neil, 1998; Jamnongijt and Hammes, 2005). In vitro, oocytes spontaneously resume meiosis when they are removed from the inhibitory follicular environment and cultured in the absence of gonadotropins (Pincus and Enzmann, 1935; Edwards, 1965; Farin and Yang, 1994). In addition to spontaneous maturation, which occurs in the absence of gonadotropins, oocytes can undergo maturation in vitro in the presence of gonadotropins (Rodriguez and Farin, 2004).

When gonadotropins are included in culture medium, GVBD is characterized by an initial inhibitory phase, followed by an acceleration in the rate of GVBD (Rodriguez and Farin, 2004). Gonadotropin-induced oocyte maturation in vitro is dependent upon an initial transcriptional event (Rodriguez and Farin, 2004). This event is vital for the resumption of meiosis and subsequent GVBD and is thought to occur within the first one to one and a half hours of culture for bovine cumulus-oocyte-complexes (COCs; Farin and Yang, 1994; Kastrop et al., 1991; Meineke and Meinecke-Tillmann, 1993; Osborn and Moore, 1983; Sirard et al., 1998).

Cumulus granulosa cells are connected to the oocyte via gap junctions, and this unit, collectively, is referred to as a COC (Jamnongjit and Hammes, 2006).

These gap junctions are necessary for cumulus cell expansion during follicular growth and for subsequent oocyte development and maturation (Jamnongjit and Hammes, 2006). The communication between the oocyte and the cumulus cells is vital for gonadotropin-induced oocyte maturation (Rodriguez and Farin, 2004).

When transcriptional inhibitors, such as the adenosine analog, 5,6-dichloro-1-B-D-ribofuranosylbenzamidazole (DRB), or α-amanitin, are included in the culture medium before the initial transcriptional event, the inhibitory phase of gonadotropin-induced maturation is prolonged (Rodriguez and Farin, 2004). As long as the transcriptional inhibitor remains in the culture medium, the oocyte does not enter the stimulatory phase of gonadotropin-induced maturation and does not undergo GVBD (Rodriguez and Farin, 2004).

Serial analysis of gene expression (SAGE) libraries comparing mRNA expression profiles from murine cumulus-oocyte complexes (COCs) cultured in FSH alone or in FSH and the presence of the transcriptional inhibitor DRB were generated (Rodriguez et al., 2006). Comparisons of these libraries allowed for the identification of a list of candidate mRNAs thought to be associated with murine COC maturation (Rodriguez et al., 2006). Among these candidates were Nuclear Receptor Subfamily 4, Member 1 (Nr4A1) and Early Growth Response 1 (Egr1). Each were shown to have significantly decreased expression when murine COCs were cultured with FSH and the transcriptional inhibitor when compared to those cultured in the presence of FSH alone (Rodriguez et al., 2006).

RNA interference (RNAi) allows for the identification of specific mRNA function without having to prepare knockout animals (McManus and Sharp, 2002; Sledz et al., 2003). With RNAi, small portions of double-stranded (ds) RNA are used to block the expression of a target mRNA homologous to the dsRNA by binding and inducing selective mRNA degradation (Fire et al., 1998). Twenty-one-to-23 nucleotide dsRNAs, known as small interfering RNAs (siRNAs), influence RNAi specificity and act as an intermediate of RNAi in mRNA silencing (McManus and Sharp, 2002). Thus, siRNAs specific to an mRNA can be synthesized and used to silence a target mRNA (McManus and Sharp, 2002; Sledz et al., 2003). Currently, few studies have used siRNAs in oocyte or embryo culture, but several laboratories have had success introducing siRNAs into culture medium (Paradis et al., 2005). Examples include use of dsRNA to reduce cyclin B1 mRNA levels (Paradis et al., 2005), inactivate mitogen-activating protein kinase (MAPK) and c-mos (Svoboda et al., 2000), and further identify the roles of MAPK in GVBD (Yu et al., 2007). Additional studies have found no evidence of discernable non-specific effects or the induction of the interferon response when culturing oocytes in the presence of dsRNAs (Paradis et al., 2005; Stein et al., 2005).

The overall objective of this study was to identify and assess the function of two candidate mRNAs, Nr4A1 and Egr1 during bovine oocyte maturation. The specific objectives of this study were to define the time required for transcription initiation during gonadotropin-induced maturation in bovine COCs, determine the

pattern of expression for 2 candidate mRNAs potentially associated with GVBD, and reduce the expression of candidate mRNAs expression using specific siRNAs to determine the effects on oocyte maturation.

MATERIAL AND METHODS

Reagents and Media

Tissue culture medium (TCM-199 with Earls salts) was obtained from Gibco BRL (Grand Island, NY). FSH from porcine pituitary glands (50 mg/vial Armour FSH standard), and 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents for culture medium were of tissue culture grade and were obtained from Sigma Chemical Company (St. Louis, MO). Tri-reagent for RNA extractions was purchased from Molecular Research Center Inc., (Cincinnati, OH) and both DNase I and Taq Polymerase were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Superscript reverse transcriptase III, dNTPs, TOPO® Cloning Kit, Lipofectamine 2000™, and Opti-MEM® I Medium were purchased from Invitrogen (Carlsbad, CA). QIAquick PCR Purification Kit, Qiaex II Kit, and QIAprep Spin Miniprep Kit were obtained from Qiagen Inc. (Valencia, CA). SMARTpool® custom siRNA pools were synthesized and purchased from Dharmacon (Lafayette, CO).

Oocyte Recovery and Culture Conditions

Ovaries were harvested from a local abattoir and transported to the laboratory in roller bottles containing 0.9% saline solution. Ovaries were then rinsed 3 times with saline solution and follicles between 2 to 10mm in diameter were aspirated using an 18 gauge needle and syringe. COCs with at least 3 complete cumulus cell layers were collected, washed 3 times in modified Tyrode's medium (TL-Hepes) and placed into appropriate treatment groups containing 0.8ml of TCM-199 supplemented with 10% heat-inactivated estrous cow serum, 1μg/ml estradiol, 200nM pyruvate, 5μg/ml FSH, and 50μg/ml gentamicin (if applicable). All cultures were maintained in a 5% CO_2 atmosphere in air with 100% relative humidity. If applicable, treatment groups contained 120μM 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) in 0.2% dimethyl sulfoxide (DMSO). DRB-containing medium was replaced at 4h intervals throughout culture. All treatment groups were assessed for cumulus expansion at 4h intervals throughout culture. At appropriate time points, COCs were either snap frozen in liquid nitrogen and stored at -80ºC prior to RNA extraction or placed on slides for assessment of stage of meiotic maturation.

Assessment of Meiotic Stage

At appointed times, COCs were removed from culture, placed in tubes with 0.4ml TL-Hepes and vortexed for 4 minutes 30 seconds to remove cumulus cells. Oocytes were then washed with phosphate-buffered saline (PBS), placed on acid-

washed slides and fixed in an ethanol-acetic acid (3:1 v/v) solution for 9 to 24hrs. Oocytes were then stained with a 1% orcein in 25% acetic acid stain, de-stained with ethanol-acetic acid, and evaluated for meiotic stage using differential interference contrast microscopy at 200X magnification.

wcRNA Extraction, Reverse Transcription and cDNA Synthesis

Tri-reagent was added to COCs frozen at -80ºC at a ratio of 800µl tri-reagent to 150 COC. RNA extraction was performed according to the manufacturer's instructions and the resulting RNA was resuspended in diethyl pyrocarbonate-treated water (DEPC water). RNA quality was assessed by examination of 18S and 28S bands following agarose gel electrophoreses.

wcRNA (2µg) was thawed on ice and treated with 60U DNase I in a 5.5µl reaction for 20min at 37ºC. This DNA-free wcRNA was then used for primer annealing in a reaction consisting of 250ng random primers, 1µl 10mM dNTP mix, and sterile DEPC water to a volume of 14µl and was incubated at 65ºC for 5min. The primer-annealed RNA was placed on ice for 1min and then reverse transcribed at 50ºC in a 20µl reaction consisting of 4µl 5X First Strand Buffer, 1µl 0.1M DTT, and 200U of SuperScript III RT. Reactions were inactivated by heating to 70ºC for 15min. All cDNA samples were then cleaned using the QIAquick PCR Purification kit as per manufacturer's instructions and stored at 4ºC in 50µl Qiagen PE buffer (Valencia, CA) until further use.

Semi-quantitative PCR Assay

Primers used for semi-quantitative PCR (sqPCR) analyses were designed using the Gene Amplify 1.2 (Madison, WI) and Oligo 4.0.2 primer analysis software (Plymouth, MA) based on the predicted bovine and murine sequences for either Nr4A1 or Egr1. Primers sets for Nr4A1 and Egr1 were designed to cross introns. Additional primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized using previously published sequences. Primers for an unrelated transcript isolated from bovine COCs (Tram6) were synthesized using unpublished data from our laboratory. GAPDH was used as a housekeeper and Tram6 was used as a positive control (see Table 1). Each PCR reaction contained 2µl 10X buffer (10mM Tris-HCL, 1.5mM $MgCl_2$, 50mM KCl), 2µl appropriate upper primer (200ng), 2µl appropriate lower primer (200ng), 3.2µl 100mM dNTP mix, 0.25µl Taq polymerase, 2µl cDNA (100ng cDNA) and DEPC water to a final reaction volume of 20µl. Reactions using Nr4A1, GAPDH, and Egr1 primers also included 4µl Q solution (Qiagen). All reactions were placed in a 96-well PCR plate, sealed using ThermalSeal™ (Excel Scientific, Wrightwood, CA), and placed into a PTC100 thermal cycler for 2min at 94ºC. PCR programs for Nr4A1, GAPDH and Tram6 used 10sec steps for denaturation at 92ºC, and primer extension at 72 ºC. For Egr1, 15sec steps were used instead. Specific annealing temperatures, cycle numbers for linear amplification and product sizes are presented in Table 1. PCR products were electrophoresed on 1.5% ethidium bromide-stained agarose gels and band intensities

were determined using the AlphaImager® (Alpha Innotech, San Leandro, CA). Relative signal intensity was calculated as the ratio of the band intensity of the target product to that of GAPDH. Data for the siRNA dose response for candidate Nr4A1 and for determining the effects of Nr4A1 siRNA treatment on oocyte maturation were indexed to the FSH control and arcsin transformed prior to analysis.

Recovery of PCR Products, Subcloning and Sequence Verification

Bands representing the PCR amplicons of interest were identified using comparison with a molecular ladder and cut out of a low sulfate agarose gel on a long wavelength light box. PCR products were extracted from the agarose gel using the Qiaex II kit (Qiagen) as per manufacturer's instructions. Four µl fresh PCR product was subcloned into a TOPO® vector according to manufacturer's instructions (TOPO® Cloning Kit, Invitrogen). Briefly, 2µl of ligation reaction was added to 50µl electro-competent *E. coli* cells, mixed gently and placed into an electro-cuvette. The cells were then electroporated at 1500V one time for 3-4milliseconds. SOC medium (250µl) was added to the electroporation-cuvette to flush out the cuvette and was shaken at 37ºC for 1h at 200rpm. 10, 30, and 50µl aliquots were placed on luria broth-ampicillin (LB-Amp) plates and incubated at 37ºC for approximately 15h. Separate colonies were transferred into 5ml LB broth with 10µl of 50mg/ml ampicillin stock and incubated for approximately 15h at 37ºC. Plasmids, containing

the inserts of interest, were purified using the QIAprep Spin Miniprep Kit (Qiagen) and used as a template to verify target insert sequences.

siRNA Treatment Conditions

Custom SMARTpool® siRNAs were generated from predicted bovine sequences for Nr4A1 and Egr1. siRNA stocks were diluted in 1X working buffer to a 40nM/µl volume, aliquoted in 25µl doses, and frozen at -80ºC until further use. For all culture experiments containing siRNAs, Lipofectamine 2000™ in Opti-MEM® I Reduced Serum Medium was used as a transfection reagent as per manufacturer's instructions. All cultures containing siRNAs were maintained in the absence of antibiotics. COCs were placed into tubes containing the appropriate dose of siRNA and were centrifuged twice at 55*g* for 3min to improve siRNA transfection. Transfected COCs were then placed into appropriate treatment wells and cultured for the appropriate duration. Following culture COCs were either snap-frozen in liquid nitrogen and stored at -80ºC until extracted for RNA or mounted on slides for assessment of meiotic stage.

RESULTS

Recovery of PCR Products, Subcloning and Sequence Verification

Primers were designed based upon predicted bovine sequences for Nr4A1 and Egr1 based on genomic DNA sequences from XM_606812 and XM_601394,

respectively. Primer sequences used for sqPCR, the length of their expected products and their annealing temperatures used for sqPCR assay are listed in Table 1. PCR products, from each of the primer sets listed, were identified, isolated, subcloned, and sequence verified. The sequences for Nr4A1 and Egr1 PCR amplicons are shown in Figure 1.

Table 1. Primer sequences used for semi-quantitative PCR of bovine COCs. Cycle number was identified to be within the linear range of amplification.

Name	Primer Sequence (5' to 3')	Product Size (bp)	Annealing Temp. (°C)	Cycle No.
Nr4A1	F: GGCTTTGCTGAACTGTCT R: GTCGGTCTGTGATGAGGA	271	62	25
Egr1	F: TGGCTCCTTTCCTCATTC R: TTTGGCTGGGGTAACTCG	317	60	33
GAPDH	F: GCCGTGAACCACGAGAAGTATAA R: CCGTCCACGATGCCAAAGT	120	60	25
Tram6	F: AGGGTTTAGAGGCTTAGG R: CGGCAACAGACGGCAGGA	289	55	27

Nr4A1 Sequence

GGCTTTGCTGAACTGTCTCCCGGAGACCAGGACCTGCTGCTGGAGTCCG
CCTTTCTGGAGCTCTTTATCCTCCGTCTGGCCTACCGGTCTAAACCAGCCG
AGGGGAAGCTCATCTTCTGCTCAGGCCTGGTGCTGCACCGCCTGCAATGT
GCCCGTGGCTTCGGGGACTGGATCGACAGCATCTTGGCCTTCTCTCGCTCC
CTGCACAGCTTGGTGGTCGACATCCCTGCCTTCGCCTGCCTCTCCGCGCTT
G**TCCTCATCACAGACCGAC**AAGGGC

Egr1 Sequence
CCCTT**TGGCTCCTTTCCTCATTCG**CCCACCATGGACAACTACCCTAAGCT
GGAGGAGATGATGCTGCTGAGCAATGGGGCTCCCCAGTTCCTCGGTGCCG
CCGGGGCCCCGGAGGGCAGCAGCGGTAGCAGCAGCGGCAGCAGCGGGGG
CGGTGGAGGTGGAGGGGGTGGCAGCAGCAGCAGCAACAGCAACAGCAGC
AGCGCCTTCAACCCTCAGGGGGAGGCAAGCGAGCAGCCCTACGAGCACC
TGACCGCAGAGTCCTTTCCTGACATCTCTCTGAATAACGAGAAGGTTCTA
GTGGAGA**CGAGTTACCCCAGCCAAA**AAGG

Figure 1. Amplicon sequences for bNr4A1 and bEgr1 candidate mRNAs. The primer sequences used for semi-quantitative PCR reactions are shown underlined in bold type.

Time Course for Transcription Initiation during Gonadotropin-Induced Maturation

To identify the time during which transcription is required for GVBD the transcriptional inhibitor, DRB (120μm), was added at initiation of culture or at 30, 60, 90, 120, 150 and 180 minutes after the start of culture to subsets of COCs. After 4 hours of culture, there was no difference in cumulus cell expansion scores, but after 20 hours of culture expansion scores increased as DRB was added as successively

later time points (Figure 2). Consistent with previous studies, when COCs were cultured entirely in the presence of DRB significantly more oocytes remained at the GV stage (95.0 ±7.5%) compared to controls (1±7.5%; P<0.05, Figure 3A). COCs maintained in medium with DRB added at 30 minutes after the initiation of culture had a significantly reduced incidence of GV-stage oocytes (70.3±7.5%) and when DRB was added at 60, 90, 120, 150 and 180 minutes after the start of culture fewer oocytes were maintained at GV stage (Figure 3A). Thus, gene transcription required for GVBD occurred between 0 and 60 minutes of culture (Figure 3B).

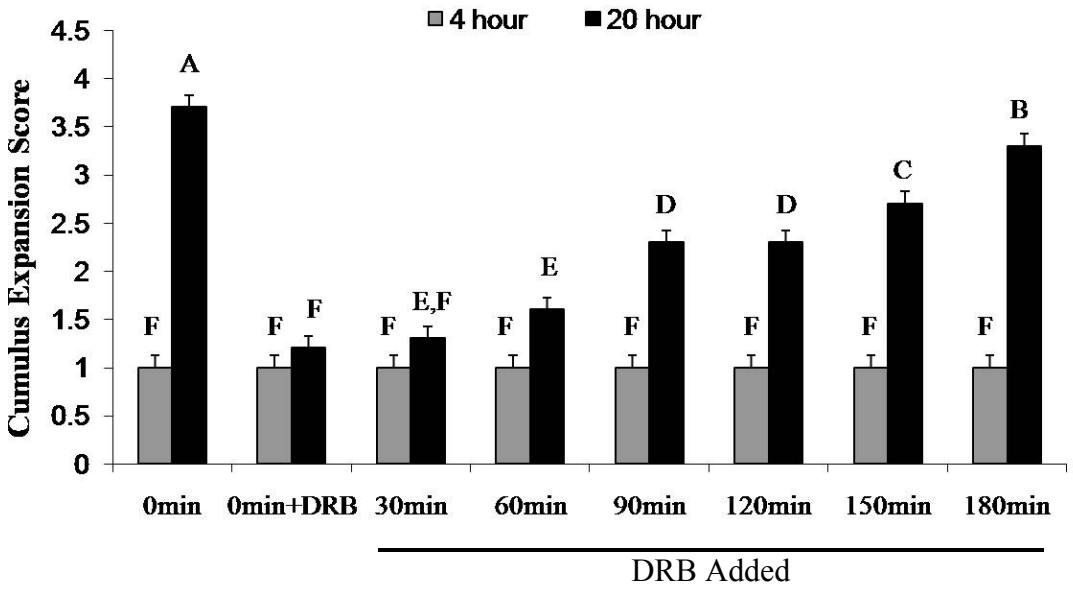

Figure 2. Cumulus expansion scores for the time course for transcription initiation required for gonadotropin-induced maturation (lsmeans±SEM). Data represent n=5 replicate experiments; ^{A,B,C,D,E,F} P<0.05.

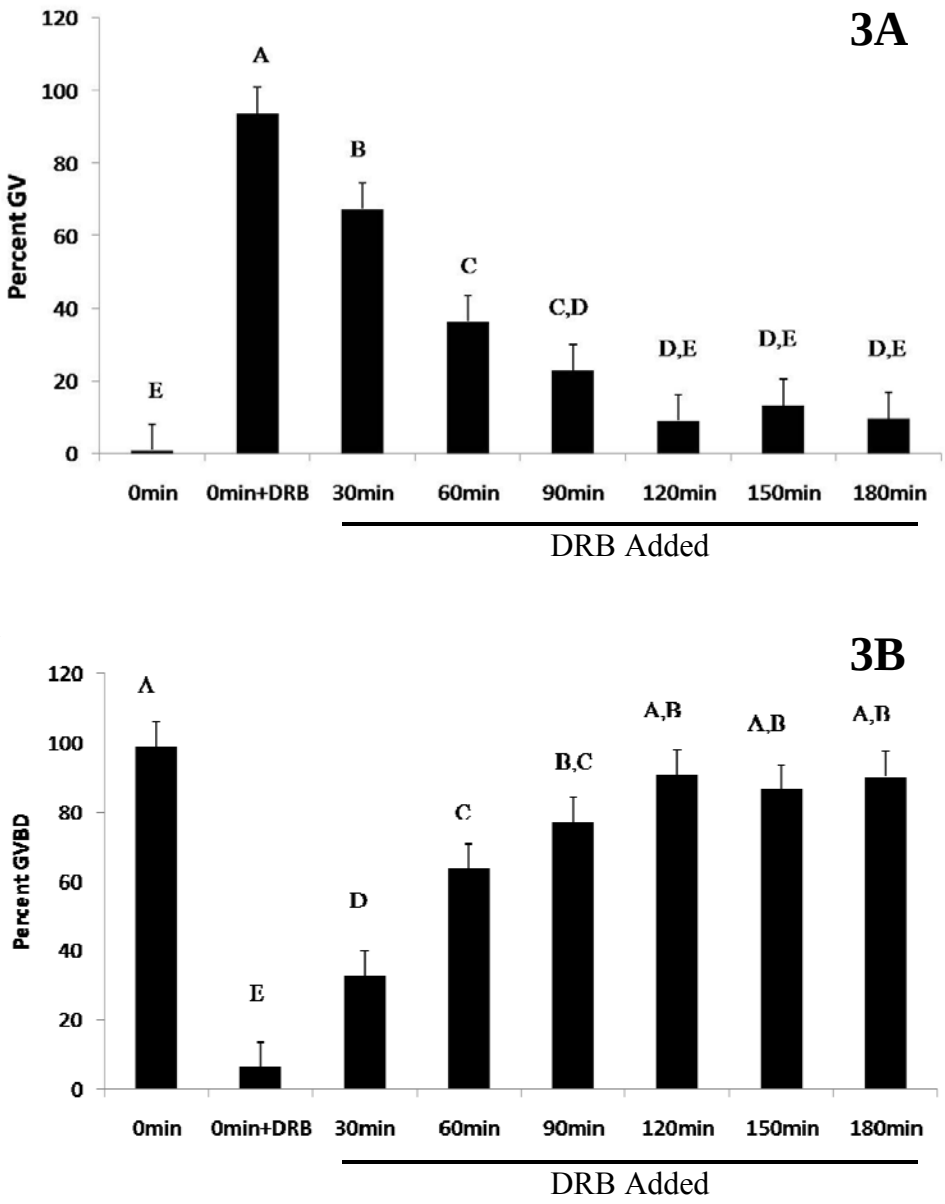

Figure 3. Time course for transcription initiation required for gonadotropin-induced maturation. **3A:** Percent of COCs at GV after 9 hours of culture (lsmeans±SEM). **3B:** Percent of COCs that underwent GVBD at 9 hours of culture (lsmeans±SEM). All data were arcsin transformed. Data represent n=5 replicate experiments; ^{A,B,C,D,E} $P<0.05$.

Determination of Pattern of Expression for Candidate mRNAs

Using data from the previous experiment, it was determined that transcription initiation began about 30 minutes after the start of culture. Therefore, subsets of COCs were collected at the start of culture, and 30, 60, 90, and 180 minutes after culture initiation and snap frozen for RNA extraction and subsequent mRNA analysis. A control group containing DRB from the start of culture was also included. In addition, a subset of COCs was cultured to 9 hours and assessed for the incidence GVBD to verify the effectiveness of the DRB treatment to arrest oocyte maturation.

Levels of Nr4A1 mRNA increased significantly ($P<0.05$) at 30 minutes after the start of culture (Figure 4), consistent with the time of transcription initiation required for GVBD (Figure 3). Nr4A1 mRNA levels slowly decreased in abundance throughout the remainder of culture (Figure 4). Levels of Egr1 mRNA did not change throughout the culture period (Figure 5).

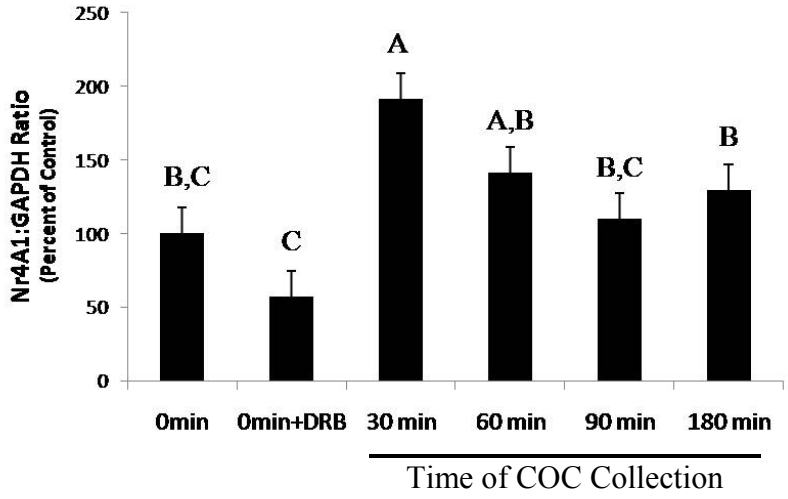

Figure 4. Semi-quantitative PCR analysis of the pattern of expression for the candidate gene, Nr4A1. Treatment groups included FSH and DRB controls, and COCs were collected at 30, 60, 90, and 180 minutes after the start of culture. All treatments were cultured in the presence of FSH. Data were expressed as the ratio of signal intensities for Nr4A1 to GAPDH with values indexed to the FSH control. Five replicate experiments were performed; ^{A,B,C} $P<0.05$.

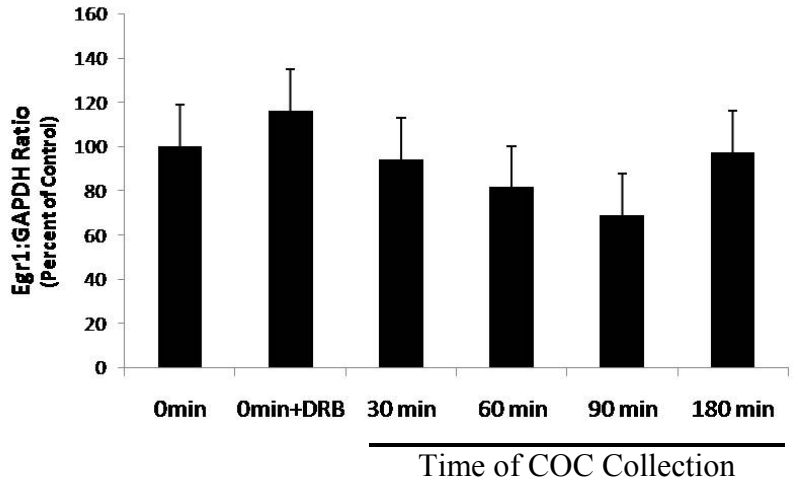

Figure 5. Semi-quantitative PCR analysis of the pattern of expression for the candidate gene, Egr1. Treatment groups included FSH and DRB controls, and COCs were collected at 30, 60, 90, and 180 minutes after the start of culture. All treatments were cultured in the presence of FSH. Data are expressed as the ratio of signal intensities for Egr1 to GAPDH with values indexed to the FSH control. Five replicate experiments were performed.

siRNA Dose Response for Candidate Nr4A1

Because levels of Egr1 did not change during culture, we focused our remaining experiments on the candidate mRNA, Nr4A1. A dose response study was performed using COCs cultured for 30 minutes with either 25, 50, or 100nM siNr4A1. Control treatments consisted of COCs cultured in the presence of FSH alone or FSH and DRB. Expression of Nr4A1 mRNA decreased in abundance in all treatment groups containing siRNAs with the greatest decreases in the 25nM and 50nM siNr4A1 treatments (Figure 6).

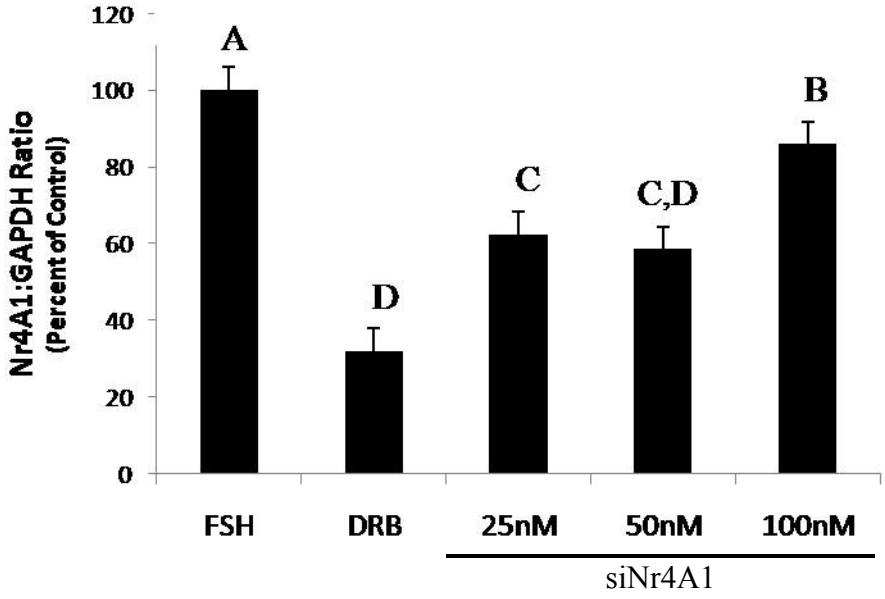

Figure 6. Semi-quantitative PCR analysis of the siRNA dose response for the candidate gene Nr4A1. Treatment groups included FSH and DRB controls, and 25, 50, or 100nM doses of siNr4A1. All treatments contained FSH. Data were expressed as the ratio of signal intensities of Nr4A1 to GAPDH indexed to the FSH control. Data were arcsin transformed. Three replicates were performed; ^{A,B,C,D} $P<0.05$.

Effects of Nr4A1 siRNA Treatment on Oocyte Maturation

Based on the results of the siNr4A1 dose response study, the 50nM siNr4A1 dose was chosen for additional studies designed to examine the effectiveness of siNr4A1 for reducing the expression of Nr4A1 mRNA and subsequent inhibition of GVBD. For assessment of the effect of siNr4A1 on Nr4A1 mRNA levels, COCs were collected after 30 minutes of culture. In addition, subsets of COCs were cultured and assessed to determine the effect of siRNA treatment on meiotic progression at either 9 or 20 hours of maturation. Treatment groups included COCs cultured with FSH alone, FSH and DRB, siNr4A1, and 50nM nonspecific siRNA. Tram6, an unrelated transcript, was used as a second unrelated mRNA control. Expression of GAPDH (data not shown) did not differ among treatment groups.

As expected, treatment with DRB resulted in reduced expression of both Nr4A1 (Figure 8) and Tram6 (Figure 9) mRNAs. In contrast, expression of Nr4A1 and Tram6 mRNAs did not differ with FSH, siNr4A1, or siNS treatment (Figures 7 and 6, respectively).

No significant difference was observed in cumulus cell expansion across treatments after 4 hours of culture (Figure 7). After 20 hours of culture, DRB depressed cumulus cell expansion, as anticipated, but no difference was observed among the FSH, siNr4A1, or siNS treatment groups (Figure 7). No significant differences were observed between the FSH and siNS treatment groups in the proportion of COCs undergoing GVBD at either 9 or 20 hours of culture (Figure 10).

As expected, significantly fewer COCs cultured in the presence of DRB underwent GVBD at both 9 and 20 hours of culture (Figure 10). In contrast to the mRNA expression data, COCs cultured with siNr4A1 showed a significant decrease in the percentage of oocytes undergoing GVBD after 9 hours of culture (62.6±4.5%) compared to oocytes in the FSH treatment group (98.4±4.5%; $P<0.05$, Figure 10). However, the percentage of oocytes undergoing GVBD at 20 hours of culture did not differ between the siNr4A1 and control treatments (100.0±4.5% vs. 100.0±4.5%, respectively; Figure 10).

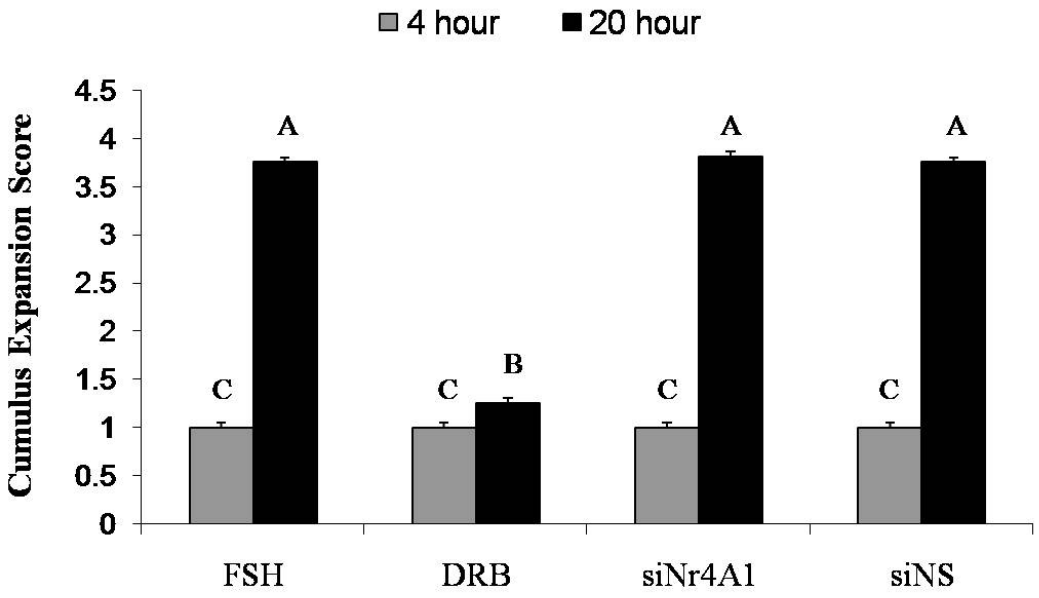

Figure 7. Cumulus expansion scores for the effects of Nr4A1 siRNA treatment on oocyte maturation (lsmeans±SEM). Data represent n=6 replicate experiments; [A,B,C] $P<0.05$.

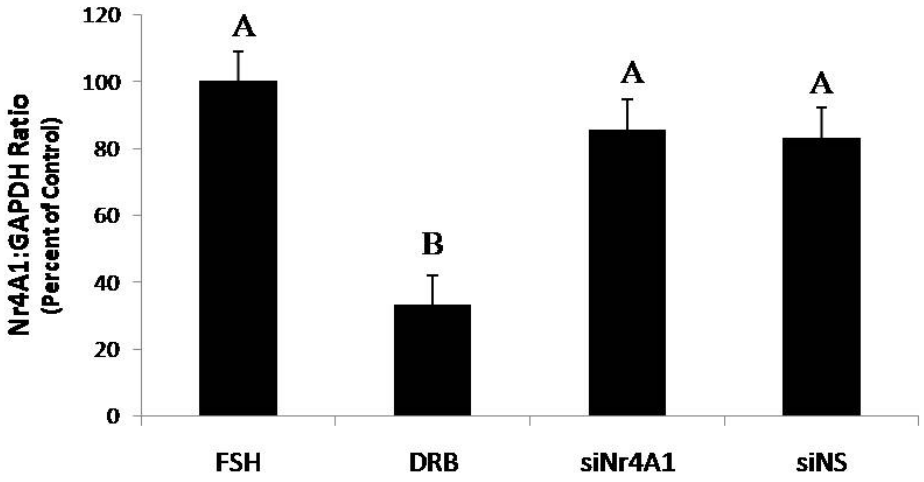

Figure 8. Semi-quantitative PCR analysis for determining the effects of siNr4A1 on Nr4A1 mRNA levels in bovine COCs. Treatment groups included FSH and DRB controls and 50nM doses of Nr4A1 or nonspecific (NS) siRNA. All treatments contained FSH. Data were expressed as the ratio of signal intensities of Nr4A1 to GAPDH indexed to the FSH control. Data were arcsin transformed. Five replicates were performed; ^{A,B} $P<0.05$.

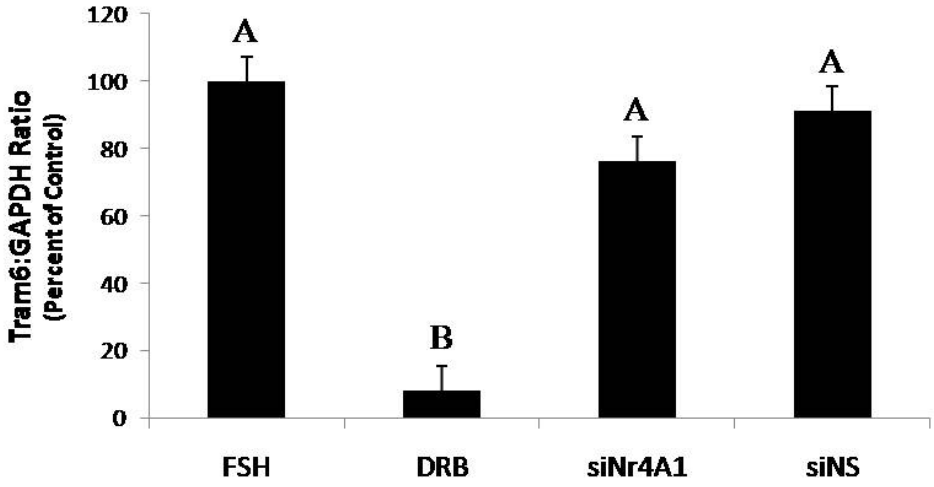

Figure 9. Effect of treatment on expression of Tram6 mRNA. Treatment groups included FSH and DRB controls and 50nM doses of Nr4A1 or nonspecific (NS) siRNA. All treatments contained FSH. Data were expressed as the ratio of signal intensities of Tram6 to GAPDH indexed to the FSH control. Data were arcsin transformed. Five replicates were performed; ^{A,B} $P<0.05$.

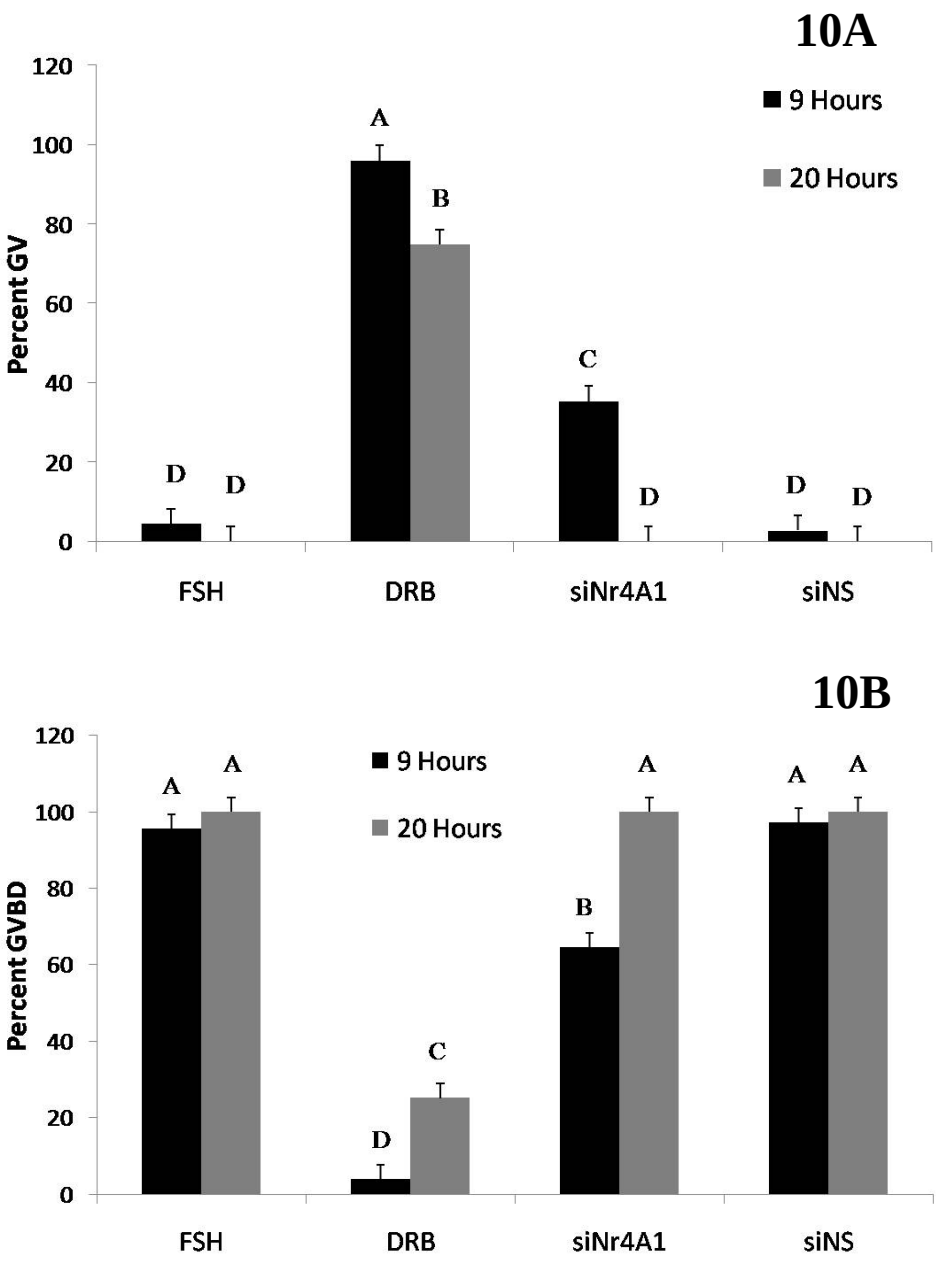

Figure 10. Effect of Nr4A1 siRNA on oocyte maturation. **10A**: Percent of COCs at GV stage after 9 or 20 hours of culture (lsmeans±SEM). **10B**: Percent of COCs that underwent GVBD after 9 or 20 hours of culture (lsmeans±SEM). Treatment groups included FSH and DRB controls, and 50nM doses of either Nr4A1 or nonspecific (NS) siRNA. All treatments contained FSH. All data were arcsin transformed. Four replicates were performed; ^{A,B,C,D} $P<0.05$.

DISCUSSION

Previous studies indicated that gene transcription of in vitro matured bovine COCs occurred within 1 to 1.5 hours after the start of culture (Farin and Yang, 1994; Kastrop et al., 1991; Meineke and Meinecke-Tillmann, 1993; Osborn and Moore, 1983; Sirard et al., 1998). In the present study, we have further refined the estimated time of transcription initiation that is required for gonadotropin-induced oocyte maturation. Based on the present work, the critical period for transcription initiation in bovine COCs actually begins about 30 minutes after the start of culture rather then at 60 to 90 minutes. This observation suggests that transcription initiation in bovine COCs occurs earlier then previously suggested. Although not statistically significant, there does appear to be a trend towards maintenance of the GV when DRB was added to culture medium at 150 minutes, indicating a possible secondary transcriptional event.

By comparing two murine SAGE libraries, a list of candidate mRNAs involved in the resumption of meiosis was identified (Rodriguez et al., 2006). Two of these candidate genes, Nr4A1 and Egr1, both demonstrated significantly decreased expression when cultured in the presence of DRB (Rodriguez et al., 2006). Nr4A1 is a parathyroid hormone-induced gene and is thought to activate cAMP-protein kinase A (Kovalovsky et al., 2002; Maira et al., 2003; Pirih et al., 2005). Nr4A1 may also be involved in the regulation of other genes, most especially those implicated in apoptosis and steroidogenesis (Mangelsdorf et al., 1995; McLean et al., 2002).

Another study determining the relationship between FSH and Nr4A1 in rat Sertoli cells suggested that Nr4A1 may in fact be activated by FSH (Schmidt et al., 2006).

Recently, Egr1 was recognized as an extremely important mRNA in oocytes, having been identified bovine granulosa cells and found to have a profound effect on oocyte developmental competence (Roberts et al., 2001). Egr1 is also thought to be induced by FSH and play an active role in follicular growth and ovulation, while closing off gap junctions during oocyte maturation (Fritz et al., 2002; Russell et al., 2003). While the mRNAs for Nr4A1 and Egr1 have been previously identified in the mouse, sequences have only been predicted in cattle based on available genomic sequence. In the present study, PCR amplicons representing bNr4A1 and bEgr1 mRNAs showed 100% identity to the predicted sequences and verified that bNr4A1 and bEgr1 mRNAs are present in bovine COCs.

Analysis of expression profiles for Nr4A1 in bovine COCs demonstrated that Nr4A1 mRNA levels increased approximately 30 minutes after the start of culture. The apparent correlation between Nr4A1 levels increasing at 30 minutes after the start of culture and the start of transcription initiation required for gonadotropin-induced in vitro maturation occurring in the same time frame supports the suggestion that Nr4A1 is not only present in bovine COCs, but also may play a role in the resumption of meiosis. In contrast to Nr4A1 mRNA, levels of Egr1 mRNA did not increase during this critical time period. Thus, it was concluded that Egr1 does not appear to play a vital role in initiating GVBD in bovine COCs. Additionally, it

should be noted that Egr1 mRNA expression was not sensitive to the transcriptional inhibitor DRB. This indicates that Egr1 is not a nascent mRNA produced during the initial transcriptional event required for GVBD in the bovine model.

With the discovery of RNAi in 1998, siRNA technology has offered a new approach for the study of specific gene function (Fire et al., 1998; McManus and Sharp, 2002). Recent concerns associated with the use of RNAi as a tool for identifying gene function in mammalian cells include the potential non-specific effects and the potential induction of an interferon response, which turns off all protein synthesis or causes random mRNA degradation within the cell (Stein et al., 2005). Although few studies have used RNAi as a tool for assessing gene function in oocytes, the studies found using RNAi in oocytes have contained no evidence of non-specific effects or of the induction of an interferon response (Paradis et al., 2005; Stein et al., 2005). It should be noted however, that these studies were done in oocytes alone, and cumulus cells in the COC may react in a different manner to siRNA treatment than the oocyte itself.

Culture of bovine COCs with a siRNA specific to Nr4A1 mRNA resulted in a significant decrease in the percentage of COCs undergoing GVBD after 9 hours of culture as compared to COCs cultured in the presence of FSH alone. Interestingly, the percentage of oocytes undergoing GVBD at 20 hours of culture did not differ between the siNr4A1 and control treatments. These findings indicate that Nr4A1

may play a role in inhibiting or delaying the resumption of meiosis and subsequent GVBD, but that this initial inhibition is not maintained throughout culture duration.

Evidence from previous studies performed on other cell types indicate that Nr4A1 may be involved in the cAMP-protein kinase A pathway (Kovalovsky et al., 2002; Maira et al., 2003; Pirih et al., 2005). Current research suggests that an increase in cAMP levels within a cell causes the activation of cAMP-dependent PKA, which in turn activates via phosphorylation the cAMP regulatory element-binding protein (CREB), which may arbitrate some of the effects of cAMP (Brindle and Montminy, 1992; Meyer and Habener, 1993; Habener er al., 1995; Monaco et al., 1995; McLean et al., 2002). It is thought that GVBD is dependent upon activation of signaling pathways and is responsible for a reduction in cAMP levels or the blocking of the inhibitory effects of cAMP (Conti et al., 2002). When cAMP levels drop or become blocked, PKA is inactivated, allowing the oocyte to undergo GVBD (Conti et al., 2002). Other data suggests that Nr4A1 may be responsible for the regulation of other genes, most especially those involved in steroidogenesis and cell death (Mangelsdorf et al., 1995; McLean et al., 2002). It may be that Nr4A1 is one of several genes responsible for the activation of signaling pathways responsible for the resumption of meiosis.

While bovine COCs cultured with siNr4A1 mRNA resulted in a significant decrease in the percentage of COCs undergoing GVBD stages after 9 hours of culture as compared to COCs cultured in the presence of FSH alone, the comparison of semi-

quantitative PCR signal intensities showed no significant differences between FSH, siNr4A1, and siNS treatment groups. Given the fact that a statistically significant physiological effect was noted, this set of results warrants attention. One possible explanation is that Nr4A1 was present in small quantities in the initial murine SAGE libraries, so it is possible that the assay was not sensitive enough to detect subtle differences in the lower levels of Nr4A1. This explanation is probably not likely as changes in Nr4A1 mRNA expression were detected in two earlier experiments.

A second possible explanation is that I gained more experience in conducting these experiments throughout my program, and may have been quicker in setting up the latter experiments. Thus, the time in which it took to get oocytes into culture may have differed between the earlier and latter experiments, and this may have affected the timecourse for detecting changes in the mRNA expression profiles.

A third possible explanation is that the pool of siRNAs used to reduce Nr4A1 mRNA expression could have caused an incomplete degradation of the mRNA. In this instance the mRNA could degraded enough to cause a statistically significant decrease in the percent of COCs undergoing GVBD at 9 hours, but remained intact enough at the location that was amplified by the Nr4A1 primers to create an inconsistent decrease in mRNA expression when measured using semiquantiative RT-PCR. Of the three possible explanations, this explanation seems most likely.

The lack of a complete inhibition of GVBD at 9 hours of culture when siNr4A1 was used could be explained by an incomplete transfection of the siRNA.

Some COCs have thicker, denser layers of cumulus cell as compared to others, and it is possible that the siNr4A1 could have penetrated the cumulus cells unevenly. Cumulus oocyte complexes with more densely packed layers of cumulus cells may not have been transfected with the siNr4A1 to the same degree as COCs with fewer cumulus layers. It is possible that the cumulus oocyte complexes that were transfected more completely were held at the GV state whereas COCs that received less of the siNr4A1 underwent GVBD.

The experiment determining the effect of siNr4A1 treatment on GVBD should be repeated and possibly all replications should be analyzed using real time PCR in order to increase assay sensitivity. Ideally, the experiment should be redone, with subsets of COCs removed from culture and snap frozen for assessment of mRNA levels at 15, 30, 45 and 60 minutes after the start of culture to create an Nr4A1 mRNA expression profile. This profile would allow one to determine when Nr4A1 mRNA levels peaked in the control treatment and then compare the expression levels to those treated with siNr4A1. Also, the experiment could be repeated using a lower dose of FSH in the culture medium. Using a lower dose of FSH could decrease the amount of PKA type I activation and could result in a more significant hold at the GV stage when using siNr4A1 if Nr4A1 mRNAs are produced after PKA type II activation. If the third explanation for the discrepancy among experiments holds true, in which the pool of siRNAs caused an incomplete degradation of the Nr4A1 mRNA, no number of additional replications of the experiments would change the results.

In summary, we have shown that gene transcription required for GVBD in bovine COCs occurs within 0 to 60 minutes of culture, that Nr4A1 mRNA is present in bovine COCs and that levels of this mRNA increase significantly after 30 minutes of culture. Furthermore, we have shown that while Egr1 mRNA is present in bovine COCs, Egr1 mRNA levels do not change throughout culture. Additionally, the expression of Nr4A1 mRNA decreased in abundance in all treatment groups containing siRNAs with the greatest decrease in the 50nM siNr4A1 treatment, and bovine COCs cultured with 50nM siNr4A1 showed a significant decrease in the percentage of oocytes undergoing GVBD after 9 hours of culture. In conclusion, Nr4A1 appears to play an active role in GVBD in bovine COCs.

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APPENDIX

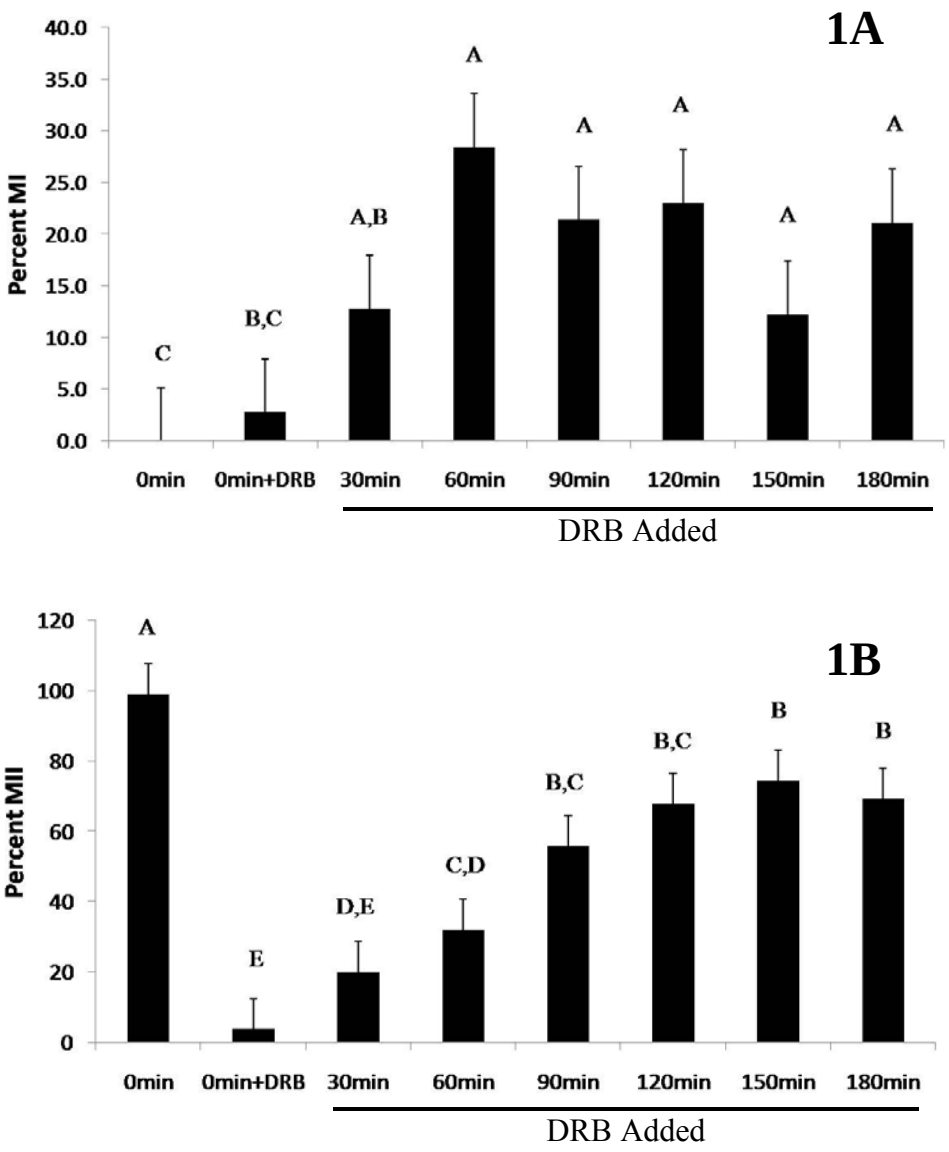

Figure 1. Time course for transcription initiation required for gonadotropin-induced maturation. **1A.** Percent of COCs at MI after 9 hours of culture (lsmeans±SEM). **1B.** Percent of COCs at MII after 9 hours of culture (lsmeans±SEM). Data shown were arcsin transformed. Data represent n=5 replicate experiments; ^{A,B,C,D,E} $P<0.05$.

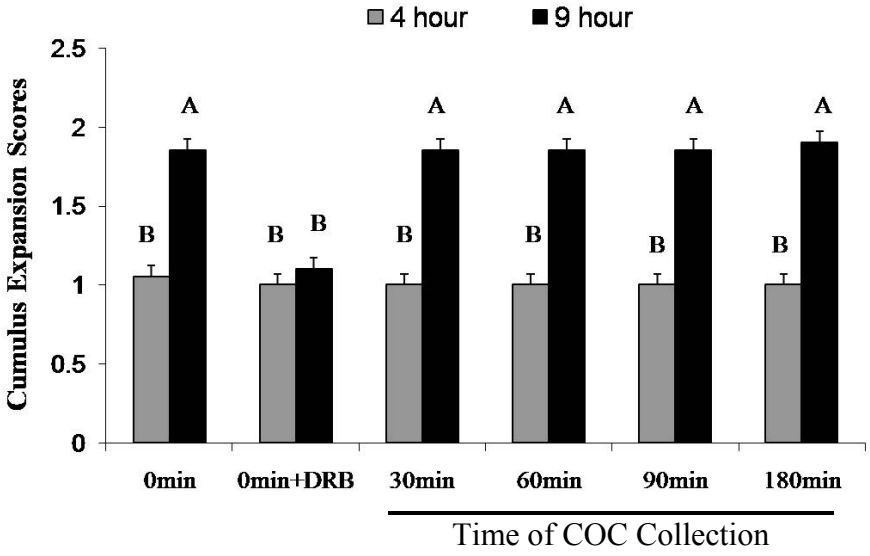

Figure 2. Effect of DRB treatment and time in culture on cumulus expansion scores in bovine COC (lsmeans±SEM). Data represent n=5 replicate experiments; [A,B] P<0.05.

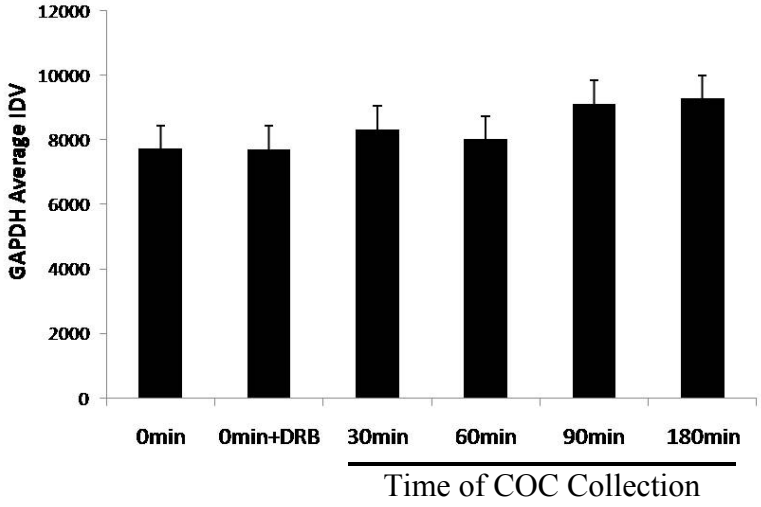

Figure 3. Semi-quantitative PCR analysis of the determination of the pattern of gene expression for the housekeeper gene GAPDH. Treatment groups included FSH and DRB controls, and COCs were collected at 30, 60, 90, and 180 minutes after the start of culture. All treatments contained FSH. Data represent lsmeans ± SEM. Five replicates were performed.

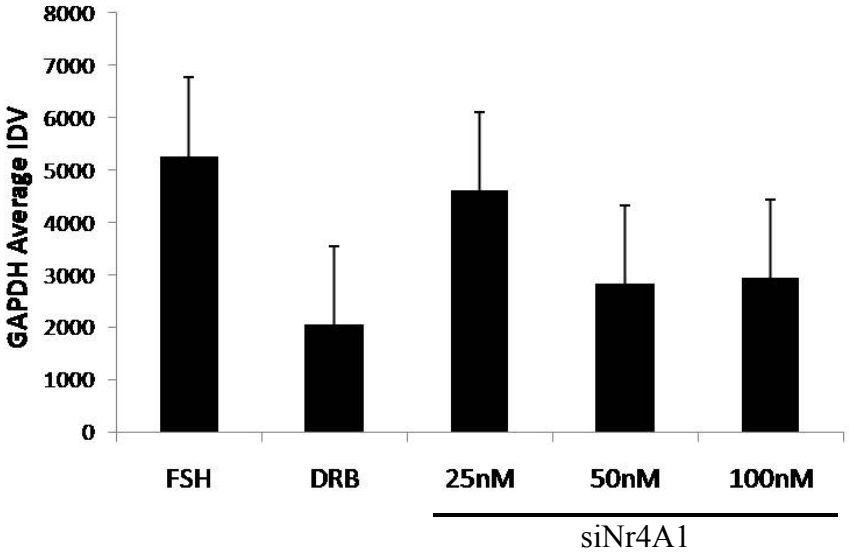

Figure 4. Semi-quantitative PCR analysis of the siRNA dose response for the housekeeper gene GAPDH. Treatment groups included FSH and DRB controls, and 25, 50, or 100nM doses of siNr4A1. All treatments contained FSH. Three replicates were performed. Data represent lsmeans ± SEM.

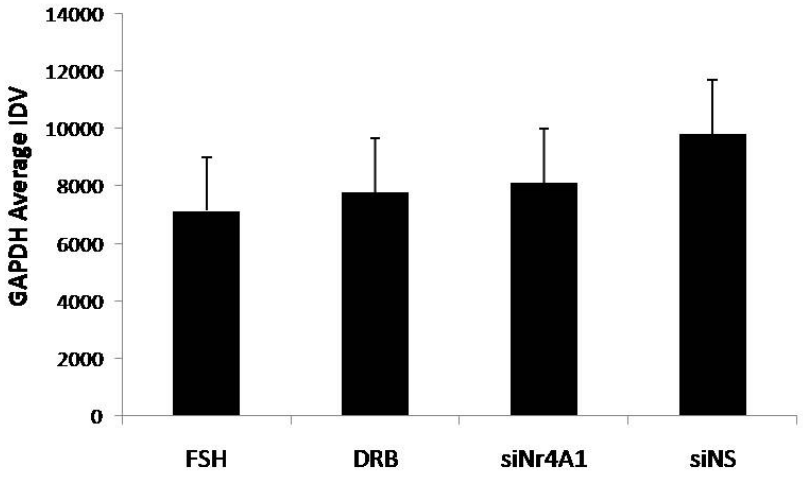

Figure 5. Semi-quantitative PCR analysis for the expression of the housekeeper GAPDH for determination of siNr4A1 treatment effect. Treatment groups included FSH and DRB controls and 50nM doses of siNr4A1 or nonspecific (NS) siRNA. All treatments contained FSH. Five replicates were performed. Data represent lsmeans ± SEM.

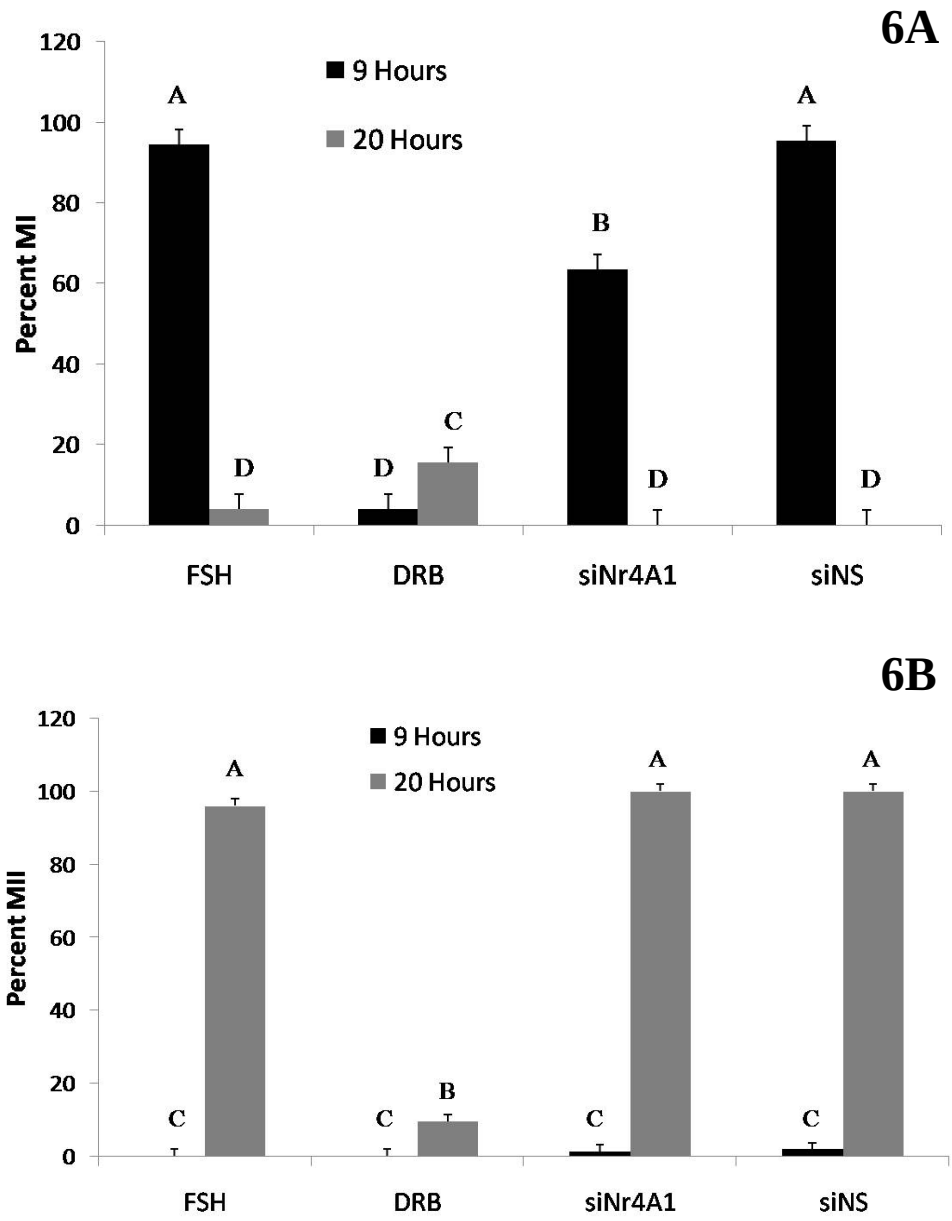

Figure 6. Effect of Nr4A1 siRNA on oocyte maturation. **6A**. Percent of COCs at MI stage after 9 or 20 hours of culture (lsmeans±SEM). **6B**. Percent of COCs that reached MII after 9 or 20 hours of culture (lsmeans±SEM). Treatment groups included FSH and DRB controls, and 50nM doses of either siNr4A1 or nonspecific (NS) siRNA. All treatments contained FSH. Data shown were arcsin transformed. Four replicates were performed; ^{A,B,C,D} $P<0.05$.

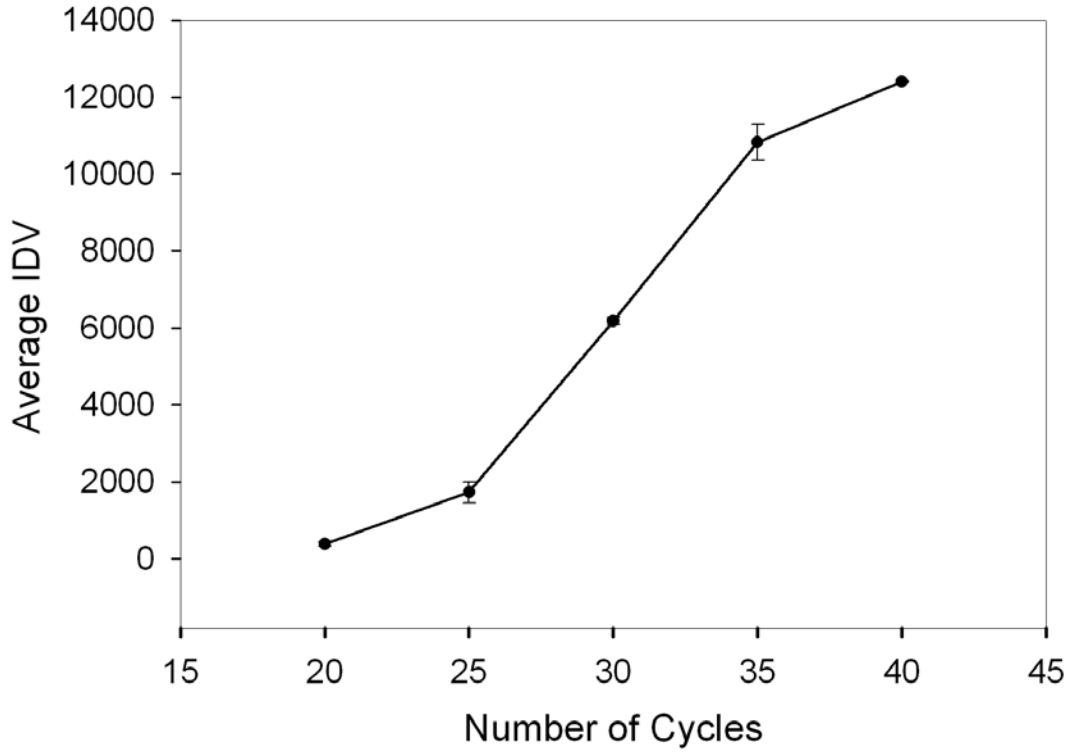

Figure 7. Graphical representation of band intensity at different numbers of cycles to determine the linear amplification range and cycle number for semiquantiative RT-PCR reactions using Nr4A1 primers. Average CV±SEM is 10.3±5.2%. Twenty-five cycles were used for Nr4A1 PCR reactions.

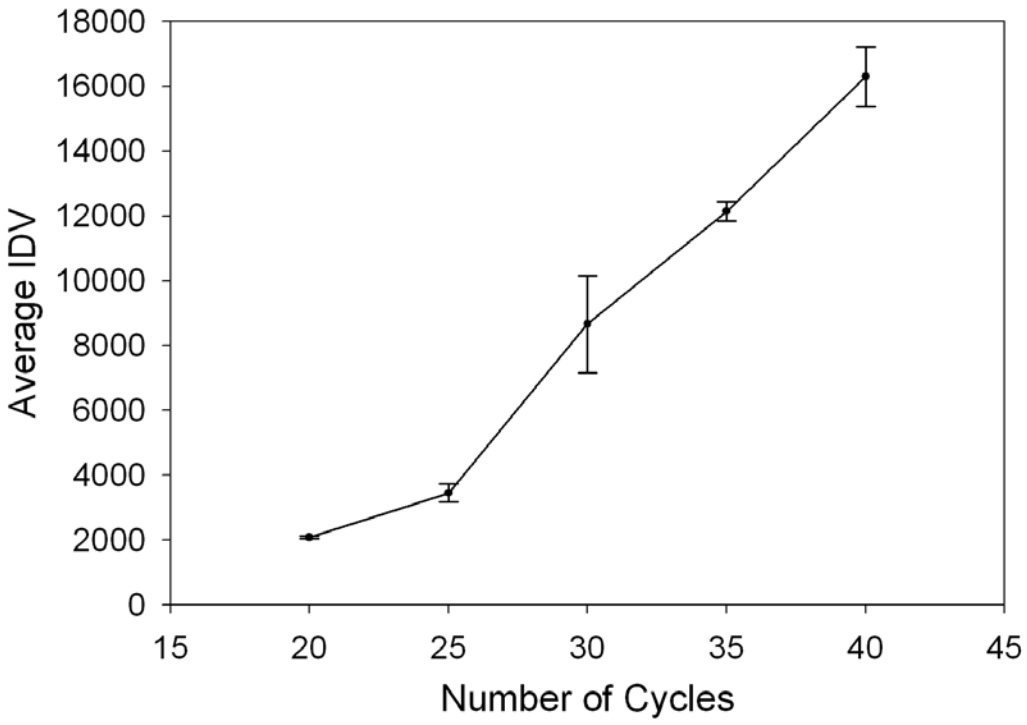

Figure 8. Graphical representation of band intensity at different numbers of cycles to determine the linear amplification range and cycle number for semiquantitative RT-PCR reactions using Egr1 primers. Average CV±SEM is 8.8±4.4%. Thirty-three cycles were used for Egr1 reactions.

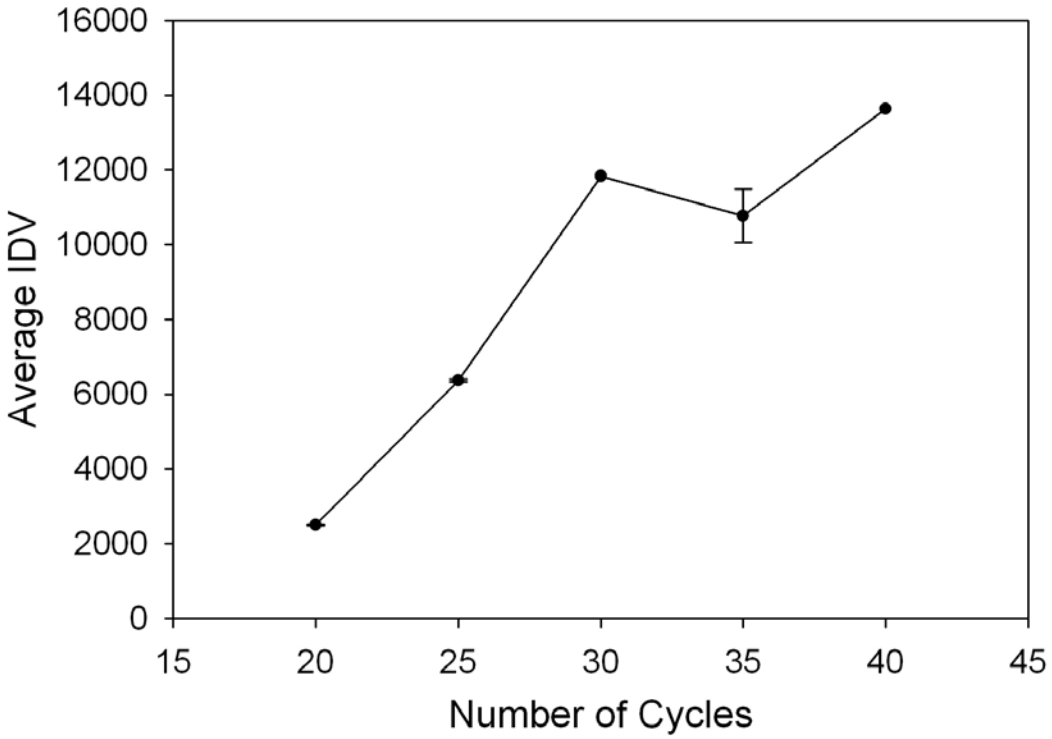

Figure 9. Graphical representation of band intensity at different numbers of cycles to determine the linear amplification range and cycle number for semiquantitative RT-PCR reactions using housekeeper GAPDH primers. Average CV±SEM is 5.0±2.5%. Twenty-five cycles were used for GAPDH reactions.

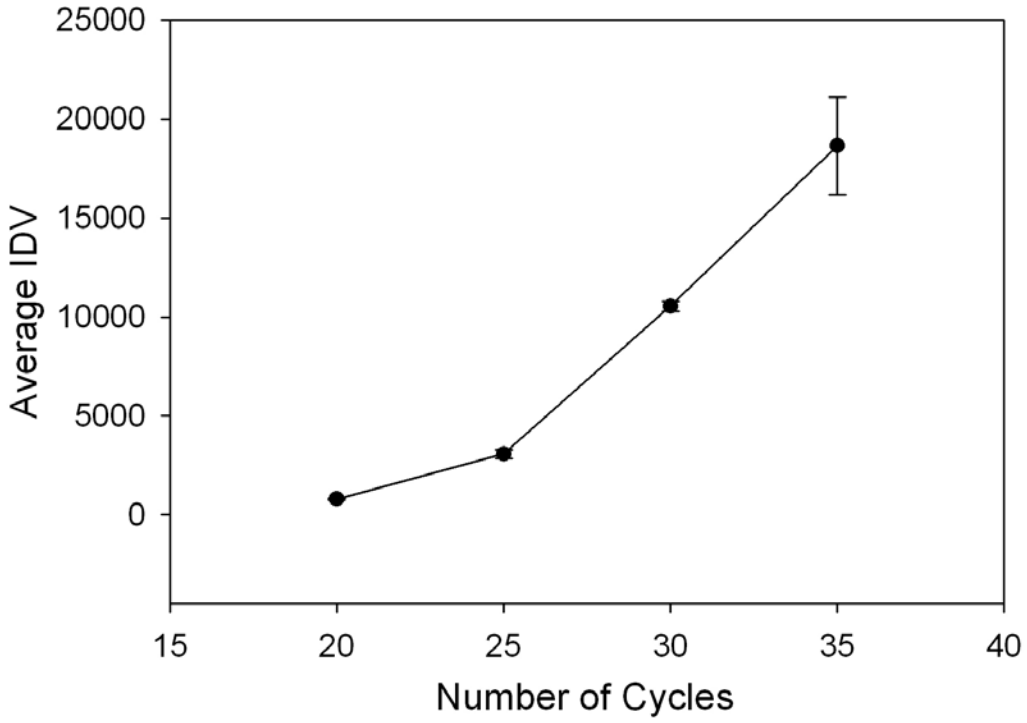

Figure 10. Graphical representation of band intensity at different numbers of cycles to determine the linear amplification range and cycle number for semiquantitative RT-PCR reactions using Tram6 primers. Average CV±SEM is 5.0±2.5%. Twenty-seven cycles were used for Tram6 reactions.

Table 1. Intra-assay coefficients of variation for semiquantitative RT-PCR assays.

Assay	Experiment			Overall CVs
	mRNA Pattern of Expression	siRNA Dose Response	Effects of siRNA Treatment	
Nr4A1	24.89%	14.78%	8.62%	16.10%
Egr1	21.45%	N/A	N/A	21.45%
GAPDH	8.69%	10.61%	13.41%	10.90%
Tram6	N/A	N/A	13.66%	13.66%