MICRORNA-21 MEDIATED POST-TRANSCRIPTIONAL GENE REGULATION IN OVARIAN FUNCTION

By

Martha Z. Carletti

Submitted to the graduate degree program in Molecular and Integrative Physiology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Dissertation Committee:

_____
Lane K. Christenson, Ph.D
Committee Chairperson

_____
David. F. Albertini, Ph.D

_____
William H. Kinsey, Ph.D

_____
Warren B. Nothnick, Ph.D

_____
Michael W. Wolfe, Ph.D

Date Defended: July 10, 2009

The Dissertation Committee for Martha Z. Carletti certifies that this is the approved version of the following dissertation:

MICRORNA-21 MEDIATED POST-TRANSCRIPTIONAL GENE REGULATION IN OVARIAN FUNCTION

Dissertation Committee:

_____
Lane K. Christenson, Ph.D
Committee Chairperson

_____
David. F. Albertini, Ph.D

_____
William H. Kinsey, Ph.D

_____
Warren B. Nothnick, Ph.D

_____
Michael W. Wolfe, Ph.D

Date Approved: July 31, 2009

Table of Contents

II. Acknowledgments

I would first like to express my sincere gratitude to my mentor, teacher, and friend, Lane Christenson. Your support and guidance over the past five years has molded me into a true scientist. You taught me not only the how of science, but the why as well; and in doing that, instilled in me a love of scientific research. I am forever grateful.

This research would not have been possible without past and present members of the Christenson lab. Not only colleagues but friends, Xiaoman, Stephanie, Lacey, Allison, and J.B., we have laughed together, complained together, and learned together, and you have made the lab feel like a second home. Xiaoman, you have been with me since the beginning, and you are always a patient and willing teacher, Thank you. Stephanie, a friend and confidante, you always stretched my knowledge base and made me think by asking thoughtful questions, Thank you. Lacey, you are an incredible friend and encourager, you made me laugh daily, and provided priceless advice on numerous occasions, Thank you.

I would like to acknowledge my committee members: Drs. David Albertini, William Kinsey, Warren Nothnick, Paul Terranova, and Michael Wolfe. The thoughtful discussions that were initiated in my committee meetings enhanced both my research and my knowledge.

A special thanks goes to those on the third floor of KLSIC who graciously loaned me supplies, reagents, and help when needed. Also thanks to Darlene Limback and Sara Turk for keeping me well fed and to Anh-Hguyet Nguyen for commiserating with me on the trials of being a mom and graduate student.

Thanks to the Madison and Lila Self Graduate Fellowship, who not only paid my salary for the past four years, but provided me with a development program that enhanced my training as a scientist.

Thanks to my parents, David and Debbie Ziegler, for encouraging me to go to graduate school, and for never doubting my ability to be both a scientist and mother. I would also like to thank my in-laws, Dan and Kathy Carletti, who never hesitated to lend a helping hand and watch Meg when I needed to come into lab on the weekend.

My biggest thanks goes to my husband, Matt. He has supported me throughout my graduate studies. He has believed in me when I didn't believe in myself, encouraged me when things didn't go as planned, and continuously reminded me what is most important in life. Few men would take on the household responsibilities as effortlessly and willingly, and I thank him for cooking dinner nearly every night, doing the laundry, and cleaning; all without complaining. Matt, I am truly blessed to have you as my husband.

Also thank to my daughter, Meg, who unknowingly motivated me to work my hardest each day and obtain this degree; and who willingly watched many more hours of tv than is probably acceptable as I wrote my dissertation.

III. List of Tables and Figures

Tables

Figures

IV. Abstract

The release of the oocyte from the ovary (ovulation) and terminal differentiation of cells of the preovulatory follicle (luteinization) are initiated by the surge of pituitary luteinizing hormone (LH) and are essential for proper reproductive function. As a result, major research efforts have been dedicated to deciphering the signaling pathways and transcriptional networks that are activated in granulosa cells following the LH surge. Remarkably however, few studies have examined the role post-transcriptional gene regulation plays in mediating LH action within preovulatory granulosa cells. Because cell and tissue function are reliant on the proper splicing, localization, and translation of mRNA to protein (i.e., post-transcriptional gene regulation) understanding this regulatory mechanism is essential for understanding ovarian (granulosa cell) function. MicroRNA (miRNA) mediate changes in post-transcriptional gene expression and have been tightly linked to a number of cell developmental and differentiation events. We identified miRNA-21 (miR-21) as an LH-induced miRNA in murine granulosa cells. The goal of this dissertation was to determine the function of miR-21 in granulosa cells of the periovulatory follicle. We found that in vitro knockdown of miR-21 in granulosa cells using antisense locked nucleic acid oligonucleotides (LNA) increased apoptotic death and decreased global translation, and these cellular events could be separated pharmacologically. Exogenous miR-21 increased global translation and our current evidence implicates changes in the activity of the Akt/mTOR pathways upstream of the critically important elongation factor-2 as the cause for this increase in protein synthesis. *In vivo* knockdown of miR-21 blocked ovulation and increased granulosa cell apoptosis.

Therefore, miR-21 functions in periovulatory granulosa cells to inhibit apoptosis and to promote global translation, thus likely promoting the formation of a functional corpus luteum and allowing for the establishment and maintenance of pregnancy.

V. Chapter One:

Introduction

1. The Periovulatory Follicle

To initiate ovulation and luteinization, luteinizing hormone (LH) acts on the dominant preovulatory follicle to form the periovulatory follicle. Periovulatory follicles are defined as those follicles that respond to the ovulatory surge of LH and proceed to ovulation. Cells of the periovulatory follicle mediate the processes of ovulation, oocyte maturation, and luteinization, and the follicle undergoes drastic molecular, phenotypic, and functional changes to allow for these to proceed. In the mouse, these changes are rapid, synchronized, and strictly regulated as 13-15 h after the LH surge ovulation occurs, followed by involution of the periovulatory follicle to form the corpus luteum (Runner MN, 1953) . Molecular changes within the periovulatory follicle occur within 1 h after LH, and phenotypic changes are evident 5-7 h after LH (Chaffin and Stouffer, 2002; Oonk et al., 1989).

Origin and Structure

The periovulatory follicle marks the last transition in the lifespan of the follicle as it converts to a new ephemeral gland, the corpus luteum. To appreciate the complexity of the periovulatory follicle, one needs to understand its origin and structure. The female contains a quiescent pool of immature primordial follicles that will either develop into a periovulatory follicle and ovulate or will degenerate through a process called atresia. Primordial follicles are comprised simply of an oocyte, arrested in the diplotene stage of meiotic prophase, surrounded by a single continuous or discountinuous layer of squamous stromal cells (Hirshfield, 1991; Zamboni, 1974).

Gradually and continuously, some of the primordial follicles are "activated" due to the actions of, among others, GDF-9, Kit ligand, and nerve growth factor beta (NGFB) to begin the development process (Dissen et al., 2001; Dong et al., 1996; Reynaud et al., 2000). The primary follicle is distinguished from the primordial follicle by a larger oocyte surrounded by cuboidal stromal cells, which are now referred to as granulosa cells. A basal lamina surrounds the granulosa cells, which separates them from the blood supply and promotes granulosa cell proliferation, polarity, and differentiation (Amsterdam et al., 1989; Luck and Zhao, 1993; Rodgers et al., 2001). The preantral follicle forms as granulosa cells proliferate into layers concentric with the inner most layers (Zamboni, 1974). In addition, the oocyte secretes glycoproteins (ZP1, ZP2, and ZP3) to assemble the zona pellucida, which is important for oocyte growth and, later, for fertilization (Bleil and Wassarman, 1980; Rankin et al., 2001; Wassarman, 2005). Those granulosa cells immediately adjacent to the oocyte are now referred to as corona radiate cells, and these cells directly interact with the oocyte through trans-zonal cytoplasmic projections that form communicative and adhesive junctions with the oolema (Anderson and Albertini, 1976; Eppig, 1982). Through these junctions maternal signals are transmitted to the oocyte, which in turn signals back to the somatic cells to direct them to perform functions necessary for oocyte development, thus forming a regulatory loop. Surrounding the corona radiata another layer of nurse cells, the cumulus cells, form junctions and communicate with the corona radiata. This combination of the oocyte, corona radiata, and cumulus cells is called the cumulus-ocoyte-complex (COC), and

the entire COC will be ovulated. As the follicle grows the number of granulosa cell layers increases, and development during this period is gonadotropin independent (Peters et al., 1975). Release of follicle stimulating hormone (FSH) from the pituitary causes the formation of a fluid-filled antrum within the follicle and the disassociation of the COC from the mural granulosa cells to form the antral follicle (Baccarini, 1971). FSH is required to stimulate/maintain antral follicle development (McGee and Hsueh, 2000), and the importance of FSH during this final transition is evident in that FSH null mice are devoid of any follicles past the preantral stage (Kumar et al., 1997). In response to FSH, granulosa cell proliferation increases and these cells secrete paracrine factors that promote fibroblast-like precursor cells surrounding the basal lamina to differentiate into theca cells (Magarelli et al., 1996; Zachow et al., 1997). Theca cells produce large amounts of androgens, and therefore differentiation involves an upregulation in genes involved in androgen biosynthesis, such as cholesterol side-chain cleavage cytochrome P450, 3β-hydroxysteroid dehydrogenase, and 17α-hydroxylase. Thecal androgens are aromatized into estrogens by mural granulosa cells (Erickson et al., 1991; Erickson et al., 1985). Therefore, following FSH stimulation the follicle produces large and increasing amounts of estrogen, and the increasing levels of estrogen feedback on the hypothalamus and pituitary to stimulate the LH surge (Kamel et al., 1987; Legan et al., 1975). FSH also acts on mural granulosa cells to increase LH receptor expression, so the granulosa cells can respond to the LH surge (Adashi, 1994; Zeleznik et al., 1974). In addition, FSH increases levels of vascular endothelial growth factor (VEGF) in the preovulatory

follicle, thus increasing blood vessel number and permeability so that LH can pervade the follicle and act on granulosa cells (Mattioli et al., 2001). LH binds to the newly acquired LH receptor on the granulosa cells to cause formation of the periovulatory follicle. LH receptors are expressed primarily by theca and mural granulosa cells, and not on cells of the COC, so the LH-induced mural granulosa cells must relay the LH signal to the COC to stimulate oocyte maturation, cumulus expansion, and ovulation (Eppig et al., 1997; Peng et al., 1991). In addition, granulosa cells of the periovulatory follicle mediate the breakdown of the surrounding basal lamina, theca cell layers, the dense, collagenous structurally supportive tunica albuginea, and the outer surface epithelium, to allow release of the entire COC from the follicle. Following ovulation, the follicle collapses and the blood supply invades the formerly avascular granulosa layer to form the corpus luteum. In rodents, the corpus luteum survives about two days if there is no mating stimulus (which will increase prolactin levels and maintain the corpus luteum), and, under the influence of prostaglandin F2α, undergoes luteolysis to form the corpus albicans (Bachelot and Binart, 2005; Horton and Poyser, 1976).

Events Stimulated by LH in the Periovulatory Follicle

Luteinizing hormone (LH) is released by the pituitary as a result of increasing levels of estradiol from the FSH-induced growing follicle. Luteinizing hormone binds to G-protein-coupled LH receptors, acquired due to the FSH stimulus, on granulosa cells of the dominant follicle to increase cyclic AMP (cAMP), cyclic GMP

(cGMP), and intracellular calcium levels (Davis, 1994; Marsh et al., 1966; Richards and Rolfes, 1980). These second messengers activate a number of cell signaling cascades, including the PKA pathway and the MAPK/ERK pathway (Babu et al., 2000; Bachelot and Binart, 2005; Cameron et al., 1996; Fan et al., 2009), inducing molecular changes that lead to the follicular and cellular events necessary for ovulation and luteinization (Duggavathi and Murphy, 2009). Although tightly co-regulated, LH surge induced events, such as ovulation and luteinization, can be considered as two separate events in that one is not required for the other to occur (Smith et al., 1994).

Although LH only binds to receptors on mural granulosa and theca cells, it causes profound changes in all cells of the follicle. The LH surge initiates cumulus expansion, re-entry of the oocyte into the cell cycle, and oocyte cytoplasmic maturation, all of which are necessary for successful fertilization and embryonic development. In addition, the LH surge initiates the breakdown of the follicle wall, thus allowing the release of the COC from the follicle through ovulation. Finally, the LH surge initiates the process of luteinization, in which the periovulatory follicle transforms into a highly vascularized transient endocrine organ. This organ, the corpus luteum, produces the high amounts of progesterone that are necessary to maintain pregnancy. Because this is such a critical time in female reproduction, these events have been well-studied, so in the following sections each event will be discussed independently.

Ovulation: Follicular Rupture

During ovulation, the periovulatory follicular wall must be breached at a specific location, the stigma, to allow the release of the COC into the oviduct (Thomson, 1919). The collagenous connective tissue of the theca externa and tunica albuginea provides strength to the follicular wall, and thus breakdown of these fibers is among the most important structural change of follicular rupture (Tsafriri, 1995). In addition, the basal membrane that separates the granulosa cells from the highly vascularized theca interna cells must be degraded (Tsafriri, 1995). In 1916, S. Schochet proposed that follicular fluid contains a "digestive enzyme" that could breakdown the follicle wall to allow for ovulation. This idea was met with a fair amount of skeptism by his colleagues, who continued to consider intrafollicular pressure the cause for ovulation as late as the 1970s, until it was finally established that antral pressure does not increase prior to ovulation (Parr, 1975). Moreover, later studies confirmed Schochet's hypothesis and LH-induced proteolytic mediators were identified in granulosa cells that activate proteolytic cascades to breakdown the follicle wall. These proteolytic mediators, prostaglandins, were first suggested to be important for follicle wall breakdown when it was shown that prostaglandin antiserum or prostaglandin synthase inhibitors, such as aspirin and indomethacin, could block ovulation (Bjersing and Cajander, 1975; Orczyk and Behrman, 1972). Levels of both prostaglandin synthase (Ptgs2) and prostaglandins increase after LH (Huslig et al., 1987; LeMaire et al., 1973). The importance of prostaglandins was further confirmed in Ptgs2 deficient mice, in which breakdown of the extracellular

matrix around the stigma is impaired (Davis et al., 1999). Prostaglandins initiate the breakdown of the follicle wall by activating plasminogen activators, which convert plasminogen in the follicular fluid to the active protease plasmin (Beers, 1975; Curry and Smith, 2006; Markosyan and Duffy, 2009). Plasmin activates the matrix metalloproteases (collagenase, stromelysin, and gelatinase), which proteolytically digest and weaken the follicle wall to allow for rupture (Reich et al., 1986; Woessner et al., 1989).

Cumulus Expansion

Cumulus cells secrete a "jelly-like matrix" composed of the mucopolysaccharide hyaluronic acid (HA) in a process called cumulus expansion following the LH surge (Austin, 1961; Eppig, 1980; Salustri et al., 1992). Cumulus expansion has been implicated in follicle cell survival, ovulation, and fertilization. Within the follicle, HA has been indicated to be important for cell growth and survival of both cumulus and mural granulosa cells as well as for oocyte quality (Kaneko et al., 2000; Ohta et al., 1999). This appears to be mediated by the binding of HA to CD44 receptor on granulosa cells and the oocyte, which activates the epidermal growth factor receptor 2 (ErbB2) receptor to initiate the phosphoinositide 3-kinase (PI3k)-dependent pathway, thus promoting cell growth and survival and preventing apoptosis (Kaneko et al., 2000; Schoenfelder and Einspanier, 2003; Yokoo et al., 2002). Expansion is also necessary for ovulation, perhaps by forming a protective and flexible meshlike structure that allows the COC to safely deform as it

passes through the follicle stigma (Russell and Salustri, 2006). Indeed, the loss of genes necessary for expansion result in reduced ovulation rates and a high incidence of oocytes trapped in luteinized follicles (Fulop et al., 2003; Varani et al., 2002). The sticky matrix of the COC may also facilitate COC pickup by the oviductal epithelial cells and its subsequent transport through the oviduct (Talbot et al., 2003). Within the oviduct, follicular molecules, such as progesterone or heparin, stuck to the expanded COC may act as a chemoattractant for sperm (Eisenbach and Tur-Kaspa, 1999). Additionally, the HA matrix may play a role in sperm capacitation (Van Soom et al., 2002), polyspermy prevention (Hong et al., 2004), or sperm acrosome activation (Tesarik et al., 1988), and an increase in expansion is associated with an increase in fertilizability (Chen et al., 1993).

Since cumulus cells and the oocyte do not express LH receptor, the molecular events that initiate cumulus expansion in response to LH are hypothesized to be mediated through the mural granulosa cells (Eppig et al., 1997; Peng et al., 1991). Granulosa cells use paracrine signals to transmit the LH signal to the COC. Epidermal growth factor (EGF)-like ligands, such as amphiregulin, epiregulin, and betacellulin, are expressed by granulosa cells in response to LH (Liu et al., 2009; Park et al., 2004). Their membrane-bound precursors are cleaved by ADAM (a disintegrin and metalloprotease) proteases, which are also upregulated in response to LH, and the mature ligands are released into the antrum of the periovulatory follicle (Conti et al., 2006; Sriraman et al., 2008a). These EGF ligands bind to ErbB receptor tyrosine kinases on cumulus cells to increase the expression of proteins necessary for cumulus

expansion, such as hyaluronidase synthase, Ptgs2, and tumor necrosis factor alpha induced protein 6 (Tnfaip6; Liu et al., 2009; Ochsner et al., 2003; Park et al., 2004; Yamashita et al., 2007). In addition, the inflammatory cytokine interleukin 6 has also recently been described as another, independent paracrine mediator of cumulus expansion that activates genes necessary for cumulus expansion (Liu et al., 2009). However, despite the importance of mural granulosa cells, the oocyte also seems to play a role in regulating cumulus expansion as removal of the oocyte from the COC complex prevents gonadotropin-induced cumulus expansion (Buccione et al., 1990). Ovarian secreted growth factors, such as GDF-9 and BMP15, have been implicated as oocyte paracrine signals that are necessary for cumulus expansion (Dragovic et al., 2005; Gueripel et al., 2006).

Oocyte Maturation

Occurring simultaneously with cumulus expansion is maturation of the oocyte, which includes both nuclear and cytoplasmic maturation. During embryogenesis, oocytes enter meiosis and arrest at the dictyate stage of prophase I (Eppig, 2003). Oocytes remain in prophase I through folliculogenesis, until the LH surge signals oocytes of antral follicles to undergo nuclear maturation (Eppig, 2003; Van De Kerckhove, 1959). During nuclear maturation the nuclear membrane (germinal vesicle) breaks down, the chromosomes condense, and the oocyte transitions out of prophase I to finish meiosis I ("meiotic resumption"), resulting in an extrusion of a polar body (Austin, 1961; Calarco et al., 1972).

Prior to formation of the antral follicle, the oocyte does not have the ability to undergo meiotic resumption, suggesting that inherent factors keep it arrested until this point (Eppig, 2003; Mehlmann, 2005). After antrum formation, however, the oocyte will spontaneously resume meiosis if removed from the follicle, suggesting that follicular factors must be responsible for its arrest (Mehlmann, 2005; Pincus and Enzmann, 1934; Racowsky and Baldwin, 1989). Indeed, loss of gap junctions between the oocyte and the corona radiata prevents the initiation of meiotic maturation (Carabatsos et al., 2000). Cyclic AMP is necessary for meiotic arrest, and elevated cAMP levels prevent meiotic resumption (Cho et al., 1974; Conti et al., 2002). Historically, cAMP from cumulus cells was thought to transit through gap junctions to the oocyte to maintain meiotic arrest (Anderson and Albertini, 1976; Piontkewitz, 1993, Webb et al., 2002). However, recent reports have indicated that cAMP does not originate from cumulus cells, but elevated cAMP levels are due to the activation of an oocyte-expressed orphan receptor, G-protein coupled receptor 3 (Gpr3; Mehlmann et al., 2004). Maintainence of oocyte cAMP levels by Gpr3 activation of adenylyl cyclase thus prevents meiotic resumption. Inhibition of Gpr3 using an inhibitory antibody causes meiosis to resume (Mehlmann et al., 2002; Mehlmann et al., 2004; Pirino et al., 2009). Elevated levels of cAMP activates protein kinase A (PKA), which phosphorylates the cell cycle mediators Cdk1 and cyclin B1 to prevent their activity and thus prevent meiotic resumption (Pirino et al., 2009). In addition, PKA also phosphorylates the phosphatase Cdc25b to prevent its activity (Pirino et al., 2009). Luteinizing hormone causes a loss of interaction

between the oocyte and the somatic cells, thus decreasing the cell signaling that activates PKA (Eppig, 1982). As PKA levels fall, Cdc25b becomes active and can dephosphorylate and thus activate mediators of the cell cycle to promote germinal vesicle breakdown, the condensation of chromosomes, and the formation of the first metaphase spindle (Pirino et al., 2009; Verde et al., 1990). Cdc25b knockout mice are sterile due to inability of the oocytes to resume meiosis (Lincoln et al., 2002).

Concomitant with nuclear maturation, the oocyte cytoplasm also matures with the acquisition of factors necessary for fertilization and subsequent early embryonic development are produced and organized (cytoplasmic maturation). One of the most morphologically obvious changes is the production and accumulation of cortical granules under the oocyte plasma membrane (Sathananthan et al., 1985). Cortical granules are synthesized from the Golgi complex, and contain at least five different proteins (Gross et al., 2000; Sathananthan et al., 1985). Some of these proteins, such as N-acetylglucosaminidase, modify the zona pellucida after fertilization to prevent polyspermy (Miller et al., 1993). Other proteins, such as p62 and p56, remain in the egg and are important for cell division in embryogenesis (Hoodbhoy et al., 2000; Hoodbhoy et al., 2001).

Cytoplasmic maturation also results in the accumulation of other molecules within the oocyte. It is well established that fertilization results in an increase in intracellular calcium levels within the egg to cause cortical granule exocytosis and the resumption of meiosis II (Kline and Kline, 1992; Miyazaki et al., 1993; Taylor et al., 1993). However, the immature oocyte does not contain enough stored calcium to

initiate these processes (Mehlmann and Kline, 1994). Therefore, following the LH surge, the oocyte increases its calcium stores, in part by increasing its amount of endoplasmic reticulum, and gains an increase in sensitivity of IP3-induced calcium release, thus preparing the oocyte so it can undergo the calcium oscillations needed during fertilization (Mehlmann and Kline, 1994; Mehlmann et al., 1995). In addition, prior to fertilization, the sperm chromatin is tightly condensed, but entry into the egg causes rapid decondensation so that the chromatin is released (Borenfreund et al., 1961; Perreault et al., 1988). Levels of glutathione, which has the ability to reduce protamine disulfide bonds, increase in the oocyte cytoplasm following the LH surge, and this increase is necessary for sperm decondensation (Perreault et al., 1988; Sutovsky and Schatten, 1997).

Luteinization

In 1940, luteinizing hormone was isolated from the anterior lobe of the pituitary gland, and was named such because it causes formation of CL in the ovary (Shedlovsky et al., 1940). The CL was first described by Renier de Graaf in 1672 in "De Mulierum Organis Generationi Inservientibus Tractatus Novus", and in this paper he proposed that CL are transient structures that develop from antral follicles and, furthermore, the number of CL corresponds to the number of embryos (translated by Jocylyn and Setchel, 1972). The idea that the CL might function as an endocrine organ was not proposed until two hundred years later by the French scientist A. Prenant (1898), and soon thereafter was confirmed when L. Frankel (1903)

demonstrated that removal of the ovaries in pregnant rabbits during the first week of gestation results in loss of pregnancy. In the 1930s, this CL-released "progestational substance" was identified as the steroid progesterone (Allen and Wintersteiner, 1934). Progesterone is necessary for implantation, uterine quiescence, lactation, (Corner, 1929; Robson, 1936), and feedback to the hypothalamus and pituitary to prevent a subsequent LH surge and ovulation in case pregnancy should occur (Parkes, 1929).

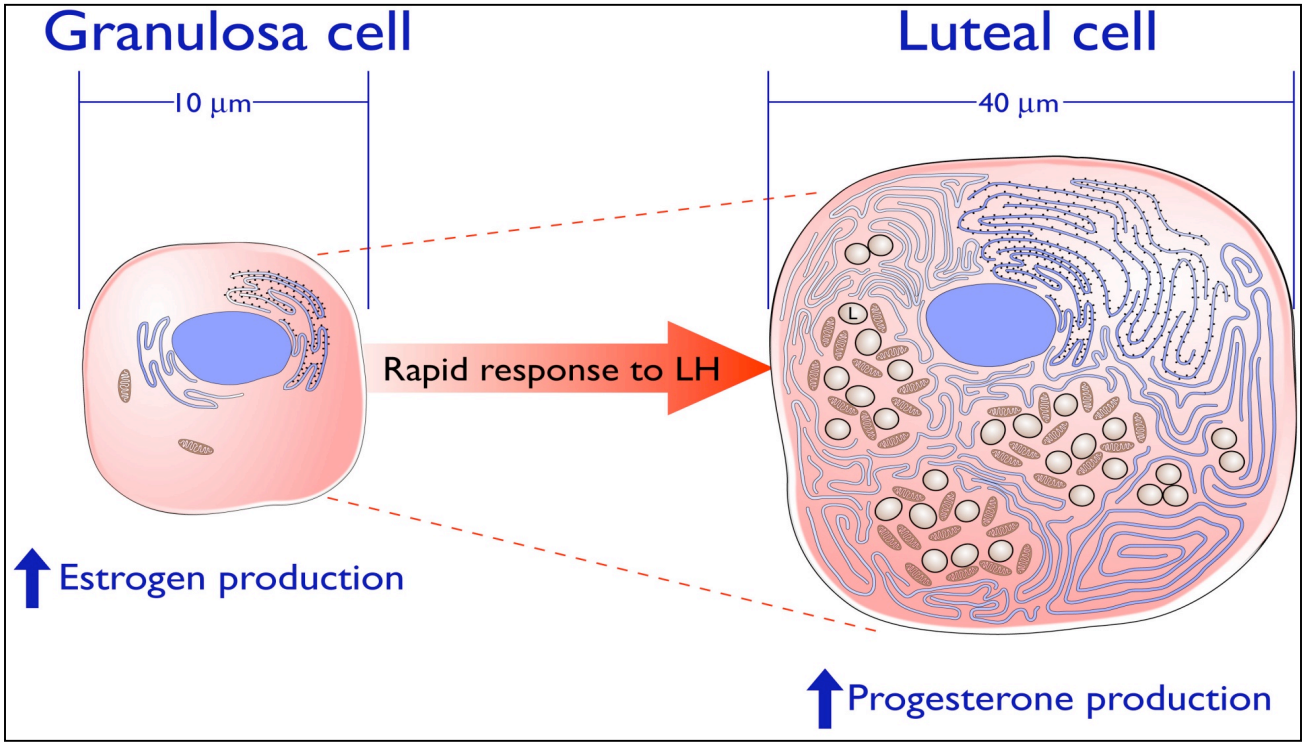
Several histological and electron microscopy studies have examined the morphological changes that occur during luteinization. At the tissue level, the basement membrane breaks down and there is an involution of granulosa and theca cells into the center of the antrum (Van Blerkom and Motta, 1978). In addition, there is rapid and dramatic angiogenesis so that the CL has an abundant blood supply in which to release progesterone into circulation (Cavender and Murdoch, 1988; Shimoda, 1993). Cords of granulosa cells surround the capillary vessels, forming interconnected spaces that allow for immediate release of progesterone into the blood (Van Blerkom and Motta, 1978). At the cellular level, granulosa cells transform into progesterone-producing luteal cells. There is a massive increase in organelles involved in steroid synthesis, including an increase in mitochondrial and smooth endoplasmic reticulum membranes and an increase in size of the Golgi apparatus (Cavazos et al., 1969; Crisp et al., 1970; Enders, 1973; Fawcett et al., 1969; McClellan et al., 1975; Priedkalns et al., 1968). This, along with an increase in large lipid droplets containing cholesterol esters, results in considerable granulosa cell hypertrophy. Within hours, the ~10um granulosa cell grows to an ~40um luteal cell

and increases in volume by 8-fold, transforming to the largest steroidogenic cell in the body (FIG V-1; Fawcett et al., 1969; Murphy, 2000). Furthermore, with the large increase in cellular membranes, the follicle can now increase serum progesterone concentration to the ng/mL range, compared to the pg/mL of estrogen it was making prior to the LH surge. It is important to note that an increase in cell size, not cell number, is responsible for this increase in steroidogenic ability, as the granulosa cell is terminally differentiated and has exited from the cell cycle (Rao et al., 1978; Robker and Richards, 1998a).

While early studies on luteinization focused on changes in morphology and general function of granulosa cells, later studies have focused on molecular changes that are occurring to mediate this transformation. A number of transcription factors have been identified as vital to the luteinization process, such as C/EBPβ and Egr1. Follicles of C/EBPβ null mice, while able to ovulate, are unable to undergo luteinization (Sterneck et al., 1997), and Egr1 null mice have defects in both ovulation and luteinization (Topilko et al., 1998), suggesting that these two transcription factors regulate luteinization. These transcription factors, and others, regulate a variety of genes that mediate the functional shift from an estrogen-producing cell to a progesterone-producing cell. The expression of genes involved in progesterone synthesis are upregulated, such as steroid acute regulatory protein (StAR; Kiriakidou et al., 1996), P450scc (Goldring et al., 1987) and 3βhsd (Couet et al., 1990), while genes involved in estrogen synthesis, such as P450 17alpha hydroxylase (Cyp17; Hedin et al., 1987) and, in most species, P450 aromatase

Figure V-1. The transition of a granulosa cell to a luteal cell involves a rapid and dramatic increase in mitochondria and smooth and rough endoplasmic reticulum, resulting in a pronounced hypertrophic response. Cells also accumulate lipid droplets which provide substrate for enhanced luteal cell progesterone synthesis.

Figure V-1


Granulosa cell
Luteal cell
10 µm
40 µm
Rapid response to LH
Estrogen production
Progesterone production

(Hickey et al., 1988), are downregulated.

The LH-induced cessation of the cell cycle is due to a loss of proteins that promote the cell cycle, including cyclins and Cdk2, and an increase in proteins that inhibit the cell cycle, including the Cdk inhibitors $p21^{cip1}$ and $p27^{kip1}$ (Hampl et al., 2000; Richards et al., 1998; Robker and Richards, 1998a). Furthermore, until onset of luteal regression, luteal cells are resistant to apoptosis (Matsubara et al., 2000). This apoptotic-resistance is due to the activiation of survival factors in granulosa cells by LH (Hu et al., 2004). While granulosa cells isolated prior to LH succumb to Fas-ligand induced apooptosis, granulosa cells isolated after LH are resistant to Fas-ligand induced apoptosis (Quirk et al., 2004). Treatment of cultured granulosa cells with the progesterone receptor antagonist RU486 inhibited this LH-induced resistance to apoptosis in some cells (Quirk et al., 2004). Therefore, the apoptotic resistance appears to be in part mediated by progesterone receptor (Quirk et al., 2004). In cultured porcine granulosa cells, LH suppressed the pro-apoptotic p53 and prevents cleavage/activation of caspase-3, and increased levels of the anti-apoptotic Bcl2 (Dineva et al., 2007). *In vivo,* the expression of the anti-apoptotic Bcl-2 family protein myeloid cell leukemia-1 (Mcl-1) and the anti-apoptotic P11, which inhibits activity of the pro-apoptotic Bad, were shown to increase following hCG in the rat, and therefore may play a role in the survival action of LH on granulosa cells (Chun et al., 2001; Leo et al., 1999). While these studies begin to examine the molecular events mediating LH-induced resistance to apoptosis, there are still many unanswered questions as to the molecular signals that mediate this resistance.

Controlled Ovarian Stimulation

Because of the cyclic nature of the ovary, and the recruitment and ovulation of just a few follicles at a time, it is difficult to isolate a large number of comparable granulosa cells from the ovary to examine and study. Therefore, researchers have adopted a "superovulation" technique in which immature animals are injected with exogenous hormones to synchronize and stimulate the growth of multiple follicles so that a large number of granulosa cells can be collected (Runner and Gates, 1954). While the technique also works on adult animals, and is in fact used on humans (and animals) during *in vitro* fertilization protocols, for laboratory studies immature animals are most often used because a larger number of cells can be obtained (Zarrow and Wilson, 1961).

Both FSH and LH have short half lives (3-4 hours for FSH, and 20 minutes for LH; (Speroff, 2004) and have to be continuously infused into an animal during ovarian stimulation (Armstrong and Opavsky, 1988; Hamilton and Armstrong, 1991). Therefore, the identification of hormones that bind to FSH and LH receptors, but have longer half-lives, provided a means for inducing superovulation with just a single injection. These hormones, equine chorionic gonadotropin (eCG, also called pregnant mare serum gonadotropin (PMSG)) and human chorionic gonadotropin (hCG) are produced by the placenta (mare or human, respectively) and have similar structure and biological activity to FSH and LH, respectively (Cole, 1930; Lamond, 1960). Like FSH, eCG stimulates follicle development, and, like LH, hCG stimulates ovulation and luteinization (Lamond, 1960). However, eCG and hCG have much

longer half-lives (6 days and 24h) than FSH and LH due to heavy glycosylation, and thus can be given as a single injection (Ladman, 1964; Speroff, 2004; Wang et al., 1995). Therefore, a superovulation protocol has been established that gives a dose of eCG to stimulate follicle development, followed by a dose of hCG to stimulate ovulation and luteinization (Runner and Gates, 1954). This superovulation protocol has been utilized in this work to obtain a large number of similar granulosa cells for study.

2. Post-Transcriptional Gene Regulation

In 1969, a paper published in Science by Gordon M. Tomkins *et al.* suggested that a "post-transcriptional mechanism" acts on messenger RNA (mRNA) to either repress or enhance translation (Tomkins et al., 1969). While they conceded that transcriptional regulation plays a large role in gene regulation, they state that control of the synthesis of mRNA, or "post-transcriptional gene regulation", is just as important in determining the final proteome of the cell. Since then, several studies have shown a lack of correlation between the steady state messenger RNA (mRNA) levels and the levels of the proteins encoded by these mRNA (correlation of 0.4, Gygi et al., 1999; correlation of 0.6, Ideker et al., 2001). This suggests that post-transcriptional gene regulation plays a major role in the establishment of a final proteome. Post-transcriptional gene regulation includes the regulation of the transport, turnover, and translation of mRNA, and infers several advantages on the cell. It allows the cell to respond rapidly to a signal, but still allows swift reversibility (Sonenberg, 2000). It allows for spatial control of a particular protein through mRNA localization and storage (Anderson and Kedersha, 2006; Colegrove-Otero et al., 2005; Hillebrand et al., 2007), and allows for cells lacking transcriptional control, such as transcriptionally quiescent oocytes or enucleated platelets a mechanism to regulate gene expression (Keene and Tenenbaum, 2002). Perhaps most interestingly, it may allow the coordinated expression of protein families, in a sense acting as a post-transcriptional operon (Keene and Tenenbaum, 2002).

Post-transcriptional gene regulation can be either global or specific. Global control can rapidly increase or repress the translation of the majority of mRNA. For example, stress causes the translation of all mRNA, except for those involved in cell survival, to decrease (Wek et al., 2006). These global effects on translation are due to regulation of the availability or activity of translational components, such as initiation factors, elongation factors, ribosomes, or tRNA (Rajasekhar and Holland, 2004; Sonenberg and Hinnebusch, 2007). Specific control of translation, on the other hand, depends on the presence of cis-acting regulatory elements in the 5' or 3' untranslated regions (UTRs) of mRNA. Trans-acting factors, including RNA-binding proteins and microRNA (miRNA)/RISC, act on these regulatory elements and can affect the translation, stability, or localization of often functionally related mRNA.

Post-Transcriptional Gene Regulation in the Ovary

The role of post-transcriptional gene regulation in the ovary remains virtually unexplored. Expectedly, it has been most studied in the transcriptionally quiescent oocyte. In 1964, Gross and Moyer showed that treatment of sea urchin eggs with actinomycin D did not affect their ability to rapidly synthesize proteins following fertilization (Gross et al., 1964). In addition, they showed that sea urchin eggs do not incorporate isotopically labeled uridine into RNA. Therefore, the oocyte is transcriptionally quiescent during maturation, fertilization and early embryogensis (up to the 2-cell stage in mouse, between the 4-and 8-cell stage in human), and thus must rely on stored mRNA during early embryonic development (Gandolfi and

Gandolfi, 2001; Gross et al., 1964; Memili et al., 1998). During folliculogenesis, the oocyte synthesizes and stores mRNA and proteins that will be used during this time period, and thus this stored pool must be kept stored and stabilized until needed (Gandolfi and Gandolfi, 2001). RNA-binding proteins have been demonstrated to be important for storing mRNA during this time period. For example, MSY2 is a germ-cell specific RNA-binding protein that accounts for 2% of the protein in the oocyte (Yu et al., 2002). It binds non-specifically to oocyte mRNA, acting as an mRNA stabilizer and translational repressor until an undefined signal causes MSY2 to release its transcripts so they can be translated into protein (Yu et al., 2002).

Within the somatic cells of the ovary, including granulosa cells, the role of post-transcriptional gene regulation has not been extensively examined. In fact, only several transcripts have been identified as post-transcriptionally regulated. Connexin 43, a major component of granulosa cell and oocyte gap junctions, is translationally repressed after the LH surge, although the specific RNA-binding protein that is responsible for the repression has not been identified (Kalma et al., 2004). The degradation of the LH receptor transcript is increased after the LH surge, and this is mediated by mevalonate kinase, which acts both as an RNA binding protein and an enzyme in cholesterol biosynthesis (Wang and Menon, 2005). In cultured bovine granulosa cells treated with FSH, the aromatase transcript has been shown to be post-transcriptionally regulated as its transcript has a longer half-life after cAMP stimulation (Sahmi et al., 2006; Sahmi et al., 2004). Finally, two miRNA, miR-17-5p

and let-7p, were recently shown to post-transcriptionally regulate TIMP1 expression during corpus luteum angiogenesis (Otsuka et al., 2008).

Recently, the role of microRNA as post-transcriptional mediators has been shown to be necessary for normal female reproductive function, as the loss of miRNA in the granulosa cells and reproductive tract leads to impaired fertility (Hong et al., 2008). Therefore, recent focus has shifted to identifying individual miRNA that are important for female reproduction.

3. MicroRNA

Lin-4 was identified in C. elegans mutants in 1981 as a mediator of *Caenorhabditis elegans* development (Chalfie et al., 1981). Unexpectedly, twelve years later, cloning of lin-4 indicated that it was not a protein-coding gene, but encoded for a small, 21-nucleotide RNA molecule (Lee et al., 1993). Furthermore, binding of lin-4 to the 3'UTR of a protein-coding mRNA, lin-14, repressed its translation (Lee et al., 1993). In 2000 another small RNA was identified in *C. elegans* (let-7(Reinhart et al., 2000)), followed by confirmation of the existence of small RNA in many diverse species, including humans (Pasquinelli et al., 2000). In 2001 the term "microRNA" was introduced as nomenclature for these small, regulatory RNA (Lagos-Quintana et al., 2001). Since then, thousands of miRNA have been identified in a multitude of organisms (microRNA.sanger.ac.uk/) and miRNA have led to an explosion in our knowledge of the role post-transcriptional gene regulation plays in organ function. It is predicted that 1-5% of genes encode for

miRNA, and they regulate the expression of as many as 30% of mRNA (Berezikov et al., 2005; Lewis et al., 2005). However, the functional importance of individual miRNA and the identity of their specific mRNA targets are just now beginning to be elucidated in rodents and human cell lines. It remains to be determined if the function of miRNA is conserved across species in the same manner as protein-encoding genes.

MicroRNA Biogenesis

MicroRNA genes reside between protein-coding genes (i.e., intergenic) and within introns of genes, and in a few cases reside within exons or comprise the entire intron (i.e., mirtrons; Berezikov et al., 2007; Saini et al., 2007). As a result miRNA can be transcribed either independently (intergenic and intronic) or in succession with an mRNA if they reside within an intron or exon (Corcoran et al., 2009). The initial long primary RNA transcript (pri-miRNA) is capped (methyl-7-GpppG) and polyadenylated, and thus the transcription of most miRNA genes is mediated by polymerase II, although a few are transcribed by polymerase III (Borchert et al., 2006; Cai et al., 2004; Kim, 2005; Lee et al., 2004b).

Within the pri-miRNA a secondary structure forms comprising an ~80-110nt stem loop. A protein complex called the microprocessor, composed of DGCR8 (DiGeorge syndrome critical region 8), Drosha, and p68/72 DEAD-box RNA helicase, binds to the stem loop and cleaves it to produce an ~70-100nt precursor miRNA (pre-miRNA; Lee et al., 2003). DGCR8 appears to act as the 'molecular ruler' by binding to the junction where the dsRNA of the stem loop meets the ssRNA,

and ‘counting’ up 11bp (one helical RNA turn; Han et al., 2006). Most pri-miRNA have a bulge or mismatched base at this site (Yi et al., 2003). The p68/72 helicases bind to the dsRNA of the stem loop and present the site to Drosha (Fukuda et al., 2007). Drosha cleaves the phosphodiester bond leaving a 2nt 3’overhang, and the resulting pre-miRNA is released into the nucleus (Lee et al., 2003). In some cases, other proteins are required for the interaction of Drosha with the pri-miRNA; for instance, KSRP (KH-type splicing regulatory protein) is an RNA-binding protein that binds to G-containing stretches in the terminal loop of the stem loop structure and is necessary for Drosha:pri-miRNA interaction for a subset of miRNA (such as miR-21; Trabucchi et al., 2009).

The pre-miRNA is exported from the nucleus by exportin 5 (Bohnsack et al., 2004; Lund et al., 2004). In the cytoplasm, pre-miRNA is further cleaved by a second RNase III enzyme, Dicer, to form a transient ~19-23nt RNA duplex (Hutvagner et al., 2001). Although Dicer is an RNase III enzyme like Drosha, it has an extra domain, the PAZ domain that allows it to act as a molecular ruler without the need for a cofactor. The PAZ domain binds to the 2nt 3’overhang at the base of the pre-miRNA, forcing the dsRNA binding domain to bind to the stem (Tahbaz et al., 2004). Dicer’s two RNAse III domains form an intramolecular dimer ~22nt from the base of the pre-miRNA, and this dimer cleaves the pre-miRNA to form an ~19-23nt RNA duplex that has 2nt overhangs on its 3’ ends (Tahbaz et al., 2004). The miRNA duplex has multiple mismatches and G:U wobble pairs, which causes one end of the duplex to be less stable at the 5’ end, and this strand is preferentially loaded onto the

RNA-induced silencing complex (RISC; Khvorova et al., 2003; Schwarz et al., 2003).

The RISC is composed of Dicer, TRBP (TAR RNA-binding protein), and one of several different Argonaute proteins (four in the human, AGO1-4; and five in the mouse, AGO1-5; Hutvagner and Simard, 2008). TRBP recruits Argonaute to the Dicer:miRNA complex, thus forming miRISC (Chendrimada et al., 2005). The 5' end of the miRNA tucks into a binding pocket on the Argonaute protein, and bases 2-8 bind to the target mRNA with perfect or close to perfect complementarity (Brennecke et al., 2005; Pratt and Macrae, 2009). These bases make up the 'seed sequence' of the miRNA, and are especially important for conferring target specificity (Brennecke et al., 2005; Lai, 2002). The seed sequence is exposed on the outside of the Argonaute protein, allowing the sequence to probe for a target through the cellular milieu and passing mRNA (Pratt and Macrae, 2009). In addition, many other proteins have been shown to interact with Argonaute proteins, but their exact roles in the miRISC are still being studied (Peters and Meister, 2007).

MicroRNA Mechanisms of Action

Binding of miRISC to target mRNA are postulated to cause: 1) mRNA cleavage and degradation; 2) mRNA deadenylation and degradation; 3) mRNA inhibition of translation initiation; 4) mRNA inhibition of translation elongation; or 5) mRNA translational enhancement. The first possibility is thought to be the most used by miRNA in plants, but in vertebrates the others are thought to be more often used. For mRNA cleavage, the miRISC must contain a catalytically active Argonaute (only

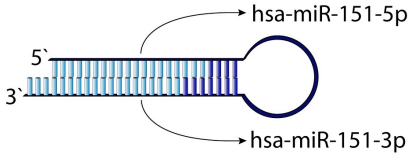
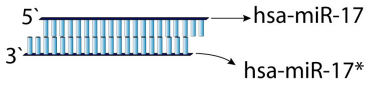
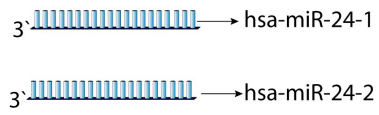
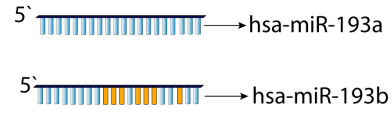
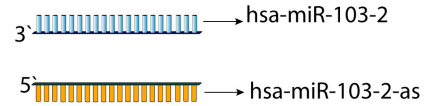
AGO2 in mammals, Liu et al., 2004), and the miRNA and mRNA must be almost a perfect complementary match. Messenger RNA degradation, however, can also be mediated by the other Argonautes even without perfect complementarity by interaction of the Argonaute protein with GW182 (Rehwinkel et al., 2005). GW182 recruits the decapping enzymes DCP1 and DCP2, and decapping of the mRNA leads to exonuclease degradation of the transcript (Rehwinkel et al., 2005). In other cases, miRNA:mRNA binding causes translational repression. Binding of miRNA to mRNA blocks translation due to RISC interaction with translation initiation factors, such as eIF6, eIF4E, and eIF4G, which prevents assembly of 80S ribosomes (Chendrimada et al., 2007). Other reports also suggest that miRNA represses mRNA translation during the elongation step, and this idea is supported in that translationally repressed mRNA were found to still associate with polysomes (Jackson and Standart, 2007; Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006). However, recent reports have also indicated that a change in proteins associated with RISC can cause a shift from translational inhibition to translational enhancement (Vasudevan et al., 2007).

Naming MicroRNA

To provide structure in naming and characterizing the thousands of recently identified miRNA, the Sanger Institute miRNA Registry was established (http://microrna.sanger.ac.uk/ ; Griffiths-Jones et al., 2006). The names of miRNA consist of four components, each conveying a specialized piece of information about the given miRNA: species, form (precursor or mature), identification number, and origin of miRNA (either processing origin or chromosomal origin). Each component is separated by dashes and is represented by the following template: xxx-miR-#-suffix. The xxx signifies the species (i.e., hsa=human, mmu=mouse). To distinguish between the precursor and mature forms of miRNA, a small case 'r' ('mir') represents the precursor form of the miRNA, while an uppercase 'R' ('miR') represents the mature form of the miRNA. Generally, an identification number is assigned in sequential order of discovery, thus recently identified miRNA have larger numbers. The suffix identifier (which may or may not be separated by a dash depending on the suffix, Table V-1) denotes either the processing origin or the chromosomal origin of the miRNA. For the processing origin, opposite arms from a single pre-miRNA are denoted '5p' and '3p'. After one arm is experimentally identified as the predominant arm, the less predominant arm is labeled with an asterisk suffix. For genomic origin, miRNA that arise from different genomic loci but have identical mature sequences are labeled with numbered suffixes, and if they arise from paralogous genomic loci and have highly similar mature sequences, they are labeled with lettered suffixes. Rarely, miRNA genes have been identified at the same chromosomal location but on

opposing DNA strands (sense versus antisense), and thus have unique mature miRNA sequences. The miRNA on the antisense chromosome is identified with an 'as' suffix. A given miRNA can have several suffixes. For example, hsa-miR-19b-1, which originates from chromosome 13, is identical to hsa-miR-19b-2 which originates from chromosome X, and both of these are paralogous (share 70% of mature sequence) to hsa-miR-19a which also originates from chromosome 13.

Table V-1. Nomenclature of MiRNA Suffixes.

Identifier (suffixes)	Meaning	Example
5p & 3p	"5p" and "3p" denote opposite arms from a single pre-miRNA hairpin. If one arm is experimentally determined to be predominantly detected, these are renamed using the * identifier.	
*	The * denotes the arm from a single pre-miRNA hairpin that is less predominantly detected within cells.	
Numbers	Numbers denote miRNA that arise from different genomic loci (unique hairpins) but have identical mature sequences.	
Letters	Letters denote miRNA that arise from paralogous genomic loci that have mature sequences with several base differences.	
'as'	"as" denotes microRNA that are found at the same chromosomal location, but have different promoters on opposing strands of the DNA (sense versus anti-sense).	

Regulation of microRNA Expression

Many miRNA are expressed in specific cell types or at specific time periods, therefore indicating that, like protein-coding genes, miRNA expression is highly regulated (Lagos-Quintana et al., 2002; Landgraf et al., 2007). However, the sequence and functional elements of miRNA promoters is only just starting to be examined. The few miRNA promoters that have been examined, however, appear to be very similar to those of protein-coding genes. Pri-miR-375, for example, is a miRNA that is expressed only in pancreatic islets (Avnit-Sagi et al., 2009). Promoter studies indicated that its promoter has transcription factor binding sites for pancreatic-specific transcription factors, such as HNF1 and Ngn3, and, in addition, chromatin immunoprecipitation studies confirmed interaction of Ngn3 with the pri-miR-375 promoter (Avnit-Sagi et al., 2009; Keller et al., 2007). In addition, approximately 50% of miRNA are found as genomic clusters, and thus are probably coordinately transcribed as one primary transcript. For example, the embryonic-stem cell (ES cell) specific miRNA gene cluster miR-290 to miR-295 is coordinately transcribed to help maintain ES cell pluripotency (Houbaviy et al., 2003). Finally, some miRNA genes lie within the intron or exon of a protein-coding gene, and thus are co-expressed with their host gene and rely on transcriptional activation of the host gene promoter for their expression (Kim and Kim, 2007; Raver-Shapira and Oren, 2007).

The processing of the primary or precursor forms of the miRNA can also be regulated, thus resulting in a form of post-transcriptional regulation. Indeed, Thomson et al. found a discreprency between the primary/precursor levels of miRNA

and the corresponding mature miRNA during mouse embryonic development (Thomson et al., 2006). Specific cases of miRNA being post-transcriptionally regulated have also been reported. In pluripotent stem cells the RNA-binding protein Lin28-homolog (LIN28) selectively binds to the pri-let-7 family members to prevent their interaction with Drosha, thus preventing let-7 mediated differentiation (Viswanathan et al., 2008). Another miRNA, miR-138, is found only in the brain, although its precursor forms are found ubiquitously in all tissues, suggesting that it is processed by Dicer only in the brain (Obernosterer et al., 2006). In another case, two RNA-binding proteins, nuclear factor 90 (NF90) and NF45, bind to pri-miRNA to prevent Drosha cleavage into pre-miRNA (Sakamoto et al., 2009). Interestingly, this binding appears to be differential, and in human embryonic kidney 293T cells, NF90 and NF45 had a higher affinity for pri-let-7a than pri-miR-21(Sakamoto et al., 2009). It would be interesting to examine whether NF90 and NF45 bind distinct pri-miRNA in different cells or in response to different treatments.

MicroRNA Functions

MicroRNA have been shown to be important in many biological processes, including cell proliferation, differentiation, and apoptosis (Asangani et al., 2008; Cloonan et al., 2008; Silber et al., 2008). In embryonic stem (ES) cells, for example, Dicer null mouse ES cells are viable, but they display major problems in growth and differentiation (Kanellopoulou et al., 2005). The ES cell specific miR-290-295 cluster has been shown to play an important role in preventing ES cell self-renewal by

indirectly decreasing the activation of Oct4 (Sinkkonen et al., 2008). In addition, to promote differentiation, miR-21 targets Nanog and Sox2, important regulators of ES cell pluripotency and self-renewal (Singh et al., 2008). MicroRNA have also been shown to be involved in the differentiation of adult stem cells during haematopoiesis, myogenesis, cardiogenesis, neurogenesis, and osteogenesis. During erythropoiesis, miR-221 and miR-222 decrease during erythroid differentiation, releasing their inhibition of C-kit protein translation, leading to the expansion of early erythroblasts (Fontana et al., 2008).

Because of their vital roles in cell growth and apoptosis, miRNA have also been associated with many disease states. MicroRNA-133 and miR-1, for example, have been associated with the increase in cardiomyocyte hypertrophy that occurs during heart failure (Care et al., 2007). Within the brain, several miRNA are upregulated in the diseased temporal lobe neocortex of Alzheimer's patients, (Sethi and Lukiw, 2009), and a single nucleotide polymorphisms effecting miRNA binding sites have been associated with Tourette's Syndrome and obsessive compulsive disorder (Abelson et al., 2005; Muinos-Gimeno et al., 2009). Within the female reproductive tract, miRNA have been shown to play a role in endometriosis and uterine leiomyomas (Marsh et al., 2008; Wang et al., 2007; Peng et al., 2008; Pan et al., 2007).

Perhaps the most studied is the role of miRNA in cancer development, progression, and prognosis. Many miRNA genes are located in genomic regions that are amplified, deleted, or rearranged in cancer (Fontana et al., 2008). Thus, it was not

surprising that many of these miRNA have been shown to play a role in cancer, and have even been given the designation "oncomir". Three of the most studied oncomirs are miR-34, miR-17-5p, and miR-21 (Esquela-Kerscher and Slack, 2006). MicroRNA-34 is downregulated in cancer, and studies have indicated that it acts as a tumor supressor and induces cell growth arrest and apoptosis by activating the p53 pathway (Yamakuchi et al., 2008). MicroRNA-17-5p and MiR-21 are both upregulated in many types of cancer and tumors, and have been demonstrated in many cases to prevent apoptosis and promote differentiation (Esquela-Kerscher and Slack, 2006).

Because miRNA have been demonstrated to be important in other developmental and differentiation systems, we propose that miRNA also play a vital role in the development and function of reproductive tissues.

Methods for Studying MicroRNA

The first step in beginning a microRNA study is to determine the microRNA that are expressed within a given tissue or at a given period. These preliminary studies can include miRNA cloning analysis or miRNA microarray analysis. MicroRNA cloning is useful to identify novel microRNA, and cloning frequency can give a general idea of abundance. Most microRNA are identified first by cloning (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), and cloning is especially useful in identifying miRNA that are novel to a specific tissue (Ro et al., 2007a; Ro et al., 2007b). With the establishment of a list of known miRNA, miRNA

microarrays were developed to allow for high throughput profiling of miRNA expression. MicroRNA microarrays provide a rapid means in establishing the miRNAome of a cell, and can provide a fairly good idea of miRNA abundance and changes between treatment groups (Li and Ruan, 2009). However, novel miRNA will be missed in a miRNA microarray study.

Once a miRNA is identified by one of these two methods, its expression pattern is confirmed using Northern blot analysis or quantitative real time PCR (qRT-PCR). As new qRT-PCR methods have been developed that allow for increased specificity, qRT-PCR has become the method for choice as it has a higher sensitivity than Northern blotting and provides a greater means for studying quantitative changes. While several qRT-PCR methods have been introduced that allow for amplifying and quantifying these extremely small miRNA, we have chosen to use a modified oligo d(T) method for analyzing miRNA in our system due to ease of use and an improved ability to normalize the data (Fiedler, 2009). In this method, a poly(A) tail is added to all RNA transcripts. The transcript is then reverse transcribed with an oligo d(T) primer that has the addition of a universal tag on its 5'end. The first strand synthesis in qRT-PCR is initiated with an miRNA-specific forward primer, and then a reverse primer specific to the universal tag finishes the amplification.

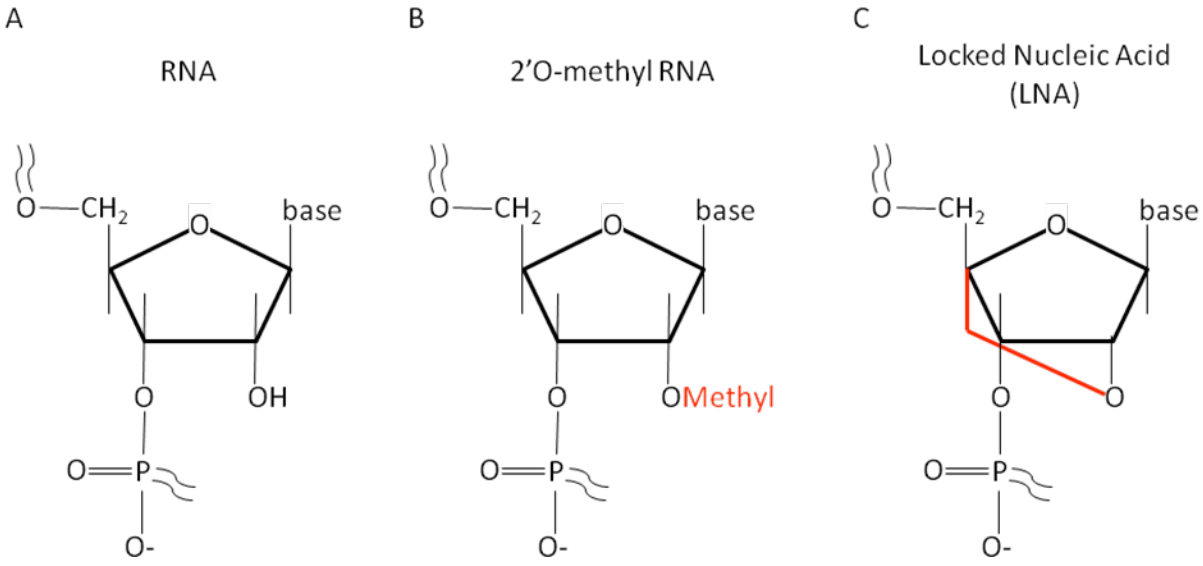
After a miRNA has been shown to be expressed in a given tissue or at a given time period, the next question is, 'what is its function'? There are two ways to ascertain the function of a miRNA: 1) inhibit the miRNA; 2) add exogenous miRNA.

MicroRNA function can be inhibited by using modified synthetic antisense oligonucleotides that bind to the miRNA and prevent its interaction with its target. Ideally, these synthetic oligonucleotides will have a high biostability and will bind to the miRNA with high affinity. 2'O-methyl oligonucleotides are RNA molecules that have a methyl group at the 2' position of the ribose (Figure V-2B) that increases nuclease resistance and thus makes the oligonucleotide more stable (Grunweller et al., 2003). The interaction of a 2'O-methyl oligonucleotide with its target miRNA is with similar affinity as a normal RNA:RNA interaction. Another option, locked nucleic acids (LNA), have a 2'-O,4'-C methylene bridge that locks the structure in a bicyclic formation (Figure V-2). This bridge dramatically increases the biostability of the molecule, and, furthermore, increases the melting temperature of the LNA:miRNA duplex by several degrees (Grunweller et al., 2003). Therefore, LNA molecules often have a more potent and lasting effect than 2'O-methyl oligonucleotides (Grunweller et al., 2003). We have utilized both molecules in this work. In addition, miRNA can be overexpressed by adding exogenous pre-miRNA. The exogenous pre-miRNA is cleaved by Dicer and incorporated into RISC in the same manner as endogenous pre-miRNA.

Knockdown or overexpression of the microRNA will cause a change in the expression of target proteins. To identify these target proteins, a two-dimensional gel approach can be used. For example, protein from cells treated with a nonspecific control LNA and protein from cells treated with an LNA to the specific miRNA can

FIGURE V-2. Synthetic antisense oligonucleotides used to knockdown miRNA function. (A) RNA molecule. (B) The 2'O-methyl oligonucleotide has a methyl group at the 2' position of the ribose (red) . (C) The locked nucleic acid oligonucleotide has a 2'-O,4'-C methylene bridge (red).

Figure V-2


A
RNA
O—CH2
O
base
O
OH
O=P
O-
B
2'O-methyl RNA
O—CH2
O
base
O
OMethyl
O=P
O-
C
Locked Nucleic Acid
(LNA)
O—CH2
O
base
O
O
O=P
O-

be isolated and labeled with either Cy3 (green) or Cy5 (red), respectively. The labeled protein is run on a single two-dimensional gel, separating the protein by both size and by isoelectric point. Unchanged proteins are visualized as yellow spots, while changed proteins are visualized as either red or green spots. The spots can be excised from the gel and analyzed by mass spectrometry to determine the identity of the protein. The changed proteins can be analyzed using bioinformatics software to find miRNA-binding sites in their 3'UTR, and the presence of a site can indicate that the protein is a direct target of the miRNA.

If a protein is predicted to be a direct target of the miRNA, luciferase assays provide an approach for confirming the interaction. The 3'UTR of the predicted target mRNA is ligated into a plasmid containing the luciferase gene. The plasmid can be co-transfected with the LNA to the miRNA, and if luciferase activity increases, the miRNA likely directly interacts with that particular 3'UTR. To confirm the interaction, various mutations can be made within the 3'UTR so the miRNA binding sites can be disrupted, and luciferase activity can be measured.

4. MicroRNA in the Ovary

Recruitment of growing follicles, atresia, ovulation, luteal tissue formation and regression are dynamically regulated events that reoccur on a cyclical basis within the ovary. Few tissues exhibit the ephemeral lifecycle seen in follicular and luteal tissues within the adult, making this system very unique. These events involve dramatic changes in cellular growth, angiogenesis, steroidogenesis, cell cycle status,

and apoptosis, and are tightly regulated at the endocrine and tissue level. Defects in regulatory control can lead to ovarian failure due to disruption of folliculogenesis, block of ovulation, and corpus luteum insufficiency, etc. Although the transcriptional regulation of ovulation and luteinization has been well studied, it stands to reason that post-transcriptional gene regulation may be critical for reproductive tissue function. Understanding the molecular events, including the role of miRNA in post-transcriptional gene regulation, that occur during these transitory periods might provide insight into how we might be able to enhance reproductive efficiencies and alleviate deficiencies.

Recently, Otsuka and coworkers, demonstrated that a Dicer1 hypomorph mouse (*Dicer1*$^{d/d}$; ~75% loss of Dicer1 mRNA levels), developed using a gene trap method, exhibited female infertility (Otsuka et al., 2008). Transplantation of wild type ovaries into *Dicer1*$^{d/d}$ females resulted in offspring, but wild type females transplanted with *Dicer1*$^{d/d}$ ovaries failed to establish pregnancies, indicating that the fertility defect was inherent to the ovary. Further characterization of *Dicer1*$^{d/d}$ mice indicated that ovulation occurred normally and that the ovulated eggs were fertilized and underwent the first embryonic cell division. In contrast, serum progesterone levels and expression of LH receptor, cytochrome p450 11a1 (Cyp11a1), and prolactin receptor, genes necessary for progesterone production and corpus luteum formation/maintenance, were all decreased in *Dicer1*$^{d/d}$ mice. Proper corpus luteum function requires a dramatic increase in vasculature, and miRNA have previously been shown to be important for angiogenesis in embryonic development (Yang et al.,

2005). Therefore, Otsuka et al. (2008) examined vasculature in these *Dicer1*$^{d/d}$ mice and found a decrease in both the number and the length of the blood vessels in the corpus luteum and correlated this with the upregulation of anti-angiogenic factors, TIMP1 and platelet factor 4, in *Dicer1*$^{d/d}$ mice. MicroRNA-17-5p and let-7p were found to regulate TIMP1 expression and loss of these in *Dicer1*$^{d/d}$ mice were predicted to cause the reduction in angiogenesis. Knockdown of these two miRNA in wild type mice impaired corpus luteum angiogenesis and decreased serum progesterone levels. Furthermore, injection of miR-17-5p and let-7p into the ovarian bursa of *Dicer1*$^{d/d}$ mice restored the vasculature within the corpora lutea and increased progesterone levels, but failed to maintain pregnancy, indicating that other miRNA might still be involved (Otsuka et al., 2008).

Our laboratory mated the anti-Mullerian hormone receptor, type 2 (Amhr2)-Cre mouse (Jamin et al., 2002) with a mouse homozygous for locus of crossover in P1 (loxP) insertions surrounding the second RNase III domain of *Dicer1* (Harfe et al., 2005). This selectively knocked out Dicer1 expression in Mullerian duct derivatives (the oviduct, uterus, and cervix) and in the granulosa cells of secondary and small antral follicles (Hong et al., 2008). The female *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice were infertile as evidenced by failure to deliver offspring over a 5 month period, but the male mice were able to sire offspring (Hong et al., 2008). *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ female mice mated normally and had normal estrous cycles, but exhibited a reduced ovulation rate (10.7 ± 0.9 and 7.0 ± 1.1, for the wild type and *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice, respectively). This effect was not due to a disruption in the secretion of

hormones, as the reduced ovulation rate was also observed in gonadotropin-stimulated immature mice (16.2 ± 1.4 and 3.7 ± 1.5, for the wild type and *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice, respectively). Moreover, these *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice had reduced ovarian weights compared to wild type controls (Hong et al., 2008). Ovulated oocytes from *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice collected at d1 post-coitus underwent *in vitro* embryonic development that mirrored that observed in the wild type mice (Hong et al., 2008). These results suggest the loss of Dicer1 expression impacts ovarian function, however, since these mice were still able to ovulate competent oocytes, the ovarian loss of Dicer1/miRNA does not explain the infertility seen in these mice.

Three other groups have also examined reproduction in *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice (Gonzalez and Behringer, 2009; Nagaraja et al., 2008; Pastorelli et al., 2009). Two of these groups did not extensively characterize ovarian defects in the *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice, but one group noted the decrease in ovulation and increase in trapped oocytes that we observed in our laboratory (Nagaraja et al., 2008). In addition, they performed TUNEL staining on the ovary to examine granulosa cell apoptosis, and they saw a significant increase in apoptotic granulosa cells in the *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice (Nagaraja et al., 2008).

To examine the importance of specific miRNA within the ovary, four studies have examined ovarian miRNA expression using several different methodologies and experimental paradigms (Choi et al., 2007; Fiedler et al., 2008; Kim et al., 2006; Ro et al., 2007b). In 2006, a computational analysis of the pig genome identified 58

miRNA, and northern blot analysis confirmed the expression of two of these miRNA (i.e., miR-31 and miR-92) within the porcine ovary, but no functional analysis of these miRNA in the ovary has been reported (Kim et al., 2006). Ro et al (2007) used a cloning technique to identify miRNA expressed in the ovaries of 2-wk-old and adult mice (Ro et al., 2007b). Combined, they identified 122 miRNA from whole ovaries of the immature and mature mice. Because whole ovarian tissue was used and the stage of the estrous cycle of the adult ovaries was unknown, the usefulness of this data set is limited. Similarly, Choi et al. (2007) cloned miRNA in newborn mouse ovarian tissue from wildtype and newborn ovary of homeobox gene (Nobox) knockout animals. Nobox is a transcription factors necessary for oocyte differentiation and survival (Choi et al., 2007). They identified 177 miRNA in the newborn ovary and found that 4 were decreased ~2-fold in the $Nobox^{-/-}$ ovaries (Choi et al., 2007).

To examine somatic cell miRNA expression and test whether LH can regulate miRNA expression, we performed a microRNA microarray on mouse granulosa cells before (0h) and 4h after hCG (Fiedler et al., 2008). We identified 196 and 206 miRNA as detectable before and 4h after hCG respectively, with 31 miRNA showing high abundance, 64 intermediate abundance, and 117 low abundance. Of the total 212 miRNA detected, only thirteen miRNA were differentially expressed between 0h and 4h post-hCG, with 3 upregulated and 10 downregulated ($p<0.05$; (Fiedler et al., 2008). Among the upregulated miRNA were miR-132 and miR-212. MicroRNA-132 has previously been shown to be transcriptionally regulated by cAMP in neuronal

cells (Vo et al., 2005), and to post-transcriptionally regulate the expression of several different genes (Klein et al., 2007). Included among the miR-132 targets was the transcriptional co-repressor C-terminal binding protein (CtBP1; Klein et al., 2007). Interestingly, CtBP1 in conjunction with steroidogenic factor-1, was recently shown to regulate adrenal steroidogenesis (Dammer and Sewer, 2008). We demonstrated that cultured granulosa cells respond to cAMP with an increase in miR-132 and miR-212 levels, and that LNA oligonucleotide knockdown of miR-132 and miR-212 caused an increase in CtBP1 protein levels, suggesting that these miRNA post-transcriptionally regulate CtBP1 expression in granulosa cells (Fiedler et al., 2008). In this array, an established oncomiR, miR-21, was also shown to be upregulated in response to LH. This dissertation examines the expression and function of miR-21 in periovulatory granulosa cells.

5. MicroRNA-21

MicroRNA-21 (miR-21) was identified as upregulated in mouse granulosa cells within 4 h following LH (Fiedler et al., 2008). In the mouse, the gene for miR-21 lies on chromosome 11 and the promoter overlaps with exon 12 of TMEM49. The transcription of miR-21 results in a 5' capped and polyadenylated pri-miR-21 transcript (Cai et al., 2004). The promoter for pri-miR-21 is one of a few miRNA promoters that has been well mapped and studied, and early reports indicate that transcription of the pri-miR-21 is regulated by promoter elements in the same way protein-coding genes are. The promoter has AP-1, NFIB, REST, STAT3, CEBP,

SRF, and p53 binding sites, and has been experimentally shown to be regulated by the first five of these (Chen et al., 2008a; Fujita et al., 2008; Loffler et al., 2007; Ramachandra et al., 2008; Singh et al., 2008). In a couple of instances, miR-21 has been shown to indirectly promote its own transcription, such that miR-21 mediated post-transcriptional downregulation of an mRNA causes an increase in miR-21 transcription (Fujita et al., 2008; Talotta et al., 2009). For example, in a RAS-inducible thyroid cell line, RAS-induced differentiation increased Jun/Fos, which acted on AP-1 sites within the miR-21 promoter to increase in miR-21 transcription (Talotta et al., 2009). In addition, miR-21 post-transcriptionally represses translation of PDCD4, a strong inhibitor of AP-1 activity (Talotta et al., 2009). In another case, in vascular sooth muscle cells, miR-21 was shown to target NFIB, a transcription factor that binds to the miR-21 promoter to inhibit its transcription (Fujita et al., 2008). Therefore, miR-21 has the ability to repress inhibitors of its own transcription, thus promoting and sustaining its own synthesis (Fujita et al., 2008; Talotta et al., 2009). DNA hypomethylation has also been indicated to increase miR-21 expression, as treatment of OVCAR3 cells with the demethylation treatment 5-aza-2'-deoxycytidine caused an increase in miR-21 expression (Iorio et al., 2007). Interestingly, miR-21 has also been shown to be regulated post-transcriptionally. The mature form of miR-21 was found to increase in vascular smooth muscle cells in response to bone morphogenic protein 4 (BMP4), but, unexpectedly, pri-miR-21 levels were unchanged. The SMAD signal transducers were found to increase the interaction of pri-miR-21 with p68 helicase, a component of the Drosha complex.

This interaction initiated Drosha-facilitated cleavage of the pri-miR-21 (Davis et al., 2008a).

Functions of miR-21 have been determined by either knocking down miR-21 or overexpressing miR-21. The loss of miR-21 increased apoptosis in several cell types, including glioblastomas (Chan et al., 2005), JHU-012 head and neck cancer cells (Chang et al., 2008), gastric cancer cell lines (Zhang et al., 2008b), and vascular smooth muscle cells (Ji et al., 2007). Furthermore, miR-21 increases cell proliferation in hepatocellular cancer cells (Meng et al., 2006), A549 lung cancer cells (Fei et al., 2008), gastric cancer cell lines (Zhang et al., 2008b), and vascular smooth muscle cells (Ji et al., 2007). Overexpression of miR-21 also causes an increase in cell growth, as seen in JHU-012 (Chang et al., 2008) and MCF-7 breast cancer cell lines (Frankel et al., 2008), and induces cellular hypertrophy in cardiomyocytes (Cheng et al., 2007; Tatsuguchi et al., 2007). MiR-21 has also been associated with an increase in cell motility and migration (Meng et al., 2006; Tsai et al., 2009). Finally, miR-21 is upregulated during embryonic stem cell differentiation and T cell differentiation (Singh et al., 2008; Wu et al., 2007). In embryonic stem (ES) cells, it prevents ES cell self-renewal and causes the downregulation (most likely indirectly) of Oct4, Sox2, and Nanog (Singh et al., 2008).

The mRNA targets of miR-21 directly correspond to its demonstrated functions. The first identified miR-21 target was the tumor suppressor PTEN in 2006 in malignant cholangiocytes (Meng et al., 2006), and the identification of other tumor suppressor genes as targets for miR-21 soon followed. The pro-apoptotic genes

tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), Sprouty, reversion inducing-cystein-rich protein with kazal motifs (RECK), heterogenous nuclear ribonucleoprotein K (HNRPK), and tumor protein p63 (Tap63) have all been identified as direct miR-21 targets (Chen et al., 2008b; Meng et al., 2006; Papagiannakopoulos et al., 2008; Thum et al., 2008; Zhu et al., 2007). However, miRNA targets can vary widely by cell type, in that a target in one cell type may not be a target in another cell type (Frankel et al., 2008; Papagiannakopoulos et al., 2008).

As would be expected from its identified functions, the dysregulation of miR-21 has been found in many diseases and cancers. In fact, miR-21 is identified as overexpressed in nearly every cancer or tumor examined, and thus has been labeled as an "oncomir" (Cho, 2007; Table V-2). The anti-apoptotic, pro-growth and pro-migration functions of miR-21 make it a prime candidate for the driving force behind the rapid metastasis and invasion that are needed for the advancement of cancer. Examples of cancers that miR-21 is upregulated in include breast, prostate, pancreatic, ovarian, cervical, heritable cancers such as Cowden's Syndrome, and viral-induced cancers such as hepadnavirus-associated hepatocellular carcinoma (Table V-2). It is also induced in vascular inflammation, LPS-induced inflammation of lung cells, and in uterine leiomyomas (Table V-2). In several cases, such as non-small cell lung cancer and pancreatic cancer, the expression of miR-21 correlated with a poorer patient outcome. Therefore, because of its upregulation in a variety of

Table V-2. MicroRNA in Disease

Ovarian Cancer	**Dahiya, N, CA Sherman-Baust, et al**. 2008. Microrna expression and identification of putative mirna targets in ovarian cancer. PLoS ONE **3** e2436. **Nam, EJ, H Yoon, et al**. 2008. Microrna expression profiles in serous ovarian carcinoma. Clin Cancer Res **14** 2690-2695. **Iorio, MV, R Visone, et al**. 2007. Microrna signatures in human ovarian cancer. Cancer Res **67** 8699-8707. **Gammell, P, N Barron, et al**. 2007. Initial identification of low temperature and culture stage induction of mirna expression in suspension cho-k1 cells. J Biotechnol **130** 213-218. **Taylor, DD, and C Gercel-Taylor**. 2008. Microrna signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol **110** 13-21.
Reproductive Tract Cancer/ Disease	**Pan, Q, X Luo, and N Chegini**. 2008. Differential expression of microrna in myometrium and leiomyomas and regulation by ovarian steroids. J Cell Mol Med **12** 227-240. **Lui, WO, N Pourmand, et al**. 2007. Patterns of known and novel small rnas in human cervical cancer. Cancer Res **67** 6031-6043. **Wang, T, X Zhang, et al**. 2007. A micro-rna signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. Genes Chromosomes Cancer **46** 336-347. **Wang, X, S Tang, et al**. 2008c. Aberrant expression of oncogenic and tumor-suppressive microrna in cervical cancer is required for cancer cell growth. PLoS ONE **3** e2557.
Breast Cancer	**Yang, Y, R Chaerkady, et al**. 2009. Identification of mir-21 targets in breast cancer cells using a quantitative proteomic approach. Proteomics **9** 1374-1384. **Iorio, MV, M Ferracin, et al**. 2005. Microrna gene expression deregulation in human breast cancer. Cancer Res **65** 7065-7070. **Frankel, LB, NR Christoffersen, et al**. 2008. Programmed cell death 4 (pdcd4) is an important functional target of the microrna mir-21 in breast cancer cells. J Biol Chem **283** 1026-1033. **Qian, B, D Katsaros, L Lu, et al**. 2008. High mir-21 expression in breast cancer associated with poor disease-free survival in early stage disease and high tgf-beta1. Breast Cancer Res Treat **Yan, LX, XF Huang, et al**. 2008. Microrna mir-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA **14** 2348-2360. **Davoren, PA, RE McNeill, et al**. 2008. Identification of suitable endogenous control genes for microrna gene expression analysis in human breast cancer. BMC Mol Biol **9** 76. **Boggs, RM, ZM Wright, et al**. 2008. Microrna expression in canine mammary cancer. Mamm Genome **19** 561-569. **Bu, Y, C Lu, et al**. 2009. Knockdown of dicer in mcf-7 human breast carcinoma cells results in g1 arrest and increased sensitivity to cisplatin. Oncol Rep **21** 13-17. **Du, J, S Yang, et al**. 2009. Bmp-6 inhibits microrna-21 expression in breast cancer through repressing deltaef1 and ap-1. Cell Res **19** 487-496. **Haverty, PM, J Fridlyand, et al**. 2008. High-resolution genomic and expression analyses of copy number alterations in breast tumors. Genes Chromosomes Cancer **47** 530-542. **Huang, GL, XH Zhang, et al**. 2008. [expression of microrna-21 in invasive ductal carcinoma of the breast and its association with phosphatase and tensin homolog deleted from chromosome expression and clinicopathologic features]. Zhonghua Yi Xue Za Zhi **88** 2833-2837. **Huang, GL, XH Zhang, et al**. 2009a. Clinical significance of mir-21 expression in breast cancer: Sybr-green i-based real-time rt-pcr study of invasive ductal carcinoma. Oncol Rep **21** 673-679. **Hui, AB, W Shi, et al**. 2009. Robust global micro-rna profiling with formalin-fixed paraffin-embedded breast cancer tissues. Lab Invest **89** 597-606. **Qi, L, J Bart, et al**. 2009. Expression of mir-21 and its targets (pten, pdcd4, tm1) in flat epithelial atypia of the breast in relation to ductal carcinoma in situ and invasive carcinoma. BMC Cancer **9** 163. **Sempere, LF, M Christensen, et al**. 2007. Altered microrna expression confined to specific epithelial cell subpopulations in breast cancer. Cancer Res **67** 11612-11620. **Wickramasinghe, NS, TT Manavalan, et al**. 2009. Estradiol downregulates mir-21 expression and increases mir-21 target gene expression in mcf-7 breast cancer cells. Nucleic

	Acids Res **37** 2584-2595. **Zhu, S, ML Si, et al**. 2007. Microrna-21 targets the tumor suppressor gene tropomyosin 1 (tpm1). J Biol Chem **282** 14328-14336. **Zhu, S, H Wu, et al**. 2008. Microrna-21 targets tumor suppressor genes in invasion and metastasis. Cell Res **18** 350-359.
Prostate Cancer	**Wang, G, Y Wang, et al**. 2008a. Transcription factor and microrna regulation in androgen-dependent and -independent prostate cancer cells. BMC Genomics **9 Suppl 2** S22. **Li, T, D Li, et al**. 2009. Microrna-21 directly targets marcks and promotes apoptosis resistance and invasion in prostate cancer cells. Biochem Biophys Res Commun **383** 280-285. **Siva, AC, LJ Nelson, et al**. 2009. Molecular assays for the detection of micrornas in prostate cancer. Mol Cancer **8** 17.
Colorectal/ Bladder Cancer	**Yantiss, RK, M Goodarzi, et al**. 2009. Clinical, pathologic, and molecular features of early-onset colorectal carcinoma. Am J Surg Pathol **33** 572-582. **Neely, LA, KM Rieger-Christ, et al**. 2008. A microrna expression ratio defining the invasive phenotype in bladder tumors. Urol Oncol **Schetter, AJ, SY Leung, et al**. 2008. Microrna expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA **299** 425-436. **Slaby, O, M Svoboda, et al**. 2007. Altered expression of mir-21, mir-31, mir-143 and mir-145 is related to clinicopathologic features of colorectal cancer. Oncology **72** 397-402. **Asangani, IA, SA Rasheed, et al**. 2008. Microrna-21 (mir-21) post-transcriptionally downregulates tumor suppressor pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene **27** 2128-2136. **Rossi, L, E Bonmassar, and I Faraoni**. 2007. Modification of mir gene expression pattern in human colon cancer cells following exposure to 5-fluorouracil in vitro. Pharmacol Res **56** 248-253.
Brain/ Pituitary Cancers	**Amaral, FC, N Torres, et al**. 2009. Micrornas differentially expressed in acth-secreting pituitary tumors. J Clin Endocrinol Metab **94** 320-323. **Chan, JA, AM Krichevsky, and KS Kosik**. 2005. Microrna-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res **65** 6029-6033. **Corsten, MF, R Miranda, et al**. 2007. Microrna-21 knockdown disrupts glioma growth in vivo and displays synergistic cytotoxicity with neural precursor cell delivered s-trail in human gliomas. Cancer Res **67** 8994-9000. **Chen, Y, W Liu, et al**. 2008b. Microrna-21 down-regulates the expression of tumor suppressor pdcd4 in human glioblastoma cell t98g. Cancer Lett **272** 197-205. **Conti, A, M Aguennouz, et al**. 2009. Mir-21 and 221 upregulation and mir-181b downregulation in human grade ii-iv astrocytic tumors. J Neurooncol **93** 325-332. **Gabriely, G, T Wurdinger, et al**. 2008. Microrna 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol **28** 5369-5380. **Papagiannakopoulos, T, A Shapiro, and KS Kosik**. 2008. Microrna-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. Cancer Res **68** 8164-8172. **Shi, L, Z Cheng, et al**. 2008a. [the mechanism of apoptosis in human u87 glioma cells induced by mir-21 antisense oligonucleotide]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi **25** 497-501. **Shi, L, Z Cheng, et al**. 2008b. Hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. Brain Res **1236** 185-193.
Lung Cancer/ Inflammation	**Markou, A, EG Tsaroucha, et al**. 2008. Prognostic value of mature microrna-21 and microrna-205 overexpression in non-small cell lung cancer by quantitative real-time rt-pcr. Clin Chem **54** 1696-1704. **Moschos, SA, AE Williams, et al**. 2007. Expression profiling in vivo demonstrates rapid changes in lung microrna levels following lipopolysaccharide-induced inflammation but not in the anti-inflammatory action of glucocorticoids. BMC Genomics **8** 240. **Lu, TX, A Munitz, and ME Rothenberg**. 2009a. Microrna-21 is up-regulated in allergic airway inflammation and regulates il-12p35 expression. J Immunol **182** 4994-5002. **Xie, Y, NW Todd, et al**. 2009. Altered mirna expression in sputum for diagnosis of non-small cell lung cancer. Lung Cancer
Heart Failure/ Disease	**Thum, T, C Gross, J Fiedler, et al**. 2008. Microrna-21 contributes to myocardial disease by stimulating map kinase signalling in fibroblasts. Nature **456** 980-984. **Urbich, C, A Kuehbacher, and S Dimmeler**. 2008. Role of micrornas in vascular diseases,

	inflammation, and angiogenesis. Cardiovasc Res **79** 581-588. **Cheng, Y, R Ji, et al**. 2007. Micrornas are aberrantly expressed in hypertrophic heart: Do they play a role in cardiac hypertrophy? Am J Pathol **170** 1831-1840. **Tatsuguchi, M, HY Seok, et al**. 2007. Expression of micrornas is dynamically regulated during cardiomyocyte hypertrophy. J Mol Cell Cardiol **42** 1137-1141. **Cheng, Y, X Liu, et al**. 2009. Microrna-21 protects against the h(2)o(2)-induced injury on cardiac myocytes via its target gene pdcd4. J Mol Cell Cardiol **Roy, S, S Khanna, et al**. 2009. Microrna expression in response to murine myocardial infarction: Mir-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res **82** 21-29.
Viral Induced Cancers	**Tsai, YH, MF Wu, et al**. 2009. The m type k15 protein of kaposi's sarcoma-associated herpesvirus regulates microrna expression via its sh2-binding motif to induce cell migration and invasion. J Virol **83** 622-632. **Cameron, JE, C Fewell, et al**. 2008. Epstein-barr virus growth/latency iii program alters cellular microrna expression. Virology **382** 257-266. **Zhang, Z, Z Li, et al**. 2008b. Mir-21 plays a pivotal role in gastric cancer pathogenesis and progression. Lab Invest **88** 1358-1366. **Connolly, E, M Melegari, et al**. 2008. Elevated expression of the mir-17-92 polycistron and mir-21 in hepadnavirus-associated hepatocellular carcinoma contributes to the malignant phenotype. Am J Pathol **173** 856-864. **Motsch, N, T Pfuhl, et al**. 2007. Epstein-barr virus-encoded latent membrane protein 1 (lmp1) induces the expression of the cellular microrna mir-146a. RNA Biol **4** 131-137.
Skin Cancers	**Bostjancic, E, and D Glavac**. 2008. Importance of micrornas in skin morphogenesis and diseases. Acta Dermatovenerol Alp Panonica Adriat **17** 95-102. **Chang, SS, WW Jiang, et al**. 2008. Microrna alterations in head and neck squamous cell carcinoma. Int J Cancer **123** 2791-2797. **Chang, SS, WW Jiang, et al**. 2008. Microrna alterations in head and neck squamous cell carcinoma. Int J Cancer **123** 2791-2797. **Wong, TS, XB Liu, et al**. 2008. Mature mir-184 as potential oncogenic microrna of squamous cell carcinoma of tongue. Clin Cancer Res **14** 2588-2592. **Zavadil, J, M Narasimhan, et al**. 2007. Transforming growth factor-beta and microrna:Mrna regulatory networks in epithelial plasticity. Cells Tissues Organs **185** 157-161. **Childs, G, M Fazzari, et al**. 2009. Low-level expression of micrornas let-7d and mir-205 are prognostic markers of head and neck squamous cell carcinoma. Am J Pathol **174** 736-745. **Fei, J, F Lan, et al**. 2008. Inhibitory effects of anti-mirna oligonucleotides (amos) on a549 cell growth. J Drug Target **16** 688-693.
Liver Cancer	**Connolly, E, M Melegari, et al**. 2008. Elevated expression of the mir-17-92 polycistron and mir-21 in hepadnavirus-associated hepatocellular carcinoma contributes to the malignant phenotype. Am J Pathol **173** 856-864. **Ladeiro, Y, G Couchy, et al**. 2008. Microrna profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology **47** 1955-1963. **Jiang, J, Y Gusev, et al**. 2008. Association of microrna expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. Clin Cancer Res **14** 419-427. **Meng, F, R Henson, et al**. 2007. Microrna-21 regulates expression of the pten tumor suppressor gene in human hepatocellular cancer. Gastroenterology **133** 647-658. **Kutay, H, S Bai, et al**. 2006. Downregulation of mir-122 in the rodent and human hepatocellular carcinomas. J Cell Biochem **99** 671-678. **Meng, F, R Henson, et al**. 2006. Involvement of human micro-rna in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology **130** 2113-2129. **Gramantieri, L, F Fornari, et al**. 2008. Microrna involvement in hepatocellular carcinoma. J Cell Mol Med **12** 2189-2204. **Selaru, FM, AV Olaru, et al**. 2009. Microrna-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3. Hepatology **49** 1595-1601.
Pancreatic/	**Zhang, Z, Z Li, et al**. 2008b. Mir-21 plays a pivotal role in gastric cancer pathogenesis and

Gastric Cancers	progression. Lab Invest **88** 1358-1366. **Chan, SH, CW Wu, et al**. 2008. Mir-21 microrna expression in human gastric carcinomas and its clinical association. Anticancer Res **28** 907-911. **Dillhoff, M, J Liu, et al**. 2008. Microrna-21 is overexpressed in pancreatic cancer and a potential predictor of survival. J Gastrointest Surg **12** 2171-2176. **Lee, EJ, Y Gusev, et al**. 2007. Expression profiling identifies microrna signature in pancreatic cancer. Int J Cancer **120** 1046-1054. **Guo, J, Y Miao, et al**. 2009a. Differential expression of microrna species in human gastric cancer versus non-tumorous tissues. J Gastroenterol Hepatol **24** 652-657. **Habbe, N, JB Koorstra, et al**. 2009. Microrna mir-155 is a biomarker of early pancreatic neoplasia. Cancer Biol Ther **8** 340-346. **Moriyama, T, K Ohuchida, et al**. 2009. Microrna-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. Mol Cancer Ther **Roldo, C, E Missiaglia, et al**. 2006. Microrna expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J Clin Oncol **24** 4677-4684. **Zhang, S, X Cai, et al**. 2008a. Effect of trichostatin a on viability and microrna expression in human pancreatic cancer cell line bxpc-3. Exp Oncol **30** 265-268. **Zhu, Z, W Gao, et al**. 2009. Genetic variation of mirna sequence in pancreatic cancer. Acta Biochim Biophys Sin (Shanghai) **41** 407-413.
Blood/ Lymphatic Cancers	**Pichiorri, F, SS Suh, et al**. 2008. Micrornas regulate critical genes associated with multiple myeloma pathogenesis. Proc Natl Acad Sci U S A **105** 12885-12890. **Wang, M, LP Tan, et al**. 2008b. Mirna analysis in b-cell chronic lymphocytic leukaemia: Proliferation centres characterized by low mir-150 and high bic/mir-155 expression. J Pathol **215** 13-20. **Fulci, V, S Chiaretti, et al**. 2007. Quantitative technologies establish a novel microrna profile of chronic lymphocytic leukemia. Blood **109** 4944-4951. **Lawrie, CH, S Gal, et al**. 2008. Detection of elevated levels of tumour-associated micrornas in serum of patients with diffuse large b-cell lymphoma. Br J Haematol **141** 672-675. **Lawrie, CH, S Soneji, et al**. 2007. Microrna expression distinguishes between germinal center b cell-like and activated b cell-like subtypes of diffuse large b cell lymphoma. Int J Cancer **121** 1156-1161. **Chen, A, M Luo, et al**. 2008a. Complementary analysis of microrna and mrna expression during phorbol 12-myristate 13-acetate (tpa)-induced differentiation of hl-60 cells. Biotechnol Lett **30** 2045-2052. **Gibcus, JH, LP Tan, et al**. 2009. Hodgkin lymphoma cell lines are characterized by a specific mirna expression profile. Neoplasia **11** 167-176. **Loffler, D, K Brocke-Heidrich, et al**. 2007. Interleukin-6 dependent survival of multiple myeloma cells involves the stat3-mediated induction of microrna-21 through a highly conserved enhancer. Blood **110** 1330-1333. **Pichiorri, F, SS Suh, et al**. 2008. Micrornas regulate critical genes associated with multiple myeloma pathogenesis. Proc Natl Acad Sci U S A **105** 12885-12890. **Robertus, JL, G Harms, et al**. 2009. Specific expression of mir-17-5p and mir-127 in testicular and central nervous system diffuse large b-cell lymphoma. Mod Pathol **22** 547-555. **Velu, CS, AM Baktula, and HL Grimes**. 2009. Gfi1 regulates mir-21 and mir-196b to control myelopoiesis. Blood **113** 4720-4728.
Misc. Cancers/ Solid Tumors/ Diseases	**Pezzolesi, MG, P Platzer, et al**. 2008. Differential expression of pten-targeting micrornas mir-19a and mir-21 in cowden syndrome. Am J Hum Genet **82** 1141-1149. **Feber, A, L Xi, et al**. 2008. Microrna expression profiles of esophageal cancer. J Thorac Cardiovasc Surg **135** 255-260; discussion 260. **Volinia, S, GA Calin, et al**. 2006. A microrna expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A **103** 2257-2261. **Gao, J, TT Yang, et al**. 2007. [cloning and identification of microrna from human osteosarcoma cell line sosp-9607]. Ai Zheng **26** 561-565. **Ji, R, Y Cheng, et al**. 2007. Microrna expression signature and antisense-mediated depletion reveal an essential role of microrna in vascular neointimal lesion formation. Circ Res **100** 1579-1588.

	Lin, Y, X Liu, et al. 2009. Involvement of micrornas in hydrogen peroxide-mediated gene regulation and cellular injury response in vascular smooth muscle cells. J Biol Chem **284** 7903-7913. **Sathyan, P, HB Golden, and RC Miranda**. 2007. Competing interactions between micro-rnas determine neural progenitor survival and proliferation after ethanol exposure: Evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. J Neurosci **27** 8546-8557. **Shen, YL, YG Jiang, et al**. 2009. Microrna expression profiles and mir-10a target in anti-benzo[a] pyrene-7, 8-diol-9, 10-epoxide-transformed human 16hbe cells. Biomed Environ Sci **22** 14-21. **Zhang, Z, H Peng, et al**. 2009. Microrna-21 protects from mesangial cell proliferation induced by diabetic nephropathy in db/db mice. FEBS Lett

cancers and diseases, miR-21 has become a target for therapeutic intervention (Tong and Nemunaitis, 2008), and is currently one of the most studied miRNAs.

6. Study Significance

Most individuals envision parenthood as an exciting part of their future. However, for seven million couples a year in the US who suffer from infertility, this future may seem impossible. Infertility is a $2 billion/year business, and causes emotional distress for the individuals involved (Albers, 1999). The number of couples suffering from primary infertility has drastically increased from 500,000 a year in 1965 to 7.7 million a year in 2006 (Chandra and Stephen, 1998; Nachtigall, 2006; Stephen and Chandra, 1998). A rise in the average age of the mother and increases in sexually transmitted diseases that affect fertility are the main causes for increased infertility (Chandra and Stephen, 1998; Stephen and Chandra, 1998). Therefore, the medical community is dealing with a growing number of infertile patients that are desperate for a solution.

On the opposite side is contraception. Almost all women desire to delay/prevent pregnancy at some point in their reproductive years. Half of all pregnancies in the United States are unplanned, and half of these unplanned pregnancies result in abortion (Henshaw, 1998). If unplanned pregnancies in the United States was reduced by just half, the number of abortions performed would decrease by 800,000 a year (Brown, 1995). In addition, children born from unplanned pregnancies are more likely to have low birth weight, increased mortality in their first year of life, and their parents are more likely to suffer from depression,

economic and marital problems (Brown, 1995). Current contraceptive choices include barrier methods, intrauterine devices, spermicides, natural family planning, sterilization, and steroid formulations. Most of these methods are not user-friendly, and hormonal contraceptives can have negative side effects and have a discontinuation rate of almost 50% after one year (Strauss and Kafrissen, 2004). Therefore, it is vital that new contraceptive choices be introduced that are user-friendly, have few side effects, and promote a high continuation rate.

The goal of this study was to further our understanding of the molecular networks that occur during ovulation and luteinization so that we can better understand how to treat infertility as well as derive new mechanisms for controlling fertility in women. Specifically, one microRNA, miR-21, was identified as upregulated in granulosa cells following LH, and thus this work further characterizes its expression after LH and explores its function both *in vitro* and *in vivo*.

In the first study, **"Rapid Effects of Luteinizing Hormone on Transcriptional Gene Expression in the Mural Granulosa Cells of Mouse Periovulatory Follicles"**, gene expression was examined immediately (1 h) after the LH surge. This study provided a novel list of genes regulated by LH, and identified a novel EGF-like factor as promoting cumulus expansion following LH. In addition, several transcription factors were identified for the first time as upregulated following LH, and further analysis of these factors will give valuable insight into the molecular pathways activated by LH in granulosa cells.

In the second study, **"MicroRNA-21 is induced by LH and blocks apoptosis in granulosa cells"**, we examined the expression and function of miR-21 after LH *in vivo*. After showing that miR-21 is increased following LH, we used an *in vitro* model of cultured granulosa cells to examine the function of miR-21, and by inhibiting miR-21 function we found that miR-21 is necessary to prevent granulosa cell apoptosis. Furthermore, *in vivo* knockdown of miR-21 increased granulosa cell apoptosis.

In the third study, **"MicroRNA-21 enhances global translation"**, we expanded our study on miR-21 function in cultured granulosa cells and found that miR-21 promotes global translation by indirectly affecting the phosphorylation of elongation factor 2 (EF2). This change in EF2 corresponded with a change in activity of the upstream Akt/mTOR pathway. We also showed that changes in EF2 phosphorylation in granulosa cells are seen *in vivo* following hCG administration.

Finally, we summarized what is currently known about the role of miRNA in reproductive tissue and disease in a comprehensive review, **"MicroRNA in the Ovary and Female Reproductive Tract"**.

Therefore, these studies move the field forward by showing that individual miRNA are important to ovarian function. These studies use an *in vivo* knockdown method to demonstrate that miR-21 is necessary for ovarian function, and use an *in vitro* model to assist with the identification of miR-21 function in granulosa cells. This understanding of miR-21 in ovarian function will help unravel the molecular, specifically post-transcriptional, events that are occurring during ovulation and

luteinization. Understanding the molecular mechanisms that underlie these processes will provide important insight into how fertility can be enhanced or inhibited.

VI. Chapter Two:

Rapid Effects of Luteinizing Hormone on Transcriptional Gene Expression in the Mural Granulosa Cells of Mouse Periovulatory Follicles

1. Abstract

Luteinizing hormone (LH) acts on periovulatory granulosa cells by activating the PKA pathway as well as other cell signaling cascades to increase the transcription of specific genes necessary for ovulation and luteinization. Collectively, these cell signaling responses occur rapidly (within minutes), however, presently no high throughput studies have reported changes before 4 h after the LH surge. To identify early response genes that are likely critical for initiation of ovulation and luteinization, mouse granulosa cells were collected before and 1 h after hCG. Fifty-seven gene transcripts were significantly ($p<0.05$) upregulated and 3 downregulated following hCG. Twenty-four of these transcripts were known to be expressed after the LH/hCG surge at later time points, while 36 were unknown to be expressed by periovulatory granulosa cells. Temporal expression of several transcripts, including the transcription factors *Nr4a1*, *Nr4a2*, *Egr1*, *Egr2*, *Btg1*, and *Btg2*, and the EGF-like ligands *Areg* and *Ereg,* were analyzed by quantitative RT-PCR, and their putative roles in granulosa cell function are discussed. Epigen (*Epgn*), another member of the family of EGF-like ligands, was identified for the first time in granulosa cells as rapidly induced by LH/hCG. We demonstrate that *Epgn* initiates cumulus expansion, similar to the other EGF-receptor ligands *Areg* and *Ereg*. These studies illustrate that a number of changes in gene expression occur *in vivo* in response to LH, and that many of the differentially expressed genes are transcription factors that we would predict in turn modulate granulosa cell gene expression to ultimately impact the processes of ovulation and luteinization.

2. Introduction

Luteinizing hormone (LH) activates a number of cellular signaling cascades within the preovulatory granulosa cell, including the canonical PKA pathway (Bachelot and Binart, 2005). These pathways induce changes in gene expression that ultimately cause phenotypic and physiological changes in the cells, culminating in ovulation and luteinization. These molecular changes include the coordinated expression of a number of genes important for, among other events, the shift from estrogen synthesis to progesterone synthesis, the structural remodeling of the follicle, and the maturation of the oocyte. A number of genes known to be critical for ovulation and luteinization have been identified downstream of the LH surge, including early growth regulatory factor-1 (*Egr1*; Espey et al., 2000), CCAAT enhancer binding protein beta (*C/EBPβ;* Sirois and Richards, 1993), cyclin D2 (Sicinski et al., 1996), steroid acute regulatory protein (*StAR;* Clark and Stocco, 1995), and progesterone receptor (Park and Mayo, 1991). Disruption of these genes in the mouse leads to either failure to ovulate or luteinization defects (Richards et al., 1998; Sterneck et al., 1997). However, while most of these critical genes have been shown to be upregulated between 2-6 hours after the LH surge, the transcriptional changes occurring in granulosa cells in the moments immediately (within 1h) after the LH surge are not well defined.

Global gene expression analyses at later time points after LH have yielded a wealth of information about genes that were not known to be involved in ovarian function. For example, Espey and co-workers (2002) have linked the expression of

many genes previously unknown to be regulated by LH in the rat ovary through the use of RT-PCR differential display (Espey and Richards, 2002). They identified the EGF-like ligand, epiregulin (*Ereg*), as being induced by LH, and subsequent studies have demonstrated that *Ereg* and several other EGF-like growth factors, amphiregulin (*Areg*) and betacellulin, are important for oocyte maturation and cumulus expansion (Espey and Richards, 2002; Park et al., 2004). A sample of 20 genes differentially regulated at various time points after LH (2, 4, 8, 12 and 24 hours) in rat whole ovary were reported; however, a comprehensive list has not yet been published (Espey and Richards, 2002). In another study, suppression subtractive hybridization analysis in mice yielded 36 ovary-specific genes, 22 of which were under hormonal regulation, as indicated by their expression 3, 6, 9, 12, 24, and 48h after hCG (Hennebold et al., 2000). McRae et al. (2005) used serial analysis of gene expression (SAGE) in mouse granulosa cells before and 12 h after hCG treatment, and identified four genes not previously known to be expressed in the granulosa cells. Finally, microarray analysis performed before and 6 h after hCG treatment in rat granulosa cells identified several more LH-regulated targets, including cutaneous fatty acid-binding protein, a factor important in lipid metabolism (Leo et al., 2001). Since the advent of global gene expression analysis methods, researchers have primarily focused on time points 3-12 h after the LH surge (Espey and Richards, 2002; McRae et al., 2005; Richards et al., 1998). In the one exception, Kawamura et al (2005) using a very comprehensive array approach identified brain-derived neurotrophic factor as an LH-regulated gene. In 2008 the same array data was used again to identify fractalkine (Zhao et al., 2008),

however, with the exception of these two genes the results of these arrays have yet to be released.

Therefore, to date, no comprehensive gene expression array analysis of the murine periovulatory granulosa cells has been published at any time point earlier than 6 h after LH. However, Northern blot and quantitative RT-PCR analyses of differential display studies and individual gene analysis studies have shown that many of the genes identified at later time points were upregulated earlier. For example, the mRNA levels of the transcription factor *Egr1* and the acute early response gene 5-aminolevulinate synthase increased within 1 h after hCG treatment (Espey et al., 2000; Park et al., 2003). The rapid expression of *Egr1* has been associated with increased transcription of a number of other genes that modulate the ovulatory and luteinization processes, and its importance in these processes is emphasized in *Egr1*-null mice that are infertile due to a lack of both ovulation and luteinization (Richards et al., 2002). This suggests that there is an unexplored realm of LH-regulation that occurs in the early minutes to hours after the LH surge, and these rapidly (and often transiently) upregulated genes may be playing a necessary, as of yet unidentified, role in ovulation and luteinization.

In this report, we utilized microarray gene expression analysis to identify rapidly LH-induced genes in mouse granulosa cells. We anticipate that the genes identified here will be pivotal to understanding the cascades of coordinated gene expression that occur in granulosa cells in response to the LH surge.

3. Experimental Procedures

Animals and Granulosa Cell Isolation

Twenty-one-day old CF-1 female mice were given intraperitoneal injections of 5 IU equine chorionic gonadotropin (eCG) (Sigma) to induce follicular stimulation. After 44 h, mice ovaries were either collected (0 h time point) or the mice were injected with 5 IU of hCG to trigger ovulation and luteinization and the ovaries collected 1 h later for array analysis. For temporal expression analysis, ovaries were collected 44 h after eCG (hereafter referred to as 0 h) or after eCG + hCG for ¼, ½, 1, 2, 4, 6, 8, or 12 h (n=4 for each time point). Granulosa cells were expressed from the ovarian tissue by follicular puncture into ice-cold PBS. The cells were pelleted by centrifugation at 700 x g, then resuspended in either 500 uL of Tri Reagent (Sigma) for array and quantitative RT-PCR analysis, or in 100 uL cell lysis buffer (Cell Signaling) for protein analysis. All procedures involving animals were reviewed and approved by the Internal Animal Care and Use Committee at the University of Kansas Medical Center and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. All experiments were performed using CF-1 female mice from Charles River Laboratories (Wilmington, MA).

RNA Isolation and Amplification

RNA was isolated with Tri Reagent following the manufacturer's protocol (Sigma), yielding >2 ug of total RNA per sample. The quantity and quality of RNA was determined using the Agilent Bioanalyzer 2001 (Agilent Inc., Palo Alto, CA),

and 0.4 ug of the RNA was subjected to two rounds of linear amplification using Gene Chip Eukaryotic Small Sample Target Labeling Assay Version II (Affymetrix).

Microarray Hybridization

Biotin-labeled cRNA was fragmented according to Affymetrix protocols. The fragmented cRNA from each sample was hybridized to individual Affymetrix 430A gene array chips using the GeneChip Fluidics Station 400 protocol (Affymetrix), and the hybridized chips were scanned using the Agilent GeneArray Scanner (Affymetrix). A scaling factor was applied to each chip using the Affymetrix Microarray Suite 5.0 software to normalize the mean raw fluorescence intensity for each chip to an average base-line fluorescence level. To allow for statistical analysis, 4 replicates were completed for both the 0 h samples and 1 h samples.

Gene Expression Analysis

Each transcript was determined to be present or absent in each sample using the statistical expression algorithm of the Affymetrix Microarray Suite 5.0 software package and was identified as expressed if it was present in at least three samples in each group. Genes that showed a statistically different expression pattern between 0 h and 1 h after hCG treatment were determined using the NIA Array Analysis Tool (Sharov, 2005). The statistical relevance of these genes was confirmed using GeneSpring GX (Agilent).

Quantitative RT-PCR

Total RNA (5 ug) isolated from additional granulosa cell samples (n=4) collected from mice at 0, ¼, ½, 1, 2, 4, 6, 8, and 12 h post-hCG were treated with

DNase I (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (Promega) as previously described (Christenson et al., 2001). Quantitative RT-PCR (qRT-PCR) was conducted as previously described (Jabara et al., 2003). Primer sets were designed for each target using Primer Express 2.0 software and qRT-PCR was carried out on an Applied Biosystems HT7900 sequence detector. To account for differences in starting material and RNA isolation, qRT-PCR was also performed on each sample with primers and probe to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems). A standard curve was run in each assay, with an arbitrary value assigned to the highest standard and corresponding values assigned to the subsequent dilutions. Each cDNA sample was run in triplicate, and the relative abundance of each target was divided by the relative abundance of GAPDH to normalize for the amount of starting material. Dissociation analysis was used to confirm the presence of a single transcript and lack of primer dimer amplification in all PCR reactions. Fold changes were calculated by dividing the normalized values of a transcript at a given time point by the normalized value of the transcript at 0h.

Immunoblotting

Granulosa cells were suspended in protein lysis buffer (Cell Signaling) and centrifuged at 16000 x g for 5 minutes to pellet the cellular membrane debris. Supernatants were transferred to a new tube and stored at -80°C until use. An aliquot of the supernatant was used for protein measurement using BSA as the standard. Each protein sample (15 ug) was denatured by diluting the sample 1:2 with sample buffer

(2.8mL distilled water; 1.0mL 0.5M Tris-HCl, pH6.8; 0.8mL glycerol; 1.6mL 10% SDS; 0.4mL 2-β-mercaptoethanol; 0.4mL 0.05% (w/v) bromophenol blue) and heating for 5 minutes at 95°C. The proteins were separated on 12% SDS-PAGE gels in 5X electrode running buffer, pH 8.3 (25mM Tris-HCl, pH 8.3, 192mM glycine, 0.1% (w/v) SDS), then transferred to PVDF membranes (Millipore) in transfer buffer (12mM Tris-HCl, pH 8.3, 96mM glycine, 20% (v/v) methanol). Blots were incubated for 1 h at RT in a 5% milk solution to block nonspecific binding, then blots were incubated overnight at 4°C with either epigen (R&D Systems, AF1127) or actin (Santa Cruz, sc-1616) antibodies. After washing and incubation with the appropriate secondary antibody, protein-antibody complexes were visualized using West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's protocol.

Cumulus Oocyte Complex (COC) Cultures

Ovaries from 19-day old CF-1 mice (Charles River) were injected with 5 IU eCG, and 44 h later the ovaries were isolated and follicles punctured with a 30-gauge needle. COCs were collected into collection media (MEM with Earle's Salts, supplemented with 25 mM HEPES, 0.25 mM sodium pyruvate, 3 mM L-glutamine, and 1 mg/mL BSA), and transferred into 4-well plates (5-7 COCs/well) containing COC expansion media (collection media with 1% FBS). COCs were cultured with one of the following treatments: COC expansion media alone, 1.26 nM EGF, 2 IU FSH, 2 IU LH, 100 nM of control protein (HUTR10002PET1), or 1, 10, or 100 nM of recombinant EPGN. Cumulus expansion was assessed following 16 h of culture using light microscopy. Quantitative analysis of cumulus expansion was determined

using MetaMorph software (Universal Imaging, Media, PA) with COCs (n=5 for each treatment) traced and the area (um^2) under the tracing ("region of interest") determined. Recombinant EPGN was generously provided by Dr. Greg Murison at Genesis Research and Development Corporation of Auckland, New Zealand.

Statistics

Means and standard errors (n=4) were determined for the normalized abundance of each target in the samples, and one-way ANOVA was used to determine whether expression was affected by time (h post-hCG) and followed by post-hoc analysis using Tukey means separation tests were completed to determine differences among means over time. Heterogeneity of variance was tested for using Bartlett's Test, if present the data was log transformed prior to ANOVA. Linear regression analysis comparing microarray and qRT-PCR data was performed to establish correlation coefficients between these methods. Statistical significance was considered as $p<0.05$.

4. Results and Figures

LH/hCG Rapidly Affects Gene Expression in Granulosa Cells

Of the 22,690 genes and ESTs on the Affymetrix microarray chips, 43% were identified as present in at least 3 of the 4 steady state samples for both 0 h and 1 h. The induction ratio for each of the 9756 genes was determined by calculating the ratio of signal detected at 1 h versus that at 0 h following hCG treatment. Seventy-three genes exhibited a statistical difference in total RNA levels between 0 h and 1 h, with

70 genes statistically upregulated 1 h after hCG and 3 genes statistically downregulated (Figure VI-1). However, due to redundancy of the oligonucleotide probes on the 430A chip, only 60 independent genes were differentially regulated (57 upregulated and 3 downregulated). Table VI-1 depicts twenty-four genes that were previously known to be expressed in the ovary, and these genes were grouped by their putative ovarian function based on previous publications (Table VI-1). Table 2 depicts the remaining 36 gene transcripts that were identified for the first time within the ovary, and more specifically within granulosa cells. In Table VI-2 genes were grouped by their molecular function within gene ontology.

Temporal gene expression profiles were determined for a subset of these identified genes using qRT-PCR on samples collected throughout the periovulatory period (0, ¼, ½, 1, 2, 4, 8, and 12 h post-hCG). The correlation coefficient on the array versus the sixteen genes analyzed by qRT-PCR at 1h post-hCG was $r=0.6825$ ($p<0.0001$).

EGF-like Ligands are Rapidly Upregulated After LH/hCG

The genes encoding the epidermal growth factor (EGF)-like ligands, *amphiregulin* (*Areg*), *epiregulin* (*Ereg*), and *epigen* (*Epgn*) were dramatically upregulated 1 h after hCG. *Areg* had the highest induction of all genes present on the array with a 81.2-fold induction 1 h after hCG, while *Ereg* was the third most upregulated transcript with a 19.9-fold induction (Table VI-1). Temporal gene expression analysis by qRT-PCR confirmed the marked upregulation of both *Areg*

(21-fold) and *Ereg* (5.5-fold) at ½ h after hCG, by 1 h *Areg* increased further (133-fold, $p<0.05$) while *Ereg* (27.5-fold) did not increase significantly until 2 h post-hCG (170-fold, $p<0.05$). As reported (Panigone et al., 2008), the expression of *Areg* and *Ereg* after hCG was rapid and transient. The expression of *Areg* (867-fold) and *Ereg* each reached peak levels at 2 h post-hCG before returning to much lower levels (i.e., equivalent to those values seen at ½ h after hCG treatment) at 6 h post-hCG where they remained through 12 h (Figure VI-2A, B; all fold changes are in comparison to levels of transcript detected in 0 h treatment group).

In addition, another member of the EGF-like ligand family, epigen (*Epgn)*, was shown for the first time to be expressed by granulosa cells. Microarray data indicated a 2.6-fold upregulation 1 h after hCG (Table VI-2), while qRT-PCR indicated a 14-fold increase at 1 h post-hCG. Quantitative RT-PCR confirmed that the expression of *Epgn*, like that of *Areg* and *Ereg*, was rapid and transient with mRNA levels peaking at 2 h post-hCG (28-fold, $p<0.05$; Figure VI-2), before returning to lower levels at 6 h post-hCG. Western blot analysis of mural granulosa cells for EPGN

FIGURE VI-1. Sixty genes were differentially expressed between 0 and 1h post-hCG.

Genes present in 3 of the 4 microarrays are categorized with the averaged log intensity for all the steady state transcripts along the horizontal axis, and the log ratio of signal intensity for each gene between 0 and 1 hour on the vertical axis (NIA Array Analysis). Upregulated genes are indicated as red spots and downregulated genes as green spots.

Figure VI-1

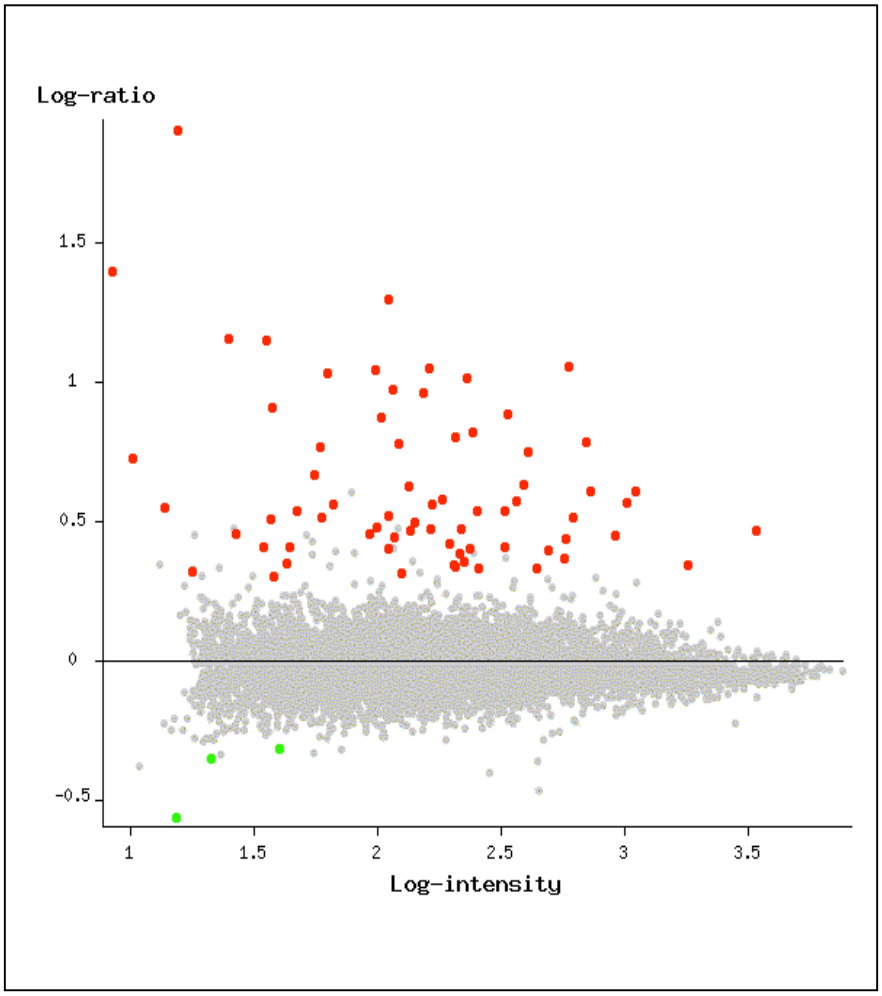


TABLE VI-1. Previously identified ovarian genes differentially expressed* in ovarian granulosa cells 1h after hCG.

Ovarian Function	Gene Symbol (Full Name)	Fold Change (1h after hCG)
COC expansion/oocyte maturation	***Areg*** *(Amphiregulin)*	81.2
	Ereg *(Epiregulin)*	19.9
	Hspa1a/b *(Heat Shock Protein 70 a and b)*	3.0
	Rhob *(Ras Homolog Gene Family B)*	2.6
	GDNF *(Glial Cell Derived Neurotrophic Factor)*	2.5
	Ifrd1 *(interferon-related developmental factor 1)*	2.3
Angiogenesis	***F3*** *(coagulation factor 3)*	14.3
Steroidogenesis	***Klf4*** *(Kruppel like factor 4)*	5.9
	Cyp19a1 *(Cytochrome p450 19a1)*	4.1
Follicular Rupture	***ADAMTS1*** *(A disintegrin and metalloproteinase 1)*	3.8
Luteinization	***Btg2*** *(B-cell translocation 2)*	7.7
	C-Fos	6.1
Broad Ovarian Functions	***Ptgs2*** *(Prostaglandin Endoperoxide Synthase 2)*	24.8
	Crem *(cAMP Response Element Modulator)*	10.4
	Egr1 *(Early Growth Response 1)*	4.0
	Nr4a2 *(Nuclear Receptor 4a2)*	3.3
	KitL *(Kit Ligand)*	2.1
Present, but Undescribed Ovarian Function	***Gadd45b*** *(Growth Arrest and Damage Inducible, beta)*	11.2
	Junb *(Jun B proto-oncogene)*	9.6
	Adrb1 *(Adrenergic beta-1 Receptor)*	5.4
	Ier3 *(Immediate Early Response 3)*	4.2
	Enc1 *(Ectodermal Neural Cortex 1)*	3.7
	Gsn *(Gelsolin)*	3.5
	Dusp6 *(Dual Specificity Phosphatase 6)*	2.5

*Genes significantly up or downregulated ($p<0.05$; NIA Array Analysis) 1h after hCG and previously shown to be present in the ovary, categorized based on described ovarian function.

TABLE VI-2. Newly identified ovarian genes differentially expressed* in ovarian granulosa cells 1h after hCG.

Function	Gene Symbol (Full name)	Fold Change (1h after hCG)
Nucleotide Binding	***Egr2*** *(Early Growth Response 2)*	14.3
	Nr4a1 *(Nuclear Receptor 4a2)*	11.3
	Plk2 *(Polo-Like Kinase 2)*	9.2
	Atf3 *(Activating Transcription Factor 3)*	8.1
	Zfp36 *(Zinc Finger Protein 36)*	3.3
	Za20d2 *(Zinc Finger A20 Domain 2)*	3.2
	Ell2 *(Elongation Factor, RNA Polymerase 2)*	3.2
	Hey1 *(Hairy/Enhancer of Split Related with YRPW Motif 1)*	2.8
	Bhlhb2 *(Basic Helix-Loop-Helix Domain Containing, class B, 2)*	2.8
	Ccnl1 *(Cyclin L1)*	2.5
	Btg1† *(B-cell Translocation Gene 1)*	2.5
	Sf3b1 *(Splicing Factor 3b, subunit 1)*	2.0
	Hist1h2bp *(Histone Cluster 1, H2bp)*	-2.1
Cell Signaling/Protein Interaction	***Ccrl2*** *(Chemokine receptor-like 2)*	6.0
	Ccng2 *(Cyclin G2)*	2.8
	Epgn *(Epigen)*	2.6
	Btg1† *(B-cell Translocation Gene 1)*	2.5
	Rasd1 *(RAS, Dexamethasone-induced 1)*	2.2
Chemical/Ion Binding, Transport	***Egln3*** *(Egl Nine Homolog 3)*	3.5
	Npn3 *(Neoplastic Progression 3)*	3.0
	Slc16a1 *(Solute Carrier Family 16, member 1)*	2.2
Enzymatic/Kinase Activity	***Hs3st1*** *(Heparin Sulfate 3-O-Sulfotransferase 1)*	3.0
	Snf1lk *(Salt Inducible Kinase 1)*	2.3
	Pim3 *(Pim-3 Oncogene)*	2.2
Unknown	***Ier2*** *(Immediate Early Response 2)*	4.3
	Errfi1 *(ERBB Receptor Feedback Inhibitor 1)*	3.8
	Osgin2 *(Oxidative Stress Induced Growth Inhibitor Family Member 2)*	3.5
	1200016E24Rik	3.3
	Tnfsf11 *(Tumor Necrosis Factor (Ligand) Superfamily Member 11)*	3.0
	Slitl2 *(Slit-like 2)*	2.6
	Cln8 *(Ceroid-Lipofuscinosis, Neuronal 8)*	2.2
	Ddit4 *(DNA-Damage Inducible Transcript 4)*	2.1
	BC023105	2.1
	BC012278	-2.2
	Eml5 *(Echinoderm Microtubule Associated Protein Like 5)*	-3.6

*Genes significantly up or downregulated ($p<0.05$; NIA Array Analysis) 1h after hCG and not previously shown to be present in the ovary, categorized based on gene ontology molecular function († denotes a gene that fits into more than one category).

FIGURE VI-2. Granulosa cell expression of amphiregulin (A; *Areg*), epiregulin (B; *Ereg*), epigen (C; *Epgn*), and ERBB receptor feedback inhibitor 1 (D; *Errfi1*) throughout the periovulatory period (i.e., 0 - 12 h after hCG). Quantitative RT-PCR data are normalized to *Gapdh* expression. Means ± SEM with different superscripts are different ($p<0.05$).

Figure VI-2

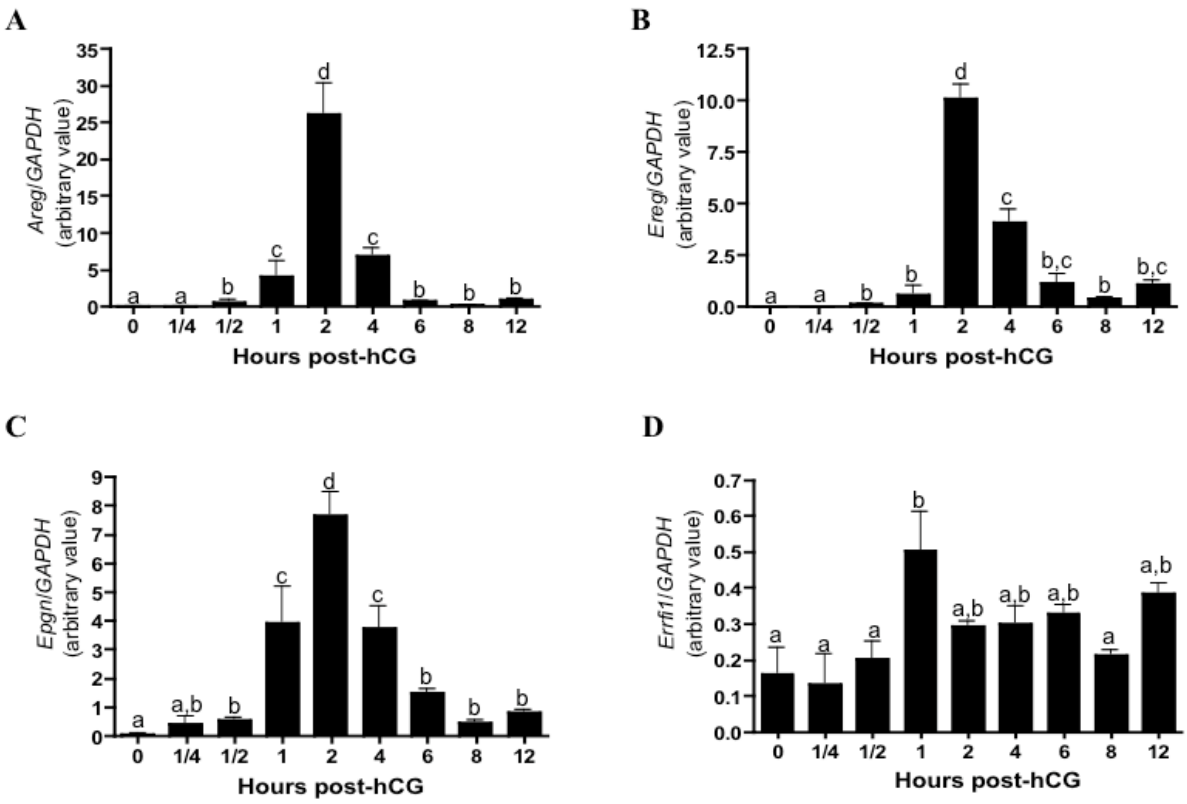
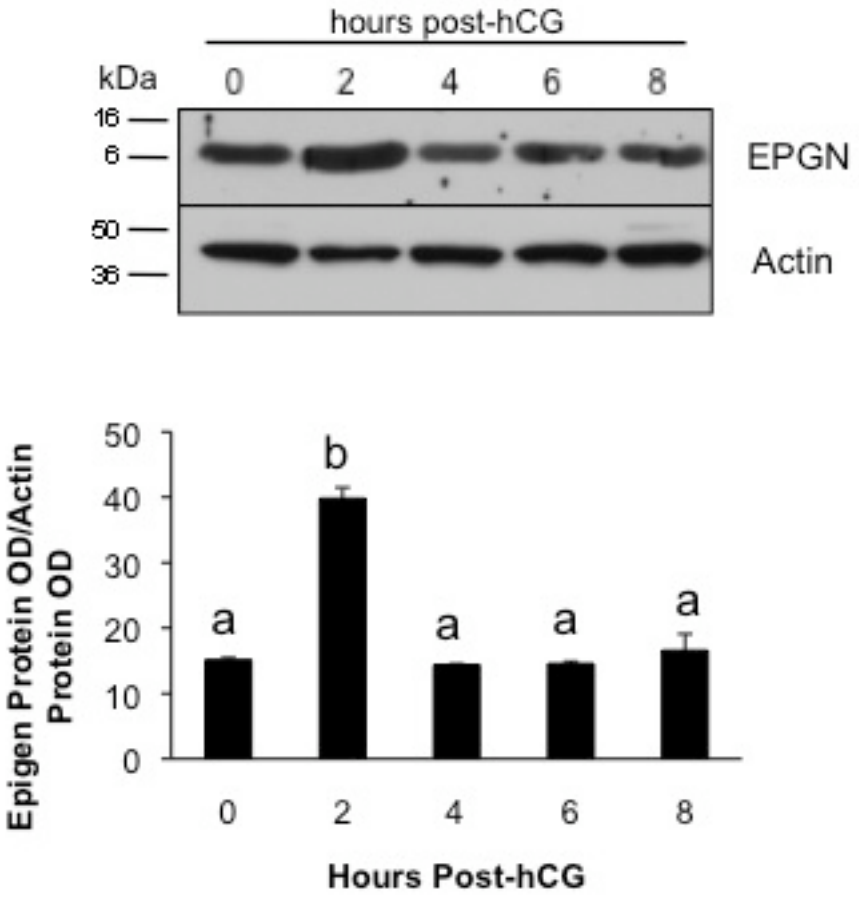

A
Areg/GAPDH (arbitrary value)
Hours post-hCG
B
Ereg/GAPDH (arbitrary value)
Hours post-hCG
C
Epgn/GAPDH (arbitrary value)
Hours post-hCG
D
Errfi1/GAPDH (arbitrary value)
Hours post-hCG
0 1/4 1/2 1 2 4 6 8 12

FIGURE VI-3. Representative EPGN Western blot of granulosa cell protein extracted from PMSG-primed ovaries at 0-8h after hCG injection, with control *Actin* below. Histogram depicts the levels of EPGN after normalization to Actin. Means ± SEM with different superscripts are different ($p<0.05$).

Figure VI-3


hours post-hCG
kDa
0
2
4
6
8
16
6
50
36
EPGN
Actin
Epigen Protein OD/Actin Protein OD
50
40
30
20
10
0
a
b
a
a
a
0
2
4
6
8
Hours Post-hCG

confirmed the presence of a rapid and transient increase (~2.6-fold) in protein expression 2 h post-hCG followed by a return to basal levels by 4 h post-hCG (Figure VI-3). Lastly, we identified that the ERBB receptor feedback inhibitor 1 (Errfi1; a.k.a., Mig6, RALT, Gene 33) was upregulated 3.8-fold in granulosa cells by microarray (Table VI-2) and 2.3-fold by qRT-PCR at 1 h post-hCG. This was the only time point that Errfi1 expression in granulosa cells was significantly different from all the other time points.

Since AREG and EREG have previously been demonstrated to stimulate cumulus expansion in response to LH, we hypothesized that EPGN might have the same effect. To test this hypothesis, we treated isolated cumulus-oocyte-complexes (COCs) with 1, 10, or 100 nM of EPGN. Like the positive controls EGF (Figure VI-4D), FSH (Figure VI-4E), which are known to cause cumulus expansion (Conti et al., 2006), EPGN significantly increased cumulus expansion compared to that of the negative controls (media alone; Figure VI-4B) or a control unrelated protein (Figure VI-4C). We observed a dose response effect of EPGN on cumulus expansion with the intermediate and highest dose causing expansion similar to that seen with positive controls (Figure VI-4G, H, I). Quantitative assessment of cumulus expansion indicated that COCs maintained for 16 h in cumulus expansion medium containing 1% FBS exhibited no change in expansion (Figure VI-5). Similarly, control protein and LH treated COCs showed no significant expansion (Figure VI-5) compared to the 16 h media alone controls. In contrast, EGF, FSH and the intermediate and high doses of EPGN treated COCs exhibited significant increases in COC expansion (Figure VI-5).

FIGURE IV-4. Isolated cumulus-oocyte complexes directly after isolation (A) or 16 h after incubation with media alone (B), 100nm control protein (C), 1.65 nM EGF (D), 2 IU FSH (E), 2 IU LH (F), 1 nM EPGN (G), 10 nM EPGN (H), or 100 nM EPGN (I). The bar represents 100 um.

Figure VI-4

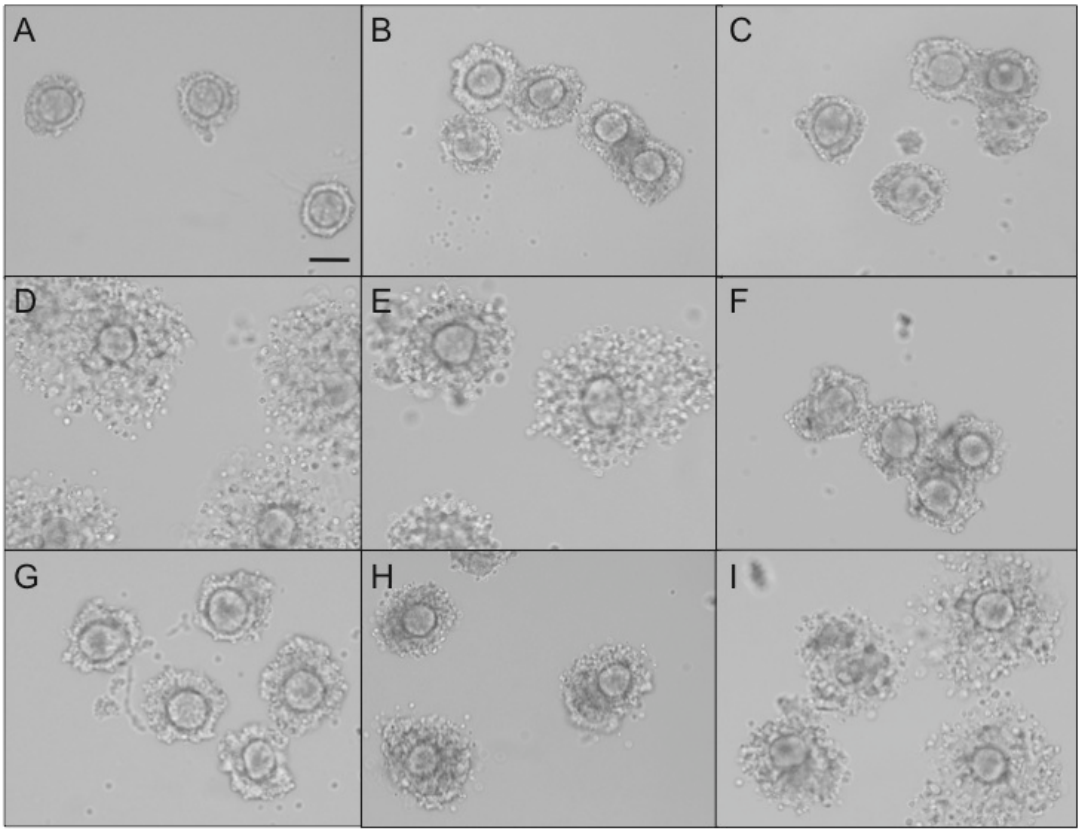
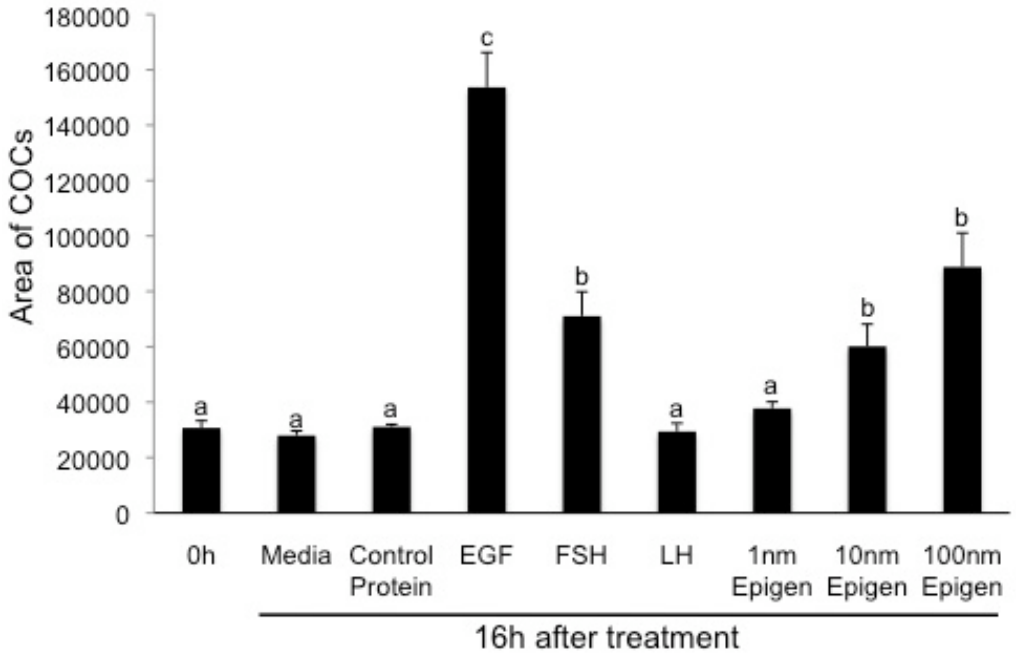


FIGURE VI-5. Quantification of COC expansion (area of the COCs, um^2) directly after isolation (0 h) or 16 h after incubation with media alone, 100 nM control protein, 1.65 nM EGF, 2 IU FSH, 2 IU LH, 1 nM EPGN, 10 nM EPGN, or 100 nM EPGN. One-way Anova was used to determine statistical differences between treatments. Means ± SEM with different superscripts are different ($p<0.05$).

Figure VI-5


Area of COCs
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
a
a
a
c
b
a
a
b
b
0h
Media
Control Protein
EGF
FSH
LH
1nm Epigen
10nm Epigen
100nm Epigen
16h after treatment

Transcription Factors and a Cell Signaling Regulator are Rapidly Upregulated After LH/hCG

Microarray analysis indicated that multiple transcription factors were upregulated in response to hCG. Microarray analysis indicated that B-cell translocation 1 (*Btg1*; Table VI-2) and B-cell translocation 2 (*Btg2*; Table VI-1) were upregulated 2.5- and 7.7-fold, respectively at 1 h post-hCG. Quantitative RT-PCR indicated that the expression of both *Btg1* and *Btg2* were significantly increased within 30 min after hCG (15-fold and 12-fold, $p<0.05$), and reached peak levels at 1 h post-hCG (31-fold and 19-fold) before returning to basal levels within 4 h (Figure VI-6A and B).

Early growth response 1 (*Egr1*) and early growth response 2 (*Egr2*) were upregulated 4- and 14.3-fold, respectively, in granulosa cells 1 h after hCG by microarray analysis (Tables VI-1 and VI-2). Temporal expression analysis using qRT-PCR indicated that their expression was also transient, with expression of both increased ($p < 0.05$) within 30 min and reached their maximal values 1 h post-hCG (45-fold and 59-fold, $p<0.05$) where it remained until returning to basal levels at 4 h post-hCG (Figure VI-6C and D). The nuclear receptor subfamily 4 group A member 1 (*Nr4a1*) and *Nr4a2* transcription factors were also identified as significantly induced 1 h after hCG administration, with increases of 11.3- and 3.3-fold, respectively (Tables VI-1 and VI-2). Quantitative RT-PCR indicated both *Nr4a1* and *Nr4a2* were increased ($p<0.05$) within 15 minutes of hCG (17- and 19-fold, respectively), before reaching their highest levels (153- and 172-fold,respectively), before returning to basal levels by 6 h after hCG (Figure VI-7A and B). Hairy enhancer of split-related with YRPW motif 1 (*Hey1)* was upregulated 11.2 fold by microarray analysis (Table

VI-2). Quantitative RT-PCR analysis confirmed the rapid upregulation of the *Hey1* transcript with peak levels of *Hey1* occurring at 2 h post-hCG (10-fold) (Figure VI-7C).

Growth arrest and damage inducible, beta (*Gadd45b*) was upregulated 2.8 fold by microarray analysis (Table VI-1). Quantitative RT-PCR analysis confirmed the upregulation of *Gadd45b* within 30 min (16-fold) where it remained at high levels through 2 h post-hCG before returning to basal levels at 4 h post-hCG (Figure VI-7D).

Steroidogenic, Angiogenic and Inflammatory Genes are Rapidly Upregulated after LH/hCG.

Microarray analysis indicated that cytochrome p450 19a1 (*Cyp19a1*) exhibited a 4.1-fold induction in granulosa cells 1 h after hCG (Table VI-1). Quantitative RT-PCR temporal analysis of *Cyp19a1* expression indicated that this induction (5.5-fold at 30 min) was transient with levels returning to basal levels by 4 h post-hCG (Figure VI-8A). Prostaglandin endoperoxide synthase 2 (*Ptgs2*) expression was upregulated 24.8-fold by microarray analysis at 1 h post-hCG (Table VI-1), however, qRT-PCR indicated a 13-fold increase at 1 h and peak expression at 4 h post-hCG (73.5-fold), before rapidly decreasing to basal levels at 6 h post-hCG (Figure VI-8B).

Kit Ligand (*Kitl*) was upregulated 2.1-fold following hCG by microarray analysis (Table VI-1), while qRT-PCR analysis indicated that it was increased 2-fold at 1 h but did not reach significance until 2 h (4.2-fold; Figure VI-8C). Microarray analysis identified coagulation factor 3 (*F3*) as upregulated 14.3-fold at 1 h post-hCG (Table VI-1). Temporal gene expression analysis

FIGURE VI-6. Expression of *Btg1* (A), *Btg2* (B) *Egr1* (C), and *Egr2* (D) transcripts 0-12h after hCG and 0-4h after hCG (insets) as quantitated by quantitative RT-PCR. Data are normalized to *Gapdh* expression. One-way Anova was used to determine statistical differences between time points; Means ± SEM with different superscripts are different ($p<0.05$).

Figure VI-6

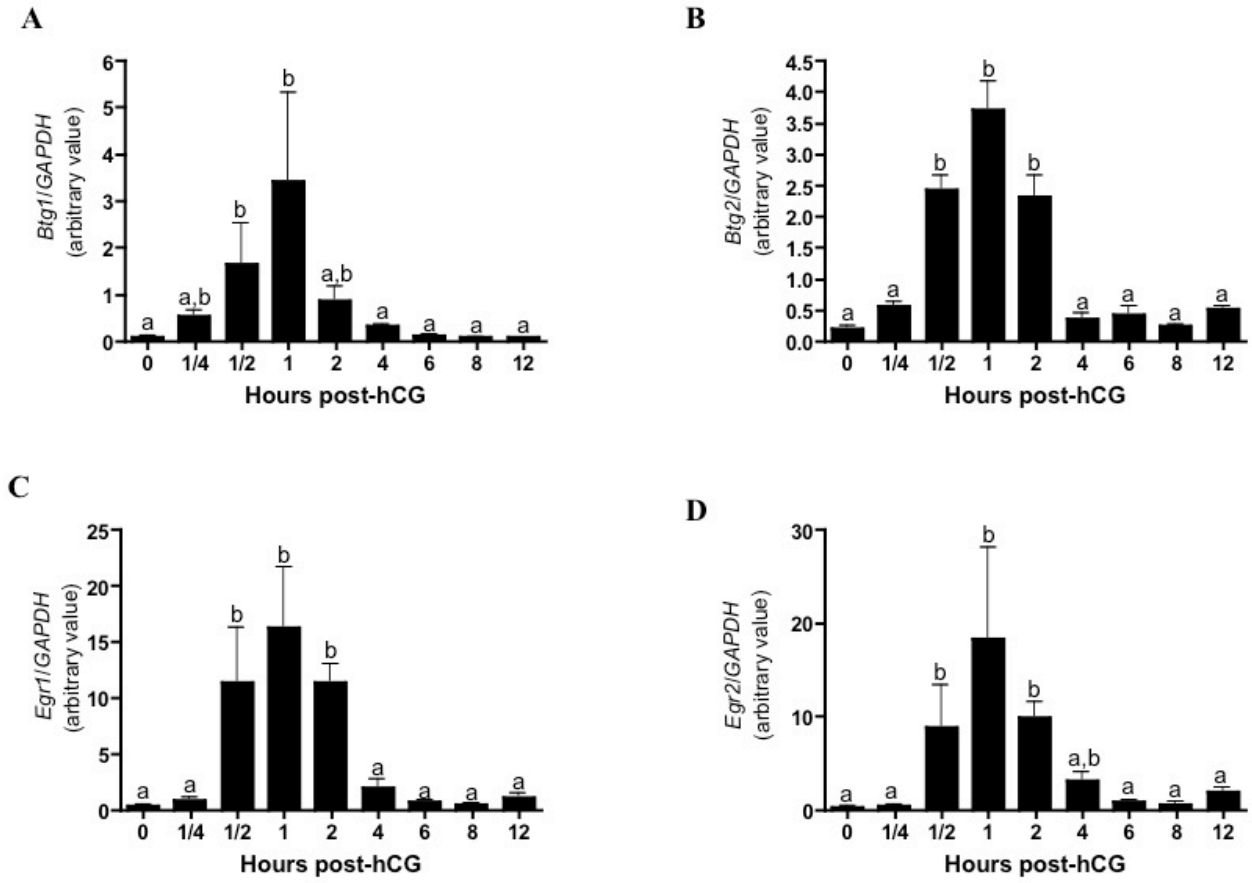

A
Btg1/GAPDH (arbitrary value)
Hours post-hCG
B
Btg2/GAPDH (arbitrary value)
Hours post-hCG
C
Egr1/GAPDH (arbitrary value)
Hours post-hCG
D
Egr2/GAPDH (arbitrary value)
Hours post-hCG
0 1/4 1/2 1 2 4 6 8 12

FIGURE IV-7. Expression of *Nr4a1* (A) and *Nr4a2* (B), *Hey1* (C), and *Gadd45b* (D) transcripts 0-12h after hCG as quantitated by quantitative RT-PCR. Data are normalized to *Gapdh* expression. One-way Anova was used to statistical differences between timepoints; Means ± SEM with different superscripts are different ($p<0.05$).

Figure VI-7

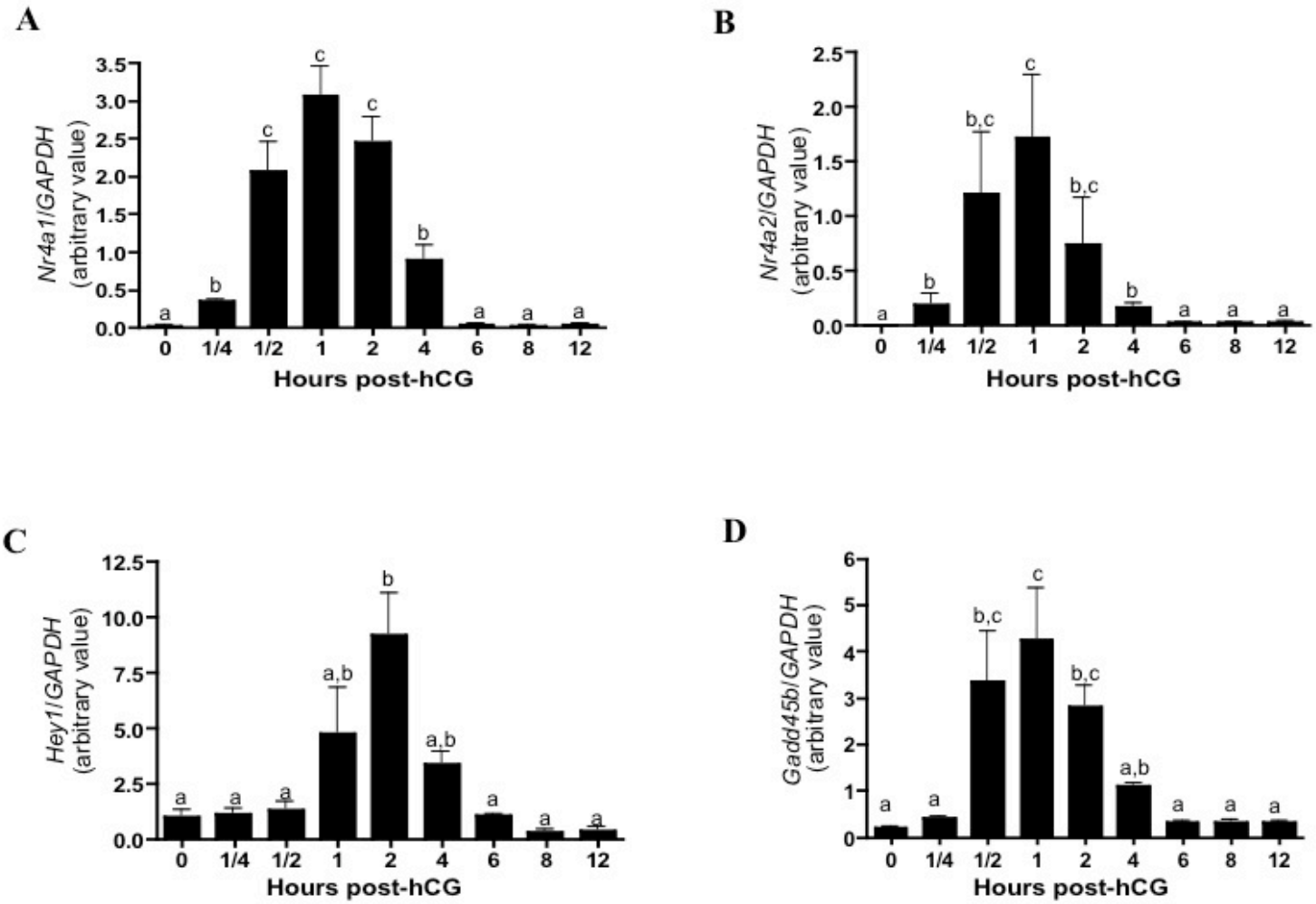
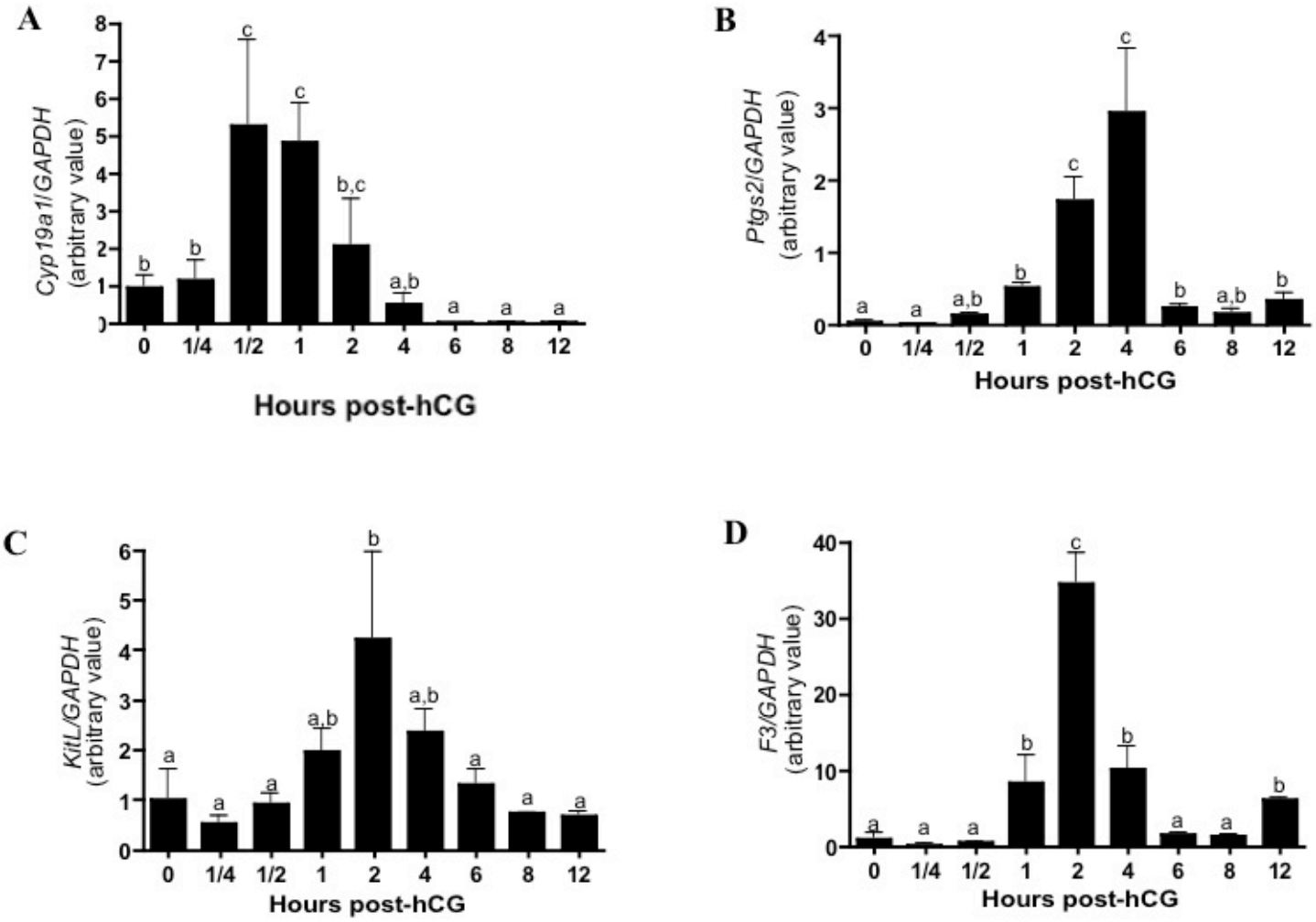

A
Nr4a1/GAPDH (arbitrary value)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a b c c c b a a a
0 1/4 1/2 1 2 4 6 8 12
Hours post-hCG
B
Nr4a2/GAPDH (arbitrary value)
2.5
2.0
1.5
1.0
0.5
0.0
a b b,c c b,c b a a a
0 1/4 1/2 1 2 4 6 8 12
Hours post-hCG
C
Hey1/GAPDH (arbitrary value)
12.5
10.0
7.5
5.0
2.5
0.0
a a a a,b b a,b a a a
0 1/4 1/2 1 2 4 6 8 12
Hours post-hCG
D
Gadd45b/GAPDH (arbitrary value)
6
5
4
3
2
1
0
a a b,c c b,c a,b a a a
0 1/4 1/2 1 2 4 6 8 12
Hours post-hCG

FIGURE VI-8. Expression of *Cyp19a1* (A) and *Ptgs2* (B), *KitL* (C), and *Coagulation factor F3* (D) transcripts 0-12h after hCG as quantitated by quantitative RT-PCR. Data are normalized to *Gapdh* expression. One-way Anova was used to statistical differences between timepoints; Means ± SEM with different superscripts are different ($p<0.05$).

Figure VI-8


A
Cyp19a1/GAPDH
(arbitrary value)
Hours post-hCG
B
Ptgs2/GAPDH
(arbitrary value)
Hours post-hCG
C
KitL/GAPDH
(arbitrary value)
Hours post-hCG
D
F3/GAPDH
(arbitrary value)
Hours post-hCG
0
1/4
1/2
1
2
4
6
8
12

indicated that *F3* was increased 88-fold at 1 h, peak expression occurred at 2 h (269-fold) before returning to basal levels at 6 h post hCG (Figure VI-8D).

5. Discussion

This study is the first to define the changes in gene expression (transcriptome) of mural granulosa cells in the moments immediately (i.e., 1 h) after the LH/hCG surge. Identification of genes that rapidly respond to the ovulatory surge of LH has provided a number of key regulatory genes (transcription factors, growth factors) previously unknown to be expressed within granulosa cells (Table VI-2). These genes may regulate key molecular and cellular events occurring within granulosa cells that facilitate the processes of ovulation (which occurs ~14 h later) and luteinization. In addition, a number of genes already known to be expressed within the ovary (Table VI-1) are now shown to be expressed specifically within granulosa cells of the periovulatory follicle. The temporal expression of genes already implicated in a variety of ovarian processes by granulosa cells specifically supports these earlier observations. Interestingly, the surge of LH/hCG predominantly caused an increase in gene expression with 57 of the 60 genes being upregulated and only 3 significantly down regulated. This fits with most other early (i.e., 4-12 h) ovarian profiling studies, and although about half of the total genes as detected as expressed were below the threshold, few reached significance. These observations would be indicative of a system whereby LH/hCG regulates mostly from a positive (stimulatory) manner the expression of mRNA transcripts associated with ovulation

and luteinization, and not the rapid destruction of specific mRNA transcript(s) to allow these cells to precede to their ultimate final terminal differentiation. However, because only a single very early time point was examined in these studies, it is possible that the loss of mRNA transcripts associated with granulosa cell functions may have not yet occurred, or that loss of these transcripts is gradual or is regulated post-transcriptionally.

Steroidogenic, Angiogenic and Inflammatory Genes

The role of Kit ligand (*Kitl)* in the ovary has been studied extensively. The expression of *Kitl* has been shown to be localized to granulosa cells, and the expression of its receptor is localized to oocytes and theca cells (Doneda et al., 2002; Horie et al., 1991; Keshet et al., 1991), so *Kitl* is most likely important for the granulosa cell – oocyte/thecal cell communication. It has been demonstrated to be important for many aspects of folliculogenesis, including the growth and survival of oocytes (Huang et al., 1993; Thomas and Vanderhyden, 2006), steroidogenesis of theca and granulosa cells (Reynaud et al., 2000), and the proliferation of granulosa cells (Reynaud et al., 2000). Previous studies have shown that *Kitl* expression increases in granulosa cells in response to hCG (Ismail et al., 1996), with levels increasing at 3h post-hCG, the earliest time point examined, and then peaking at 6 h post-hCG. Our results indicate that *Kitl* increases within 1 h, suggesting that its effects on ovarian function could be broader than previously envisioned.

Coagulation factor F3 (*F3*) is a cell surface glycoprotein that is a potent initiator of the coagulation protease cascade. F3 has previously been found in follicular fluid, along with high levels of its inhibitor, tissue factor pathway inhibitor (TFPI*;* Shimada et al., 2001). Surprisingly, there is little information about the role of coagulation during the ovulatory process. In the chicken, coagulation factor XIIIA was identified as secreted from theca cells and acts to stabilize the follicle wall during oocyte growth and may aid in the clotting of small capillaries that are ruptured during ovulation (Recheis et al., 2000). F3 may be playing a similar role in preventing microbleeding during ovulation, especially since it undergoes such a rapidly and dramatic upregulation in response to LH.

It is well established that the LH surge initiates a shift in granulosa cell steroidogenesis. A major player in this shift is the dramatic decrease in the expression of *Cyp19a1* (P450 aromatase), which catalyzes the formation of estrogens from androgens (Hickey et al., 1990; Stocco, 2008). This loss of *Cyp19a1* dramatically reduces the amount of estrogen produced by granulosa cells, and, in combination with an increase in progesterone-producing steroidogenic enzymes (i.e., CYP11A1 and 3βHSD), results in the shift from an estrogen-producing cell to a progesterone-producing cell. Similar to our observations of a rapid and transient increase in *Cyp19a1* expression, Hickney et al. (1988) observed an almost identical pattern in rat granulosa cells following *in vivo* hCG administration. The transient increase in *Cyp19a1* occurring within granulosa cells is likely a hold over effect of pulsatile LH secretion occurring in the later stages of folliculogenesis, however, after

the initial response to the increasing LH, the surge levels of LH induce other cell signaling cascades / transcription factors that ultimately repress *Cyp19a1* expression, leading to the conversion of an estrogen-producing granulosa cell to a progesterone-producing luteal cell.

Prostaglandin-endoperoxide synthase 2 (PTGS2) is an inducible enzyme that catalyzes the key step in the production of prostaglandins from arachidonic acid. Prostaglandins have been shown to be involved in a wide array of ovulatory and luteal events, including follicular rupture, angiogenesis, and progesterone production (Sakurai et al., 2003; Tsafriri, 1996). Targeted deletion of *Ptgs2* confirmed its critical role in the processes of ovulation and luteinization and female fertility (Lim et al., 1997). The expression of *Ptgs2* was previously shown to be rapidly (within 2 h post-hCG) and transiently increased within granulosa cells (Huslig et al., 1987; Sirois et al., 1992). Our study confirms these observations and indicates that *Ptgs2* expression occurs even earlier (at 1 h post-hCG) in granulosa cells.

Transcription Factors

Early growth response 1 (*Egr1)* has previously been demonstrated to be induced by hCG in granulosa cells, and it is hypothesized that this early response gene binds to the promoter of later LH-responsive genes, such as the proteases cathepsin L and ADAMTS-1, thus impacting the structural events that occur during the periovulatory period (Espey et al., 2000; Russell et al., 2003). Studies of *Egr1* knockout mice suggest that it is vital to the processes of ovulation and luteinization

(Topilko et al., 1998). To the best of our knowledge, this is the first study to show expression of *Egr2* in periovulatory follicles and its induction by LH. In adipose tissue, EGR2 has been shown to increase C/EBPβ expression (Chen et al., 2005). In granulosa cells, C/EBPβ plays a necessary role in ovulation and luteinization (Christenson et al., 1999; Sterneck et al., 1997), and future studies may find that EGR2 also stimulates C/EBPβ expression in LH-induced granulosa cells, thus allowing these downstream events to occur. In peripheral nerve cells, EGR2 has been shown to indirectly activate the transcription of genes important for lipid and cholesterol biosynthesis (Leblanc et al., 2005). While *Egr2* has been knocked out in mice, its effect on reproduction has not been reported (Jacquin et al., 1996). Further studies on *Egr1* and *Egr2* will give insight into their regulatory roles that impact ovulation and luteinization.

The transcription factors *Btg1* and *Btg2* were also rapidly upregulated within granulosa cells following hCG administration. *Btg2* is upregulated during differentiation in neuronal cells, and is important for inhibiting proliferation during this process (Montagnoli et al., 1996). Both BTG1 and BTG2 have been shown to play roles in terminal differentiation events, such as the differentiation of hematopoetic cells in response to retinoic acid (Passeri et al., 2006) and myoblast differentiation (Rodier et al., 1999), and both are anti-proliferative (Rouault et al., 1996; Rouault et al., 1992). Whole ovary expression of *Btg2* after hCG adminstration was examined in the rat, and was found to not change, however, the earliest time point examined was 12 h after hCG treatment (Schmidt et al., 2006). This is the first

study to describe the rapid and transient upregulation of *Btg1* and *Btg2* after hCG within granulosa cells, and due to their role in DNA methylation it is interesting to speculate that these two proteins could play a vital role in the terminal differentiation of granulosa cells as they transform into luteal cells.

NR4A1 (NUR77) and NR4A2 (NURR1) are transcription factors that have previously been shown to be upregulated by hCG/LH in the rat and in the human granulosa tumor-like cell line KGN (Park et al., 2003; Wu et al., 2005). Here we show that they are also upregulated in the mouse within 15 minutes of hCG treatment. NR4A1 and NR4A2 are orphan nuclear receptors that generally act as transcriptional activators or repressors, although they can also affect apoptosis by binding to B-cell lymphoma protein 2 in the mitochondria (Li et al., 2006). *Nr4a1* is upregulated in the rat by the actions of LH-induced protein kinase C zeta (Park et al., 2007). NR4A1 binds the *Cyp19a1* (aromatase) promoter to repress its expression, which may explain why *Cyp19a1* expression levels decrease after the LH surge (Wu et al., 2005). Consistent with our array data, the LH/hCG surge indicates that *Cyp19a1* expression increases at ½ h after hCG (Table VI-1 and Figure VI-7A), perhaps before *Nr4a1,* whose transcript is increased at 15 minutes (Figure VI-6A), is able to be converted to protein and subsequently repress *Cyp19a1* expression. NR4A1 upregulates *3β-hydroxysteroid dehydrogenase* expression within granulosa cells and this enzyme is necessary for progesterone synthesis (Havelock et al., 2005). The promoters for several other steroidogenic enzymes, including *Cyp11a1*, *Cyp11b1*, and *Cyp17* have binding sites for NR4A1 and NR4A2 (Morohashi et al., 1992; Park et al., 2003;

Zhang and Mellon, 1997), suggesting they may regulate the changes in steroidogenesis that occur during the differentiation of the granulosa cell to a luteal cell.

Expression of another transcription factor, *Hey1*, was identified as 2.8-fold upregulated within 1 h post-hCG. HEY1 is a transcriptional repressor that is transcriptionally activated by the cell membrane receptor Notch. In response to Notch-mediated induction, HEY1 plays an essential regulatory role in angiogenesis (Fischer et al., 2004). During the follicle to corpus luteum transformation, there is a considerable increase in angiogenesis, resulting in a highly vascularized corpus luteum. While the pro-angiogenic vascular endothelial growth factor (VEGF) has been shown to play an important role in these processes, the Notch pathway, including HEY1, may also play a vital role since here we show it is also upregulated after LH.

Cell Signaling

GADD45b is a direct activator of the MEKK4 kinase, and thus an activator of the p38/JNK pathway. Recent reports have indicated that this pathway is activated in response to LH-induced protein kinase C activation, and culminate in the expression of genes important in ovulation and luteinization (Sriraman et al., 2008b). GADD45b has also been indicated to play a role in inhibiting the cell cycle through its interaction with the Cdk1/cyclinB1 complex, and has been proposed to play a role in terminal differentiation and apoptosis (Abdollahi et al., 1991; Vairapandi et al., 2002;

Zhang et al., 1999). Therefore, GADD45b may be playing an important role in promoting the differentiation of the granulosa cell to the luteal cell.

Growth Factors

The importance of the EGF-like growth factors, AREG and EREG in cumulus expansion and oocyte maturation are established (Conti et al., 2006; Shimada et al., 2006). These two proteins are released from mural granulosa cells in response to LH and are postulated to then act on the LH receptor-deficient cumulus cells and the oocyte to initiate oocyte maturation and cumulus expansion (Conti et al., 2006). In our study, *Areg* had the highest upregulation among the genes expressed in granulosa cells with a 133-fold observed increase 1 h after hCG. Amphiregulin has previously been shown to be highly upregulated at 4 h post-hCG, and remained elevated (compared to 0 h) through 12 h post-hCG by semi-quantitative RT-PCR analysis (Shimada et al., 2006). While our results confirm this marked upregulation at 4 h it points to even a greater increase (>800-fold) at 2 h post-hCG. Our quantitative RT-PCR analysis indicated that, like Shimada et al., (2006), amphiregulin levels remained above basal levels at 8 and 12 h post-hCG.

Similar to granulosa *Areg* expression, *Ereg* and *Epgn*, two other EGF-like ligands, also exhibited robust and transient increases in expression in response to the LH/hCG surge. *Ereg* expression has previously been examined in granulosa cells (Shimada et al., 2006), this however is the first report of *Epgn* expression with ovarian tissue. Phylogenetically, *Epgn* is most related to *Ereg*, although it has lower

affinity than EREG or AREG for their common receptor ERBB1 (Kochupurakkal et al., 2005). Its lower affinity, however, makes it a more potent mitogen, in that the ERBB1 receptor is less likely to be downregulated and inactivated in response to the low affinity binding of EPGN (Kochupurakkal et al., 2005). EPGN has been shown to stimulate epidermal cell proliferation *in vitro* and induce differentiation of mammary, prostate, pheochromocytoma and endothelial cells (Strachan et al., 2001). We hypothesize that EPGN, as another member of the EGF-like ligands, is important for cumulus cell function. To test this hypothesis, we treated cumulus-oocyte-complexes (COCs) with EPGN and found an increase in cumulus cell expansion (Figure VI-4 and VI-5). While knockout *Areg* or *Ereg* mice do have delayed cumulus expansion, they remain fertile, indicating that these genes can compensate for each other (Hsieh et al., 2007; Lee et al., 2004a; Luetteke et al., 1999; Panigone et al., 2008). Here evidence is provided that indicates EPGN may be able to replace AREG or EREG function. In addition because of its unique biochemical properties EPGN may be critical for maintaining cellular signaling as its lower affinity would not cause down regulation of the EGF receptor protein.

In addition to identification of the novel EGF-ligand, EPGN, within granulosa cells, we also identified ERBB receptor feedback inhibitor 1 (*Errfi1*; a.k.a., Mig6, RALT, Gene 33) as upregulated in granulosa cells. ERRFI1 binds to the activating kinase domain of the EGF receptor, thus preventing its activity (Ferby et al., 2006; Zhang et al., 2007). The increase in this inhibitor of EGF receptor signaling suggests

that signaling through this pathway is much more dynamically regulated than previously envisioned.

In conclusion, microarray analysis of mouse granulosa cells before and 1 h after hCG has allowed for the global identification of rapidly differentiated genes in response to LH. The protein product of one upregulated gene, the EGF-like ligand, EPGN, was shown to stimulate cumulus expansion, and thus can join the other members of the EGF-like ligands, EREG, AREG, and betacellulin (BTC) in initiating/modulating the cumulus expansion process in response to LH. These results allow a better understanding of the rapid molecular events that occur in granulosa cells after LH, and provide a starting point for future research to analyze the roles these newly identified genes play in ovulation and luteinization, and to analyze the early role of genes previously shown to be important in these processes. Further studies are needed to elucidate the role many of these genes play in ovarian biology.

VII. Chapter Three:

MicroRNA-21 is Induced by LH and Blocks Apoptosis in Granulosa Cells

1. Abstract

MicroRNA (miRNA) play important roles in many developmental processes including cell differentiation and apoptosis. Transition of proliferative ovarian granulosa cells to terminally differentiated luteal cells in response to the ovulatory surge of luteinizing hormone (LH) involves rapid and pronounced changes in cellular morphology and function. MicroRNA-21 (miR-21) is one of three LH-induced miRNA in murine granulosa cells, and here we examine the function and temporal expression of miR-21 within granulosa cells as they transition to luteal cells. Quantitative RT-PCR analysis indicates that *in vivo* hCG/LH administration induced granulosa cell pri-miR-21 expression 10-fold within 2 h with mature miR-21 levels peaking ~4 h later. To examine function, granulosa cells were transfected with a miR-21 blocking oligonucleotide (LNA-21) that decreased mature miR-21 levels 27-fold. LNA-21 depletion of miR-21 activity in cultured granulosa cells induced apoptosis (annexin V positive staining, caspase 3 cleavage). LNA-21 treatment showed no changes in global granulosa cell gene expression, indicating that miR-21 likely affects translation and not mRNA degradation. *In vivo* granulosa cell cleaved caspase 3 (i.e., apoptosis) levels decline 6 h after an ovulatory surge of hCG/LH, coincident with the rise in miR-21 expression. Furthermore, ovarian bursal injection of a phosphothioate LNA-21 oligonucleotide increased ovarian apoptosis. A number of miR-21 apoptotic target transcripts have been identified in other systems; currently none appear to play a role in the induction of ovarian granulosa cell apoptosis. This study is the first to

implicate miR-21 (an oncogenic miRNA) as playing a clear physiologic role in ovarian function.

2. Introduction

Luteinizing hormone (LH) acts on ovarian granulosa cells to induce both ovulation and luteinization. The interval between ovarian recognition of the LH surge and follicular rupture (ovulation) is termed the periovulatory period, and lasts ~13-15 h in the mouse. In response to the ovulatory surge of LH, granulosa cells not only stimulate the resumption of oocyte meiosis, cumulus expansion, and breakdown of the follicular wall in preparation for ovulation, but also rapidly differentiate to form luteal cells, the primary endocrine cell in the corpus luteum. Luteinization is a terminal differentiation process and is characterized by the rapid transition in morphology and function of the granulosa cell, with phenotypical changes becoming evident within 5-7 h of the ovulatory surge of LH/hCG (Chaffin and Stouffer, 2002; Oonk et al., 1989). Proper luteinization and sustained progesterone secretion are necessary for maintenance of pregnancy, and thus survival of periovulatory granulosa cells is obligatory for pregnancy. Thus, in addition to its differentiative effects, LH also acts as a survival factor by preventing apoptosis of the differentiating granulosa cells (Chaffin et al., 2001; Robker and Richards, 1998a, b). While several anti-apoptotic LH-induced factors have been identified in periovulatory granulosa cells, including progesterone receptor, pituitary adenylate cyclase-activating polypeptide, and atrial natriuretic peptide, it is expected that a conglomeration of factors are necessary for the molecular changes that need to occur to ensure both the survival and differentiation of these cells (Dineva et al., 2007; Lee et al., 1999; Shao et al., 2006).

MicroRNA (miRNA) are emerging as important mediators of differentiation, proliferation, and apoptotic events (Ademokun and Turner, 2008; Bueno et al., 2008; Chivukula and Mendell, 2008; Gangaraju and Lin, 2009; He et al., 2007; Jovanovic and Hengartner, 2006). Studies have implicated miRNA in the differentiation of mouse embryonic stem cells (Singh et al., 2008), effector and memory T cells (Wu et al., 2007), and many other tissues/cells (Hashimi et al., 2009; Ketting, 2009; Yang et al., 2009a). Recently, conditional-deletion of Dicer within granulosa cells using the anti-Mullerian hormone receptor-2 promoter–Cre (Dicer cKO) demonstrated that miRNA are essential for normal ovarian function (Hong et al., 2008; Nagaraja et al., 2008). Female Dicer cKO mice exhibited reduced ovulation rates, presence of trapped oocytes within luteinized follicles and were infertile (Hong et al., 2008; Nagaraja et al., 2008). Our laboratory recently identified thirteen differentially regulated miRNA in murine granulosa cells collected before and 4 h after hCG/LH surge (Fiedler et al., 2008). Three of these miRNA were highly upregulated and two of these, miR-132 and miR-212, were shown to be transcriptionally co-regulated and induced by cAMP in cultured granulosa cells (Fiedler et al., 2008). The remaining highly upregulated miRNA, miR-21, increased 7.5-fold by microarray analysis in response to *in vivo* hCG treatment and is the subject of manuscript.

Enhanced miR-21 expression is evident in multiple types of cancer, including breast, pancreatic, colorectal, and esophageal (Cho, 2007; Dillhoff et al., 2008; Feber et al., 2008; Schetter et al., 2008; Verghese et al., 2008). Therefore, it has received a substantial amount of scientific attention to determine the signaling pathways that

dictate its regulation as well as to identify its target gene transcripts (Davis et al., 2008a; Fujita et al., 2008; Thum et al., 2008). Studies using locked nucleic acid-(LNA) or 2'O-methyl-oligonucleotides complementary to miR-21 demonstrate that loss of miR-21 action leads to increased cell apoptosis in a variety of cell culture systems (Chan et al., 2005; Si et al., 2007). Several miR-21 target transcripts have been identified to explain its anti-apoptotic effect, including programmed cell death 4 (PDCD4), tropomyosin 1 (TPM1), phosphatase and tensin homologue (PTEN), maspin, reversion-inducing-cysteine-rich protein with kazal motifs (RECK), sprouty, and heterogeneous nuclear ribonucleoprotein K (HNRPK) and tumor protein p63 (Tap63; (Frankel et al., 2008; Hu et al., 2008; Meng et al., 2006; Papagiannakopoulos et al., 2008; Thum et al., 2008; Zhu et al., 2007). Although many different cell types undergo apoptosis in response to inhibition of miR-21 action, the miR-21 targets implicated vary widely for different cells (Frankel et al., 2008; Papagiannakopoulos et al., 2008).

In this study, we examine the temporal expression of miR-21 in murine granulosa cells in response to LH/hCG, and examine the function of miR-21 in cultured granulosa cells and in vivo by depleting mature miR-21 function with LNA oligonucleotides complementary to miR-21. We provide evidence that miR-21 acts as an anti-apoptotic factor in cultured murine granulosa cells and in ovarian tissue. Furthermore, we show that miR-21 expression is transcriptional and post-transcriptional regulated in ovarian granulosa cells.

3. Experimental Procedures

Animals and isolation of granulosa cells: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. All experiments were performed using CF-1 female mice from Charles River Laboratories (Wilmington, MA). Granulosa cell isolation was performed as previously described (Fiedler et al., 2008). Briefly, for *in vivo* expression analysis, 19-day old female CF-1 mice were injected i.p. with 5 I.U. of eCG (Calbiochem, San Diego, CA, FSH analog) to stimulate follicular development, and ovaries were collected at 12, 24, 36, and 48 h later. To stimulate ovulation and luteinization, mice were injected 48 h after eCG with hCG (Sigma, St. Louis, MO, LH analog), and then collected 1, 2, 4, 6, 8, or 12 h later. Granulosa cells were obtained from the ovarian tissue by follicular puncture into PBS. For *in vitro* analyses, ovaries from 26-day old CF-1 female mice were collected, incubated for 15 min in M-199 media containing 0.5M sucrose, rinsed with M-199 media, and the cells were expressed by follicular puncture into M-199 media (Campbell, 1979).

Ovarian bursal injections were completed as previously described (Otsuka et al., 2008). Briefly, female mice (19 days of age) were injected i.p. with 2 I.U. eCG, 24 h later, mice were anesthetized by i.p. injection of a 1:1 mixture of ketamine (0.75mg/animal; Ketaset, Fort Dodge, IA) and xylazine (0.75mg/animal, Anased, Lloyd Laboratories, Shenendoah, IA). The ovaries and associated fat pad were

exteriorized through incisions in the dorsal abdominal wall. To deliver treatments, a Hamilton syringe (10 ul) outfitted with a 33-gauge needle (45° beveled tip) was passed through the fat pad into the ovarian bursa. Each animal served as its own control with one ovary receiving the blocking oligonucleotide and the other vehicle control or a nonspecific oligonucleotide. Animals were then closed and 20 h later (i.e., 44 h post-eCG) were injected i.p. with 2 I.U. hCG. In order to not mask a knockdown effect with excessive ovarian stimulation, a "physiologic" dose of 2 I.U. of eCG and hCG was administered to ovarian bursa treated mice. Ovaries were fixed overnight in freshly made 4% paraformaldehyde (PFA).

In vitro granulosa cell culture: Isolated granulosa cells were seeded at $2.5x10^4$ cells per well into 6-well fibronectin-coated tissue culture plates for gene expression and apoptosis analyses and $1.5x10^6$ cells per 10 cm tissue culture dish for protein analyses. An aliquot of the isolated granulosa cells was placed in Trizol (Invitrogen, Carlsbad, CA) prior to culture to determine "before culture" expression values. Cells were cultured in DMEM/F12 (10%FBS, 1% gentamicin) at 37°C with 5% CO_2. To determine if plating affects miR-21 expression, RNA from cells were collected in Trizol at 6, 12, 24, 48, and 72 h after plating, another group of cells were cultured for 48 h in serum-containing medium and then cultured for 24 h in serum-free medium prior to RNA collection. Granulosa cells treated with 8-bromo-cAMP (Sigma) were cultured for 48 h in medium containing serum, then with serum-free medium for 24 h prior to treatment with 8-bromo-cAMP (1mM) for 1, 2, 4, 6, 8, or 12 h.

Oligonucleotide transfections were performed 48 h after plating, with the medium containing serum replaced with serum-free medium directly before transfection.

Oligonucleotide transfections: The control nonspecific scrambled (anti-NS) and anti-21 2'-O-methyl RNA oligonucleotides were purchased from Ambion (Houston, TX). Locked nucleic acid (LNA) oligonucleotides were synthesized with a mix of LNA/DNA nucleotides: LNA-21 5'-TCA GTC TGA TAA GCT A-3', and the control nonspecific (LNA-NS), 5'-CGG CAG TAT GCG AAT C-3' (underlined bases denote the LNA nucleotides; Integrated DNA Technologies, Coralville, IA). The *in vivo* LNA oligonucleotides, LNA-21pt and LNA-NSpt, (IDT) were identical in sequence and LNA base location to the in vitro oligonucleotides, the only difference being the addition of a nuclease-resistant phosphothioate backbone. The nonspecific scrambled control, LNA-NSpt, was synthesized with a rhodamine attached to the 5' end to allow for visualization of injection efficiency and tissue incorporation.

Oligonucleotides (42nM) were mixed with Lipofectamine 2000 reagent (Invitrogen) and then added to the isolated granulosa cells 48 h after culture at a final concentration of 42 nM. Cells were transfected for 4, 8, 12 and 24 h then collected for protein, RNA, or apoptosis assays. Granulosa cell protein lysates were obtained by resuspending cells in 200uL cell lysis buffer (Cell Signaling, Danvers, MA) with 1mM phenylmethylsulphonyl fluoride (PMSF). Cells were centrifuged at 1000 x g for 5 min to pellet the cellular membrane debris and supernatants were stored at -80°C until use. For RNA, cells were resuspended in 500uL Trizol and RNA was isolated following the manufacture instructions.

For in vivo transfections, the LNA-21pt and LNA-NSpt oligonucleotides (0.4nM) were mixed with Lipofectamine 2000 prior to bursal injection. Saline plus Lipofectamine was used as the vehicle control.

Apoptosis assay: Granulosa cells were trypsinized, washed twice with cold PBS, and resuspended in 1x-binding buffer (BD Pharmingen, San Jose, CA) at a concentration of $1x10^6$ cells/mL. One hundred uL ($1x10^5$ cells) of the solution was transferred to a 5mL polystyrene tube, and incubated with 5uL of PE-conjugated annexin V (BD Pharmingen) and 1uL live/dead fixable violet dead cell stain (Invitrogen) for 10 minutes at room temperature in the dark. One mL of 1x binding buffer was added to the cells, centrifuged at 1000 x g for 5 minutes and the supernatant removed. Cells were resuspended in 1% paraformaldehyde / PBS. The samples were analyzed by flow cytometry (BD Biosciences LSR II, San Jose, CA) using FACSDiva research software (BD Biosciences). Annexin V positive cells were considered apoptotic.

RNA isolation and quantitative RT-PCR: Total RNA was isolated using Trizol following the manufacture's instructions. Total RNA (250ng) was reverse transcribed using stem-loop primers specific to miR-21 (Applied Biosystems, Carlsbad, CA; (Fiedler et al., 2008). The resulting cDNA was used in the quantitative PCR reactions using miR-21 primers and probe (Applied Biosystems). For analysis of pri-miR-21, TMEM49, and GAPDH expression, 1ug total RNA was reverse transcribed using oligo-(dT) primers, and for pri-miR-21 and TMEM49 the following primers were used for quantitative PCR: pri-miR-21, forward: 5'-GAC ATC GCA TGG CTG TAC CA-3'; reverse: 5'-CCA TGA GAT TCA ACA GTC AAC ATC A-

3'; TMEM49, forward: 5'-TGG CAT CGT CAA AGC ATT GT-3', reverse: 5'-CCG CTG CAC ATA CTG TTG GT-3'. The TMEM49 primers spanned intron 4 to prevent any possible amplification of the pri-miR-21 transcript, which starts in intron 10. GAPDH and snoRNA U6 were used to normalize miR-21 expression. GAPDH primers and probe were purchased from Applied Biosystems and the snoRNA U6 primers were: forward: 5'-CTC GCT TCG GCA GCA CA-3', reverse: 5'-AAC GCT TCA CGA ATT TGC GT-3'.

Microarray analysis: Cultured granulosa cells were transfected with LNA-NS or LNA-21 for 24 h as above. Total RNA was isolated using Trizol and was biotin-labeled and fragmented according to Affymetrix protocols. Fragmented cRNA from each sample (n=2) was hybridized to individual Affymetrix 430A gene array chips using the GeneChip Fluidics Station 400 protocol (Affymetrix, Santa Clara, CA), and the hybridized chips were scanned using the Agilent Gene Array Scanner (Affymetrix). A scaling factor was applied to each chip using the Affymetrix Microarray Suite 5.0 software to normalize the mean raw fluorescence intensity for each chip to an average base-line fluorescence level.

The microarray data was analyzed using the Partek Genomic Suite (Partek, St. Louis, MO). Probe level intensity measurements were background corrected, normalized and summarized using RMA (Robust Multi-array Analysis; (Irizarry et al., 2003) after adjusting probe intensities for fragment length, GC content, and sequence allele position. Probe level fold changes and significances were computed using the least square mean (LS Mean) of each term of the linear contrasts performed

between LNA-21 and LNA-NS within the context of a 2-way mixed model ANOVA. List of genes involved in various KEGG pathways were identified through DAVID (Dennis et al., 2003), GenePattern (Kuehn, 2008) and oncological implications with relevant PubMed IDs for the most part were also identified through DAVID. The Ingenuity Pathway Analysis application (IPA, Ingenuity Systems, Redwood City, CA) was used to examine changed networks in the upregulated and downregulated genes (>1.5-fold).

Immunoblotting: Granulosa cell protein lysate concentrations were determined using the Bio-Rad protein assay (Hercules, CA). Each lysate (15 ug) was denatured by diluting the sample 1:2 with sample buffer (2.8mL distilled water, 1.0mL 0.5M Tris-HCl, pH6.8; 0.8mL glycerol, 1.6mL 10% SDS, 0.4mL 2-β-mercaptoethanol and 0.4mL 0.05% (w/v) bromophenol blue) and heating for 5 minutes at 95°C. Proteins were separated on 12% SDS-PAGE gels in 5X electrode running buffer, pH 8.3 (25mM Tris base; 192mM glycine; 0.1% (w/v) SDS), and transferred to PVDF membranes (Millipore, Billerica, MA) in transfer buffer (12mM Tris-HCl; 96mM glycine; 20% (v/v) MeOH). Blots were incubated for 1 h at RT in a 5% milk solution to block nonspecific binding. Blots were then incubated overnight at 4°C with one of the following antibodies: caspase 3 antibody (Cell Signaling), sprouty 2 (Santa Cruz, Santa Cruz, CA), tropomyosin 1 (Santa Cruz), PDCD4 (ProSci, Poway, CA), PTEN (Sigma) and actin (Santa Cruz). After washing and incubation with the appropriate secondary antibody, protein-antibody complexes were visualized using West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's protocol.

MicroRNA in situ hybridization: Double 5' and 3'-digoxigenin (DIG)-labeled pre-designed LNA probes for detection of miR-21 (same sequence as LNA-21) as well as a double-DIG-labeled scrambled control (5'-GTG TAA CAC GTC TAT ACG CCC A-3') were purchased from Exiqon (Vedbaek, Denmark). *In situ* hybridization was performed on 4% PFA fixed and paraffin-embedded tissue as previously described in detail (Nuovo et al., 2009). Following deparaffinization, samples were subjected to pepsin (1.3mg/mL) digestion for 30 min. The tissues were then incubated overnight with the LNA probes. Tissue sections were then incubated for 30 min at 37°C in anti-digoxigenin-alkaline phosphatase conjugate (Roche, Nutley, NJ), followed by visualization with NBT/BCIP chromogen (Enzo Diagnostics, Farmingdale, NY).

In situ apoptosis assay: Tissue sections (5um) were deparaffinized and apoptotic cells were detected using the ApopTag Red *In Situ* apoptosis detection kit (Chemicon, Billerica, MA). Briefly, samples (n=3) were digested with proteinase K (20ug/mL) for 15 min and then terminal deoxynucleotide transferase (TdT) was added to catalyze the addition of digoxigenin-dNTPs onto the DNA strand breaks. For negative control, the TdT enzyme was inactivated by adding stop buffer to the enzyme to chelate the divalent cationic enzyme cofactor prior to applying to the tissue sections. Incubation with anti-digoxigenin-rhodamine allowed visualization of apoptotic cells. Slides were also labeled with the nuclear binding dye Hoechst to visualize ovarian morphology. Fluorescence was examined on an Olympus1x71 inverted microscope.

4. Results and Figures

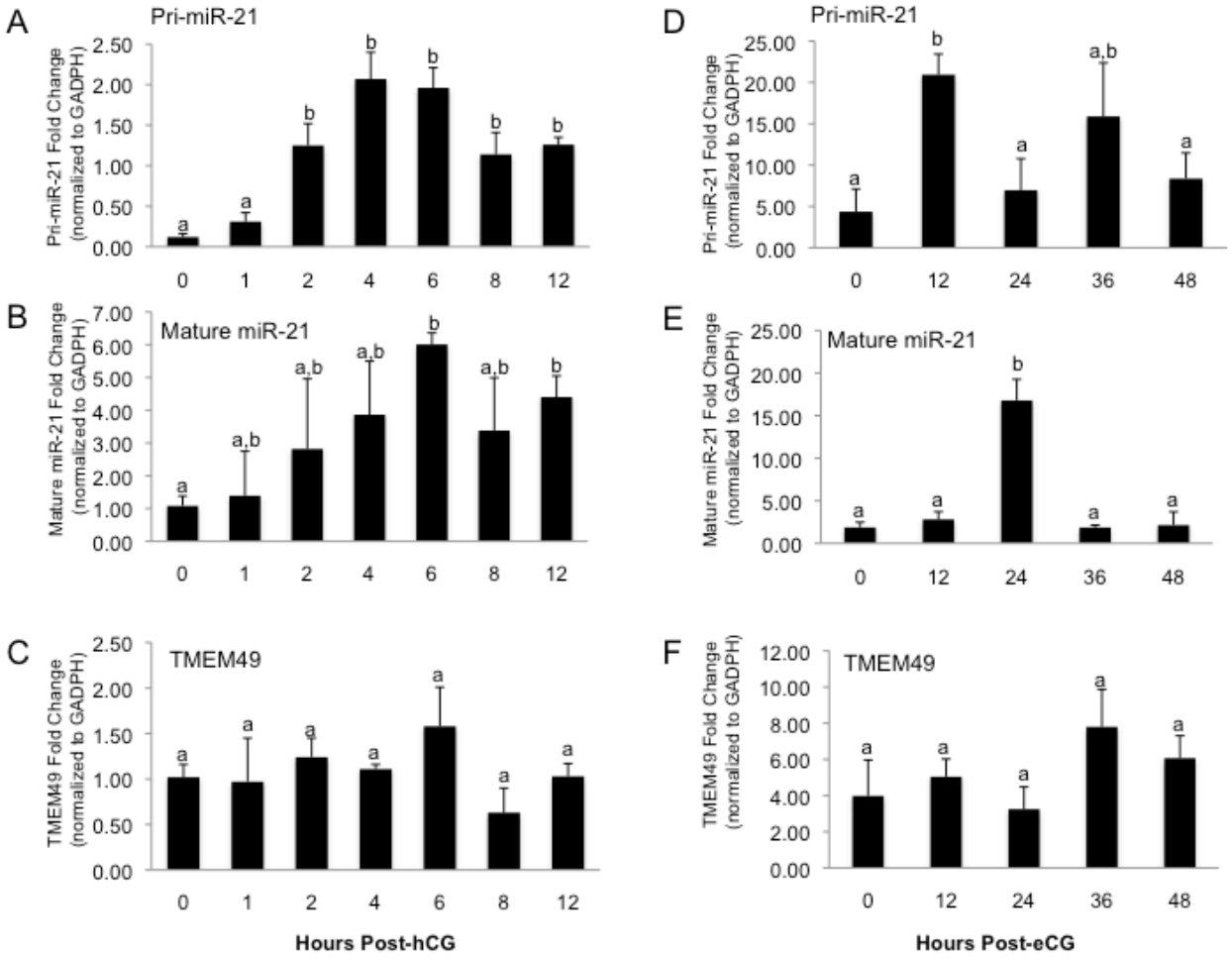
MiR-21 expression increases after in vivo *hCG/LH and eCG/FSH administration.*

Mural granulosa cells collected from periovulatory follicles exhibit an increase in miR-21 expression, confirming earlier array analyses (Figure VII-1; Fiedler et al., 2008). To determine if transcription of miR-21 increased in response to hCG, the primary-miR-21 transcript (pri-miR-21) levels were examined by qRT-PCR. Pri-miR-21 expression progressively increased through 2 h post-hCG, reached a maximal 10-fold induction, and then remained elevated through 12 h post-hCG (Figure VII-1A). *In vivo* expression of mature (21 basepair) miR-21 trailed that of pri-miR-21 levels by several hours, with mature miR-21 levels reaching their maximum 5.8-fold induction at 6 h post-hCG, miR-21 levels remained elevated through 12 h post-hCG (Figure VII-1B). Despite having its own promoter, a recent study indicated that miR-21 might be co-expressed with the gene TMEM49 in which it resides (Hu et al., 2008). Expression of TMEM49 within granulosa cells showed no change following *in vivo* hCG administration (Figure VII-1C).

To further examine miR-21 expression in the ovary, miR-21 levels were also examined during the preovulatory period (0-48 h post-eCG). Levels of pri-miR-21 increased 4-fold at 12 h following eCG, and then returned to basal levels (Figure VII-1D). Mature miR-21 levels followed pri-miR-21 levels, with levels increasing 6.4-fold at 24 h post-eCG before returning to basal levels at 48 h (Figure VII-1E). Once again, TMEM49 expression exhibited no change (Figure VII-1F).

FIGURE VII-1. *Induction of miRNA-21 by eCG and hCG.* Quantitative RT-PCR of pri-miR-21 (A, D), mature miR-21 (B, E), or TMEM49 (C, F) in murine granulosa cells with eCG alone (0h) or 1, 2, 4, 6, 8, or 12h following hCG injection (A, B, C) or following 12, 24, 36, or 48 h eCG injection (D, E, F). All qRT-PCR data (n=3) were normalized to GAPDH; One-way ANOVA was used to determine statistical differences between time points and ^{a,b} means ± SEM with different superscripts are different ($p<0.05$).

Figure VII-1.

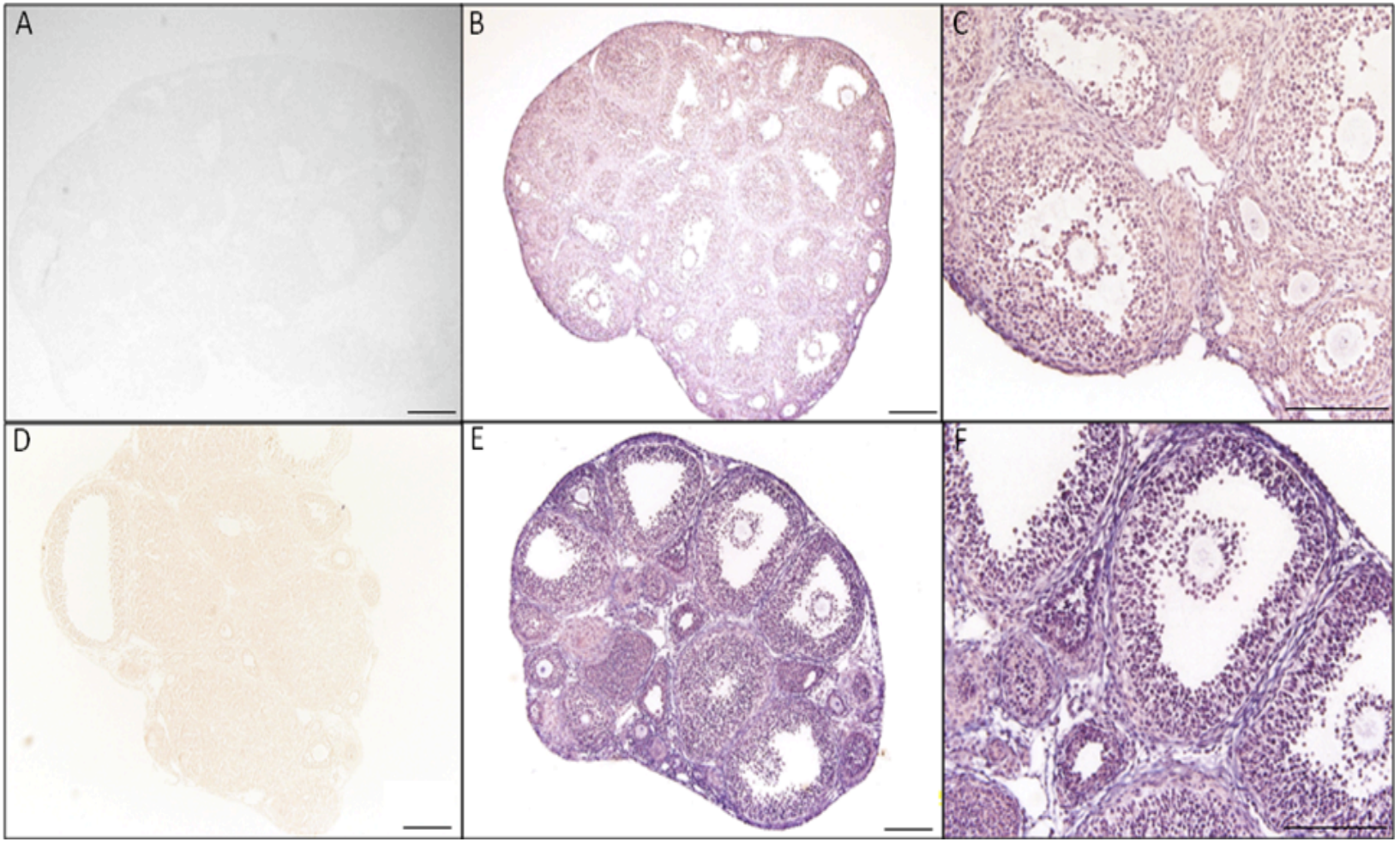

A
Pri-miR-21
Pri-miR-21 Fold Change (normalized to GADPH)
B
Mature miR-21
Mature miR-21 Fold Change (normalized to GADPH)
C
TMEM49
TMEM49 Fold Change (normalized to GADPH)
Hours Post-hCG
D
Pri-miR-21
E
Mature miR-21
F
TMEM49
Hours Post-eCG

To localize and confirm upregulation of miR-21 within granulosa cells *in situ* hybridization was conducted before and 6 h after hCG treatment. *In situ* hybridization revealed miR-21 was expressed mostly in individual granulosa and cumulus cells, although some individual stroma cells expressed low levels at 0 h post-hCG (Figure VII-2B, C). Oocytes of primary and early antral follicles also expressed miR-21, with expression localized to the nucleus, and levels were unchanged in response to hCG (Figure VII-2B, C, E). At 6 h post-hCG, miR-21 expression dramatically increased in cumulus and granulosa cells, especially of antral follicles (Figure VII-2E, F) and while expression increased in some stroma cells, it remained undetectable in others (Figure VII-2E, F).

In contrast to *in vivo* miR-21 expression, which demonstrated a clear transcriptional response, mature miR-21 levels in cultured granulosa cells increased 5- fold upon plating with no corresponding increase in pri-miR-21 levels (Figure VII-3A). Granulosa cell expression of mature miR-21 increased ($P<0.05$) within 12 h of plating and reached its greatest levels at 48 h (Figure VII-3A). Since subsequent cAMP treatments and transfection experiments were done with cells removed from serum-containing media and placed in serum-free media at 48 h post-plating, we examined the expression of miR-21 in granulosa cells cultured with serum for 48 h, then put in serum free media for the next 24 h. Removal of serum had no significant effect on mature miR-21 levels (Figure VII-3A). Pri-miR-21 levels did not change in response to plating (Figure VII-3B). In addition, treatment of granulosa cells with the cell permeable 8-bromo-cAMP (i.e., the primary second messenger utilized by LH),

FIGURE VII-2. *MiR-21 is upregulated in periovulatory granulosa cells after hCG.* In situ hybridization with a locked nucleic acid (LNA) complementary to miR-21 before (B, C) and 6h (E, F) after hCG. A control scrambled LNA resulted in no signal at either time point (A, D). Similar results were obtained in four different replicates. Bar = 500 um.

Figure VII-2.



had no affect on miR-21 expression across time (Figure VII-3C).

Differential knockdown of miR-21 by LNA and 2'-O-methyl oligonucleotides leads to functional differences.

To analyze the function of miR-21, granulosa cells were treated with either a 2'O-methyl oligonucleotide complementary to miR-21 (anti-21) or a control scrambled 2'-O-methyl oligonucleotide (anti-NS). Mature levels of miR-21 decreased 9-fold with addition of the anti-21 (Figure VII-4A). The specificity of this anti-21 was confirmed as the levels of two other miRNAs, miR-132 and miR-212 were not affected (data not shown). Similarly, knockdown of miR-132 and miR-212 had no effect on miR-21 expression (Fiedler et al., 2008). LNA oligonucleotides have been shown to block miRNA expression and function to a greater degree than 2'-O-methyl oligonucleotides (Marquez and McCaffrey, 2008; Orom et al., 2006). Consistent with these observations, granulosa cells treated with LNA-21 had a 27-fold reduction in miR-21 levels when compared to a scrambled LNA (LNA-NS; Figure VII-4B).

The 3-fold difference in miR-21 knockdown between the different miR21 inhibitors was associated with a marked difference in cell morphology as only the LNA-21 treated cells rounded up and pulled off of the dish, a well-described "late step" in the process of apoptosis (Falcieri et al., 1994; Figure VII-5A). To confirm apoptosis, cells were stained with annexin V and live/dead violet stain, fixed and then analyzed by flow cytometry. Annexin V labeling indicated that granulosa cells began undergoing apoptosis within 8 h of LNA-21 treatment and by 24 h of treatment 42%

FIGURE VII-3. *MiRNA-21 levels increase in response to plating and do not change in response to cAMP.* Quantitative RT-PCR of mature miR-21 (panel A) and pri-miR-21 (panel B) before culture/plating (BC), and 6, 12, 24, 48, 72h or SF which represent cells 72h after plating, with the last 24h in serum-free media. Mature miR-21 levels in granulosa cell before culture (BC) and after 72h plating (minus serum for the last 24 h) and then treated with 8-bromo-cAMP for 1, 2, 4, 6, 8, or 12h of 8-bromo-cAMP treatment are shown in panel C. Data (n=3) was normalized to GAPDH; one-way (A, B) and two-way ANOVA (C) was used to determine statistical significance; ^{a,b}means ± SEM with different superscripts are different ($p<0.05$).

Figure VII-3.

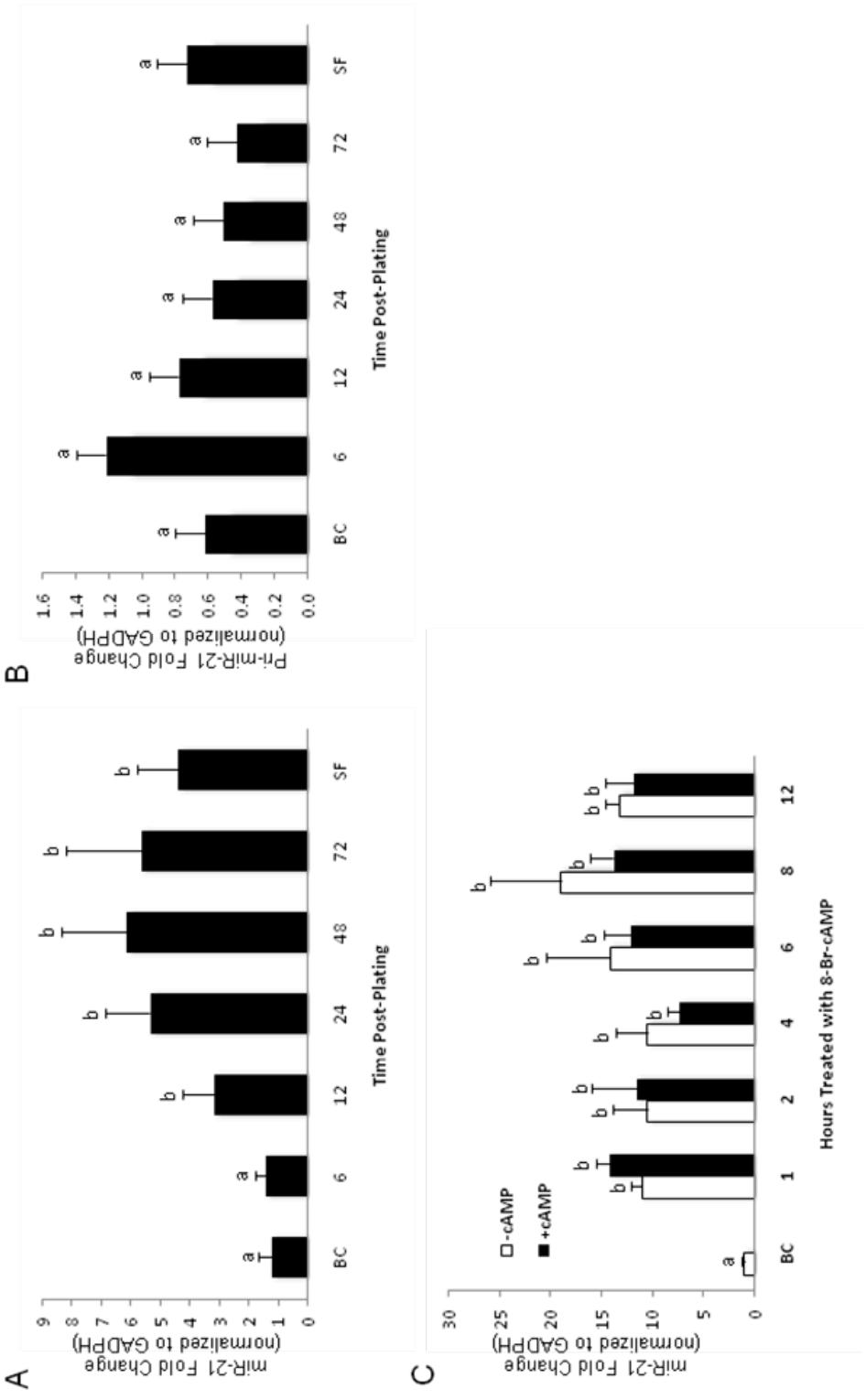
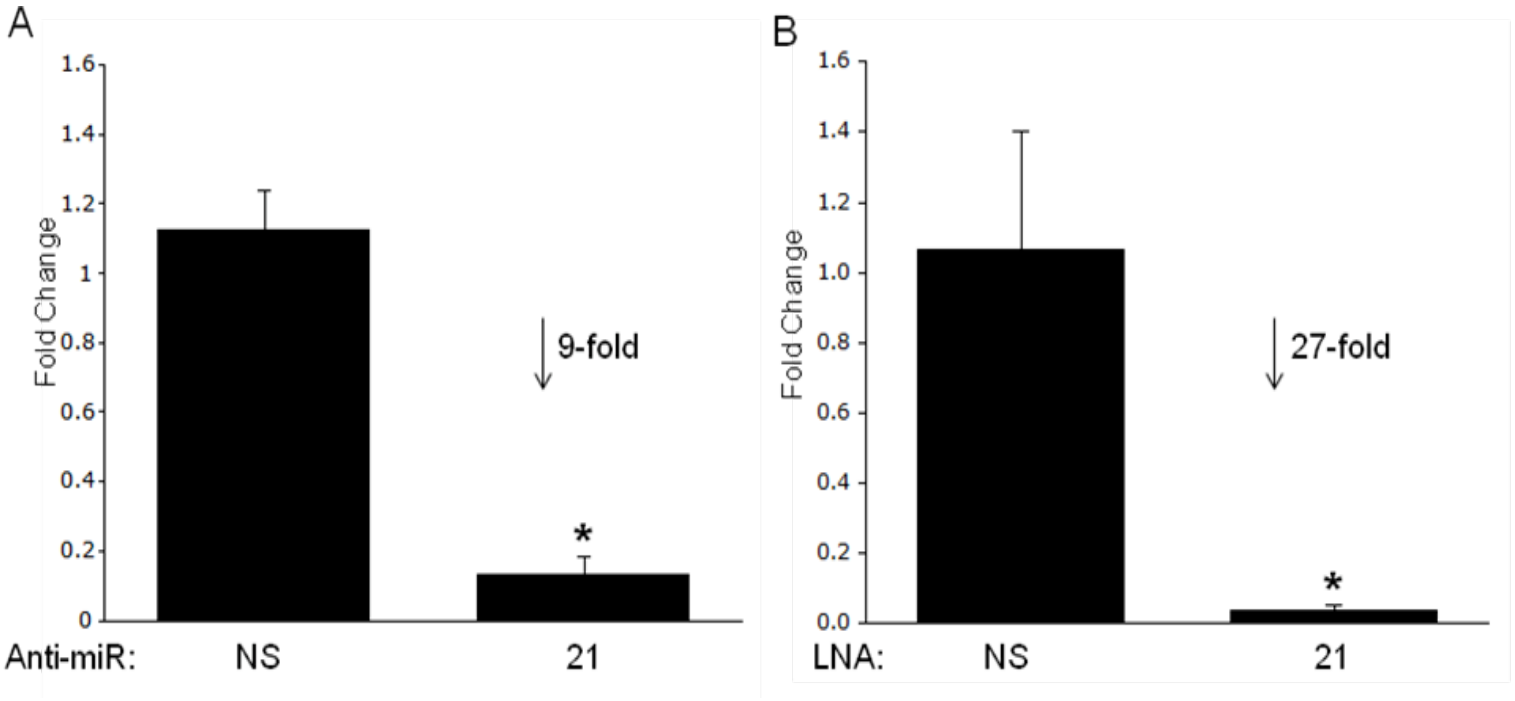

A
miR-21 Fold Change (normalized to GADPH)
Time Post-Plating
BC
6
12
24
48
72
SF
B
Pri-miR-21 Fold Change (normalized to GADPH)
Time Post-Plating
C
-cAMP
+cAMP
miR-21 Fold Change (normalized to GADPH)
Hours Treated with 8-Br-cAMP
1
2
4
6
8
12

FIGURE VII-4. *Knockdown of miR-21 by 2'-O-methyl oligonucleotides or locked nucleic acid (LNA) oligonucleotides in cultured granulosa cells.* Quantitative RT-PCR of mature miR-21 in cultured granulosa cells following transfection with 2'-O-methyl "anti-21 or anti-NS oligonucleotides (Panel A) or with LNA-21 or LNA-NS (i.e., control) oligonucleotides (Panel B). Data (n=3) was normalized to GAPDH; t-tests were used to determine statistical significance within the oligonucleotide treatment groups; *means ± SEM with different superscripts are different ($p<0.05$). Two-way ANOVA comparison of relative values across treatment groups indicated a 3-fold decline ($p<0.05$) in miR-21 transcript levels between anti-21 and LNA-21 treatment groups, with no difference between anti-NS and LNA-NS treated cells.

Figure VII-4.


A
Fold Change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
9-fold
*
Anti-miR:
NS
21
B
Fold Change
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
27-fold
*
LNA:
NS
21

of the cells had undergone apoptosis (Figure VII-5B). No change in apoptosis was observed in the control LNA-NS treated cells (Figure VII-5B). Flow cytometry analysis also indicated that granulosa cells transfected with anti-21 or precursor-miR-21 (which increases miR-21 levels) had no effect on apoptosis at 24 h and 48 h post-transfection (data not shown). To further confirm the increase of apoptosis in LNA-21 treated cells, immunoblotting was performed for cleaved caspase 3, an active mediator of apoptosis (Earnshaw et al., 1999). Cleaved caspase 3 levels were increased in LNA-21 treated cells within 8 h after transfection, but were not increased in anti-21 treated cells (Figure VII-5C). Dose response analysis using decreasing concentrations of LNA-21 (20nM, 5nM, and 1.25nM; Figure VII-6A) displayed decreasing levels of cleaved caspase 3 (Figure VII-6B). While the 1.25nM dose of LNA-21 showed a similar knockdown to anti-21 (9-fold), there was still a slight increase in cleaved caspase 3 levels (Figure VII-6A,B).

Known apoptotic miR-21 target transcripts were unaffected by LNA-21 treatment of granulosa cells.

MicroRNA-21 has been implicated in the post-transcriptional regulation of 8 apoptotic genes in other cell systems (Frankel et al., 2008; Hu et al., 2008; Meng et al., 2006; Papagiannakopoulos et al., 2008; Thum et al., 2008; Zhu et al., 2007). Western blot analyses examined the expression of four of these proteins at 4 h, 8 h, and 24 h after transfection with the LNA-21 oligonucleotide (Figure VII-7). RECK and maspin were also analyzed by Western blot, but expression of both appeared extremely low in granulosa cells as evident by the almost nonexistent signal

FIGURE VII-5. *Induction of apoptosis upon LNA-21 treatment of cultured murine granulosa cells.* Bright field image of rounded up cells after 24h treatment with LNA-21 (panel A). LNA-NS and LNA-21 transfected cells were double-stained with annexin and live/dead violet stain and were analyzed by fluorescence activated cell sorting (FACS) 0-24h after transfection (panel B). Line graph summaries the percentage of live cells 0-24h after transfection, and data (n=3) was analyzed by t-test; *means ± SEM with different superscripts are different ($p<0.05$) between the LNA-21 and LNA-NS at that time point. (C) Representative Western blot (n=3) of caspase 3 and cleaved (active) caspase 3 in LNA-NS and LNA-21 transfected granulosa cells 4, 8, and 24h after transfection, or 24h after transfection with anti-NS or anti-21.

Figure VII-5.

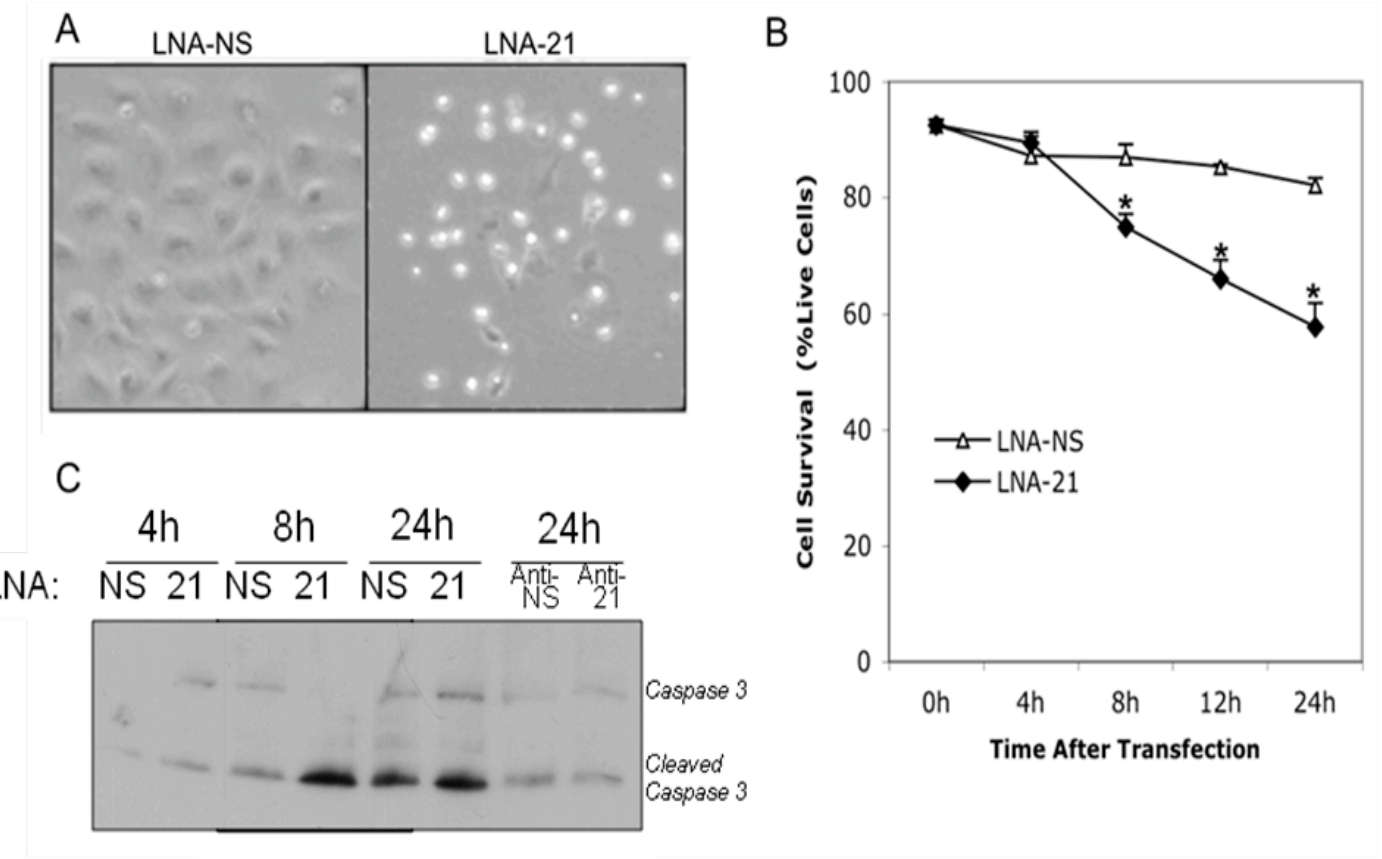
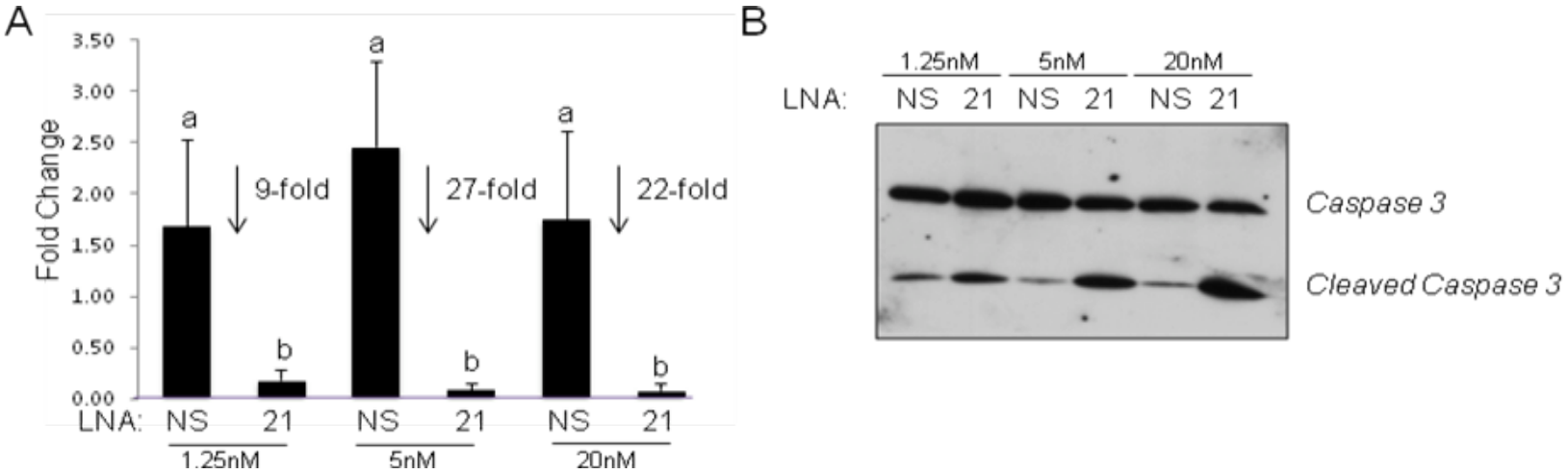

A
LNA-NS
LNA-21
B
100
80
60
40
20
0
Cell Survival (%Live Cells)
LNA-NS
LNA-21
0h
4h
8h
12h
24h
Time After Transfection
C
4h
8h
24h
24h
LNA:
NS
21
NS
21
NS
21
Anti-NS
Anti-21
Caspase 3
Cleaved Caspase 3

FIGURE VII-6. *Reduced concentrations of LNA result in a decrease in the amount of caspase 3 cleavage.* Quantitative RT-PCR of mature miR-21 in cultured granulosa cells with 1.25nM, 5nM, or 20nM LNA-NS or LNA-21 (panel A). Cells transfected with 1.25nM LNA-21 had comparable fold reductions in mature miR-21 levels to those transfected with 42nM anti-21. Data (n=3) was normalized to GAPDH; two-way ANOVA was used to determine statistical significance; ^{a,b}means ± SEM with different superscripts are different ($p<0.05$). Representative Western blot (n=3; panel B) of caspase 3 and cleaved (active) caspase 3 in granulosa cells transfected with 1.25nm, 5nm, or 20nm LNA-NS or LNA-21.

Figure VII-6.


A
Fold Change
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a
b
9-fold
27-fold
22-fold
LNA: NS 21 NS 21 NS 21
1.25nM
5nM
20nM
B
1.25nM
5nM
20nM
LNA: NS 21 NS 21 NS 21
Caspase 3
Cleaved Caspase 3

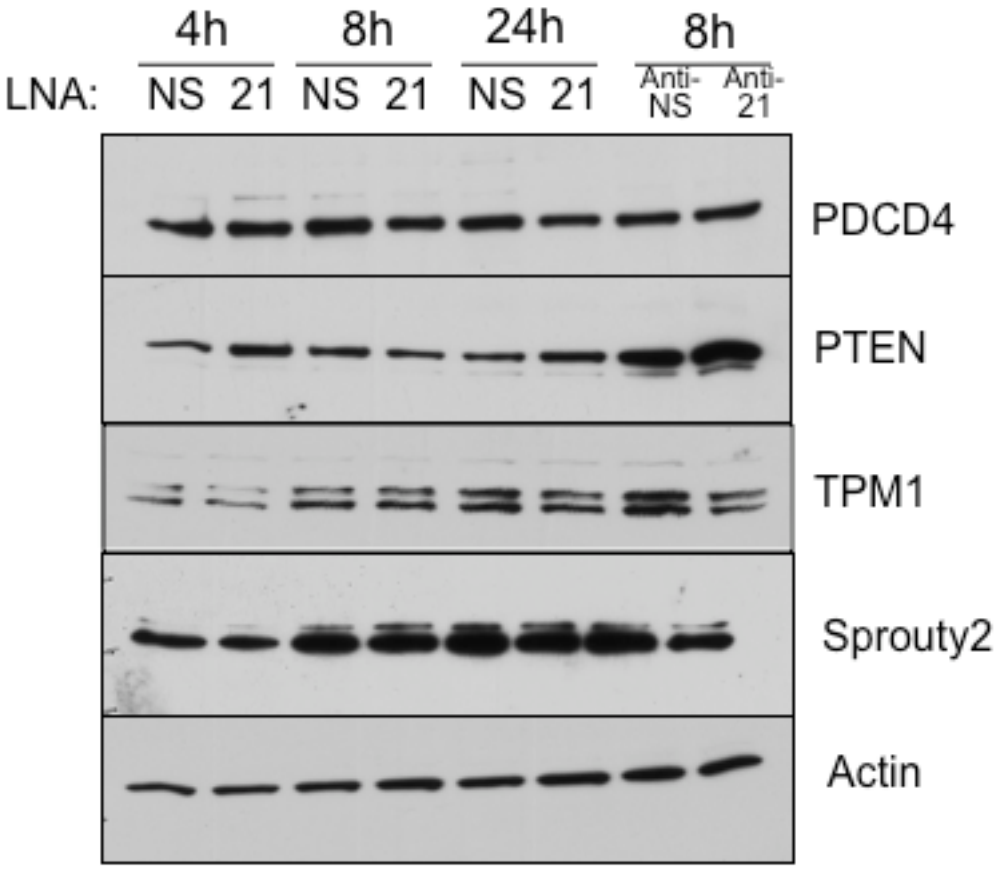
and lack of differences between LNA-NS and LNA-21 samples (data not shown). With the exception of PTEN which exhibited a slight increase 4 h after treatment with LNA-21, none of the other proteins exhibited any change in protein expression (Figure VII-7).

To determine whether the change in PTEN levels could affect granulosa cell apoptosis, granulosa cell PTEN levels were decreased by transfection with PTEN siRNA (Supplemental Figure VII-1A). After PTEN siRNA transfection (24 h), cells were transfected with LNA-NS or LNA-21. However, apoptosis still occurred in LNA-21 treated cells even in the presence of PTEN siRNA (Supplemental Figure VII-1B). To further rule out the role of PTEN in apoptosis in LNA-21 treated granulosa cells, oligonucleotides were designed to "block" the miR-21 sites on the PTEN 3'UTR. Since basal miR-21 levels are high in plated granulosa cells, and presumably play a role in granulosa cell survival, the prevention of miR-21 from binding to PTEN should result in an increase in basal PTEN levels and possibly lead to apoptosis. We did not observe a change in apoptosis (Supplemental Figure VII-1C).

Loss of miR-21 fails to elicit changes in mRNA expression. Microarray analyses of granulosa cells treated with LNA-21 (n=2) and the control LNA-NS (n=2) were completed to identify miR-21 target transcripts that might be post-transcriptionally regulated through a mRNA-degradation mechanism. Overall, 203 upregulated and 115 downregulated genes exhibited >1.5-fold change and 50 upregulated and 12 downregulated exhibited >2-fold change. Statistical analyses,

FIGURE VII-7. *Granulosa cell expression of known miR-21 targets are not affected by LNA-21 knockdown.* Representative western blots (n=3) for PDCD4, PTEN, TPM1, Sprouty2, and control actin after 4h, 8h, or 24h of transfection with LNA-NS or LNA-21, or 8h after transfection with anti-NS or anti-21.

Figure VII-7.


4h
8h
24h
8h
LNA:
NS
21
NS
21
NS
21
Anti-NS
Anti-21
PDCD4
PTEN
TPM1
Sprouty2
Actin

however, failed to detect any transcripts that exhibited a significant change in response to loss of miR-21 action.

To determine whether a particular cell biologic pathway is overrepresented we compiled all of the genes with predicted >1.5- fold differences and mapped them to KEGG pathways to perform pathway analysis using GenePattern. We identified apoptosis/cell death pathway as one of the significant and important pathways through this analysis. We used classification and regression tree methodology to identify the list of genes in various pathways. Table VII-1 contains the list of pathways identified using our approach. We also used Ingenuity Pathway Analysis to validate our results. Both analyses identified the apoptosis/cell death pathway as significantly overrepresented as shown in Table VII-1.

Caspase 3 cleavage decreases in granulosa cells after administration of an ovulatory surge of LH/hCG. Levels of cleaved caspase 3 were analyzed by western blot following LH/hCG administration *in vivo*. Uncleaved caspase 3 levels were unchanged following hCG, except for a slight increase at 4 h post-hCG (Figure VII-8). Levels of cleaved caspase 3, however, decreased at 6 h post-hCG and remained low at 8 h post-hCG (Figure VII-8).

In vivo knockdown of miR-21 causes an increase in apoptosis. To examine the effect of miR-21 on ovarian apoptosis, miR-21 function was knocked down *in vivo*. Following follicular stimulation (i.e., 24 h of eCG), an LNA-21pt oligonucleotide targeting miR-21 was injected into one ovarian bursa, while the other ovary received vehicle or the fluorescent-tagged nonspecific LNA-NSpt. Twenty-two h post-

Table VII-1: Overrepresented Pathways in genes changed >1.5-fold between LNA-NS and LNA-21 treated cultured granulosa cells.

Pathway Name	p-value	Number of genes
cell cycle	2.59E-11	67
cell division	1.22E-10	35
cell cycle&mitotic	2.02E-10	30
dna replication	9.93E-10	30
s phase	2.71E-09	33
m phase	1.37E-07	20
cell cycle checkpoint	2.95E-06	15
g1 phase	2.36E-05	20
dna repair	2.36E-05	22
apoptosis	**3.15E-05**	**51**
replication complex	3.87E-05	7
g2 phase	3.87E-05	11
transcription	3.50E-04	79
synthesis of dna	2.32E-03	5
signal transduction	3.22E-03	45
g2/m transition	3.22E-03	7
signaling cascade	3.68E-03	13
cytokine receptors	4.44E-03	12
integrin-mediated cell adhesion	5.00E-03	6
proteasome	7.01E-03	26

FIGURE VII-8. *Cleaved caspase 3 is decreased in vivo following LH/hCG.* Representative Western blots (n=3) of caspase and cleaved caspase 3 and actin after 0, 1, 2, 4, 6, and 8 h after hCG administration.

Figure VII-8.

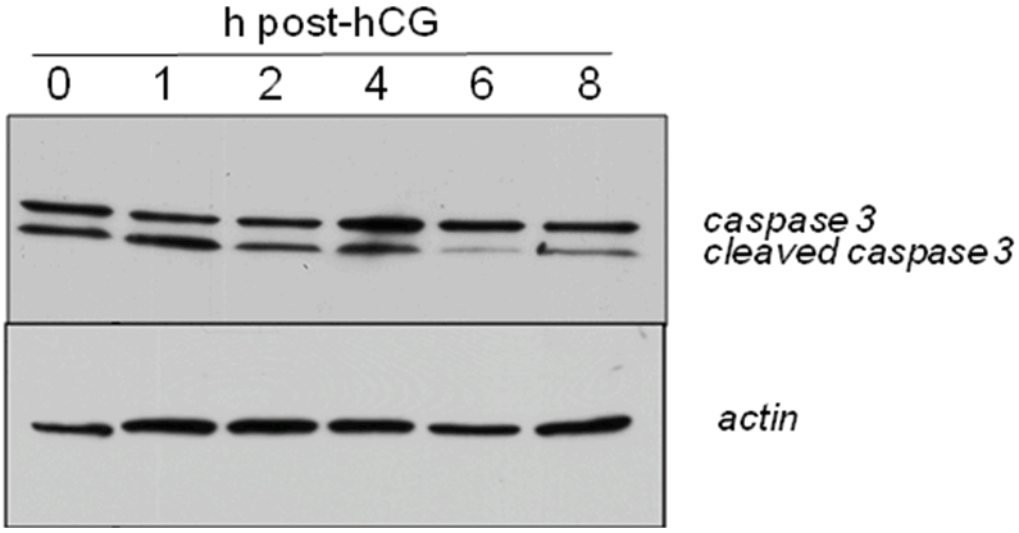


FIGURE VII-9. *Ovarian bursal injections of LNA-21pt cause an increase in apoptosis.* Representative fluorescence images (n=4) of mouse ovaries exposed to saline (A) or LNA-21pt (D) stained with the DNA-binding dye Hoechst. TUNEL assay to detect apoptotic cells in the saline (B) and LNA-21pt (E, F) treated ovaries. Negative control TUNEL assay (C; non-working enzyme) in saline treated mouse ovary. Panel F is a 10X magnification of E. (G) Negative control TUNEL assay (non-working enzyme) of the LNA-21pt injected mouse ovary in G and H. Bar = 500 um.

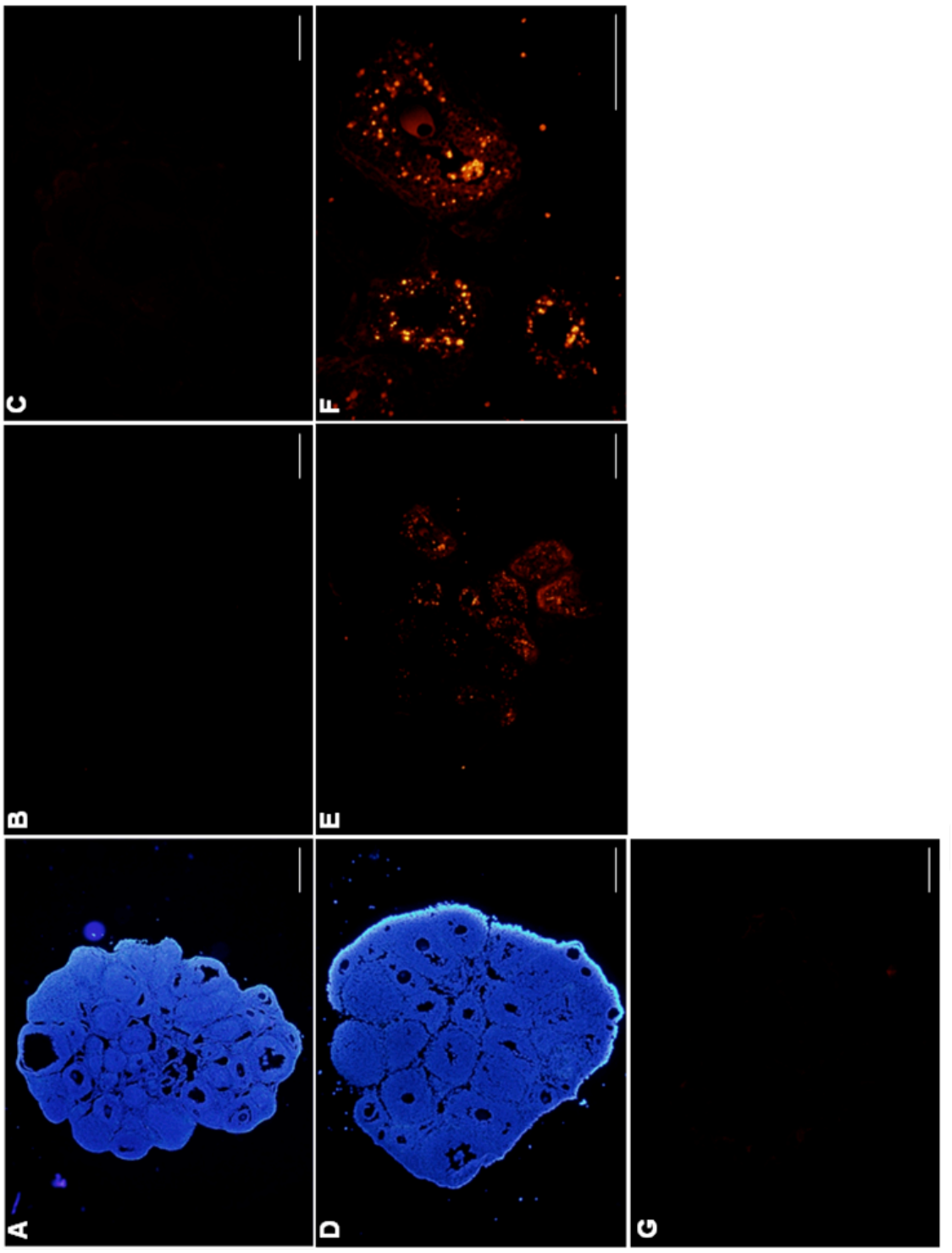


Figure VII-9.

surgery, animals were injected with hCG to stimulate ovulation and luteinization. Ovaries were collected 16 h post-hCG and were analyzed for apoptosis (Figure VII-9). Apoptosis was significantly increased in LNA-21 treated ovaries compared to saline treated ovaries (Figure VII-9). Transfection efficiency was measured by distribution of the fluorescent-labeled LNA-NS (Supplemental Figure VII-2). While labeling was found throughout the ovary, there were punctate regions of increased fluorescence (Supplemental Figure VII-2). When tested for apoptosis, there was no increase in TUNEL fluorescence between the LNA-NS negative control and the TUNEL-labeled LNA-NS samples (Supplemental Figure VII-2).

In addition, ovaries treated with LNA-21 displayed decreased ovulation rates compared to LNA-NS or saline treated controls (Figure VII-10). Histological analysis of LNA-21 treated ovaries showed an increased incidence of trapped oocytes within luteinized follicles, and in two cases there was an increased accumulation of red blood cells around the oocyte of the luteinized follicle (Figure VII-11).

FIGURE VII-10. *Ovulation rate is decreased in LNA-21 treated ovaries.* Number of cumulus-oocyte-complexes within the clutch ("ovulation rate") of LNA-NS/saline treated ovaries or LNA-21 treated ovaries. * indicates significance ($p<0.05$).

Figure VII-10

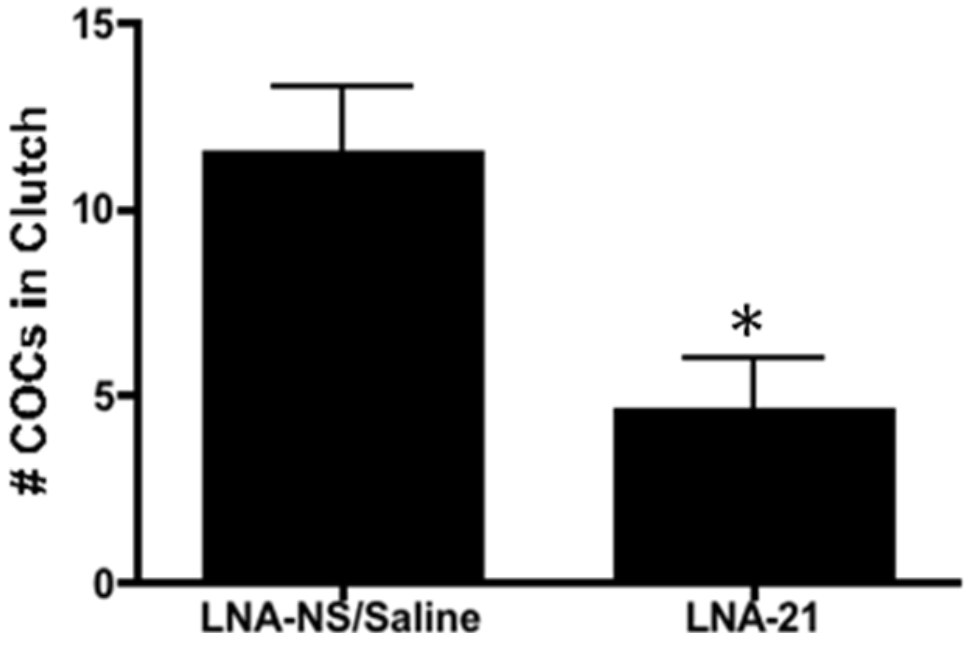
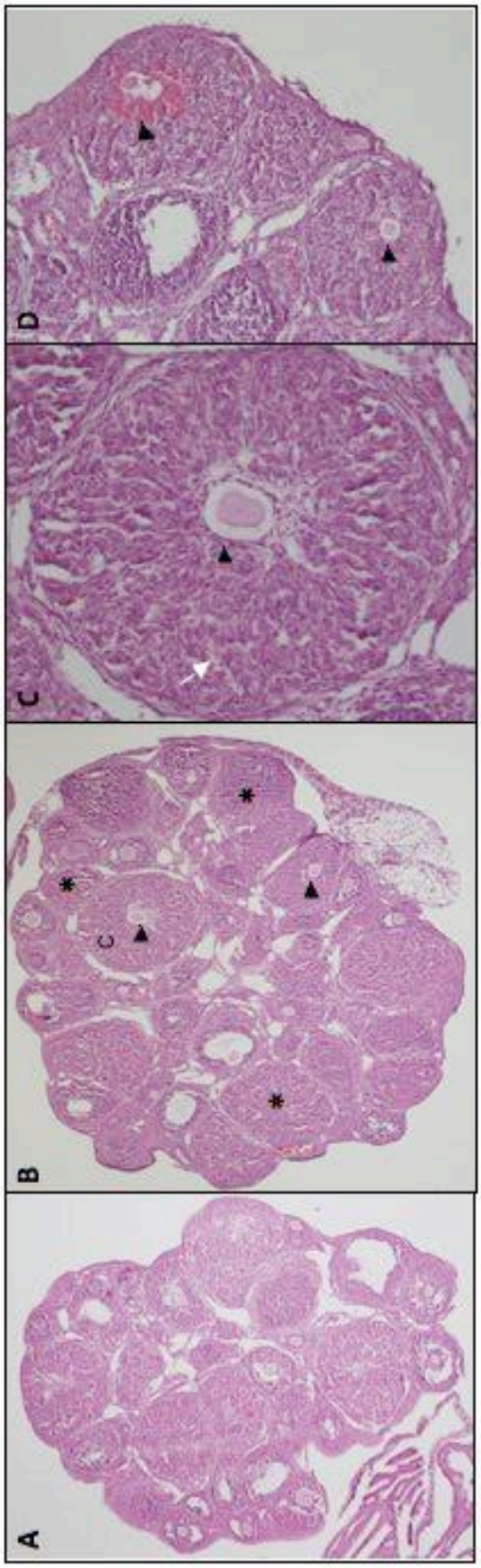


FIGURE VII-11. Ovarian sections from LNA-NS (A) and LNA-21 (B-D) treated ovaries. Panel B, asterisks are luteinized follicles that have a trapped oocyte in another section, arrowheads are trapped oocytes that are visible in this section. Panel C shows a magnified view of trapped oocyte (c) from panel B. Panel D, arrows point to two trapped oocytes in another LNA-21 treated oocyte, and white arrow points to an accumulation of red blood cells.

Figure VII-11



5. Discussion

Recent *in vitro* studies in cultured cancer cells, cancer cell lines, and embryonic stem cells suggest that miR-21 expression is important for differentiation and apoptosis (Chan et al., 2005; Chen et al., 2008a; Si et al., 2007; Singh et al., 2008; Zhang et al., 2008b). Here we show for the first time that miR-21 also plays a role in apoptosis in a physiological system. We show that miR-21 is upregulated in granulosa cells in response to LH. During LH-induced terminal differentiation of the granulosa cell, it is necessary to block apoptosis to allow for the formation of a functional corpus luteum (Chaffin et al., 2001; Robker and Richards, 1998a, b). We show here that the loss of miR-21 in periovulatory granulosa cells causes an increase in apoptosis, and thus may be important for the prevention of apoptosis that occurs during luteinization.

To examine the function of miR-21 in granulosa cells, miR-21 action was knocked down in cultured granulosa cells using 2'O-methyl or LNA oligonucleotides. Consistent with other investigations using these two different types of inhibitors, we observed a greater effect (3-fold) with the LNA oligonucleotide (Marquez and McCaffrey, 2008; Orom et al., 2006; Papagiannakopoulos et al., 2008). The increased effectiveness in knockdown was associated with the appearance of the apoptotic phenotypic response associated with loss of miR-21 in other cell types (Chan et al., 2005; Si et al., 2007). One possible explanation for this may be that basal miR-21 levels are much higher in granulosa cells, compared to cell lines examined by other investigators, and the reduction induced by 2'O-methyl oligonucleotides was

insufficient to knockdown miR-21 expression to levels that cause apoptosis. Results similar to ours were recently described in glioblastoma cells, in which only the LNA-21 oligonucleotides caused significant amounts of apoptosis, while the 2'O-methyl oligonucleotides had little effect (Papagiannakopoulos et al., 2008). However, when we tested lower doses of LNA-21 at a dose that reached an equivalent (i.e., 9-fold loss in miR-21) to the 2'O-methyl, we still detected apoptosis (cleaved caspase 3: Figure VII-6). It presently is unclear why similar levels of knockdown of miR-21 would not generate similar phenotypical responses; this is an interesting phenomenon and suggests that blocking miRNA action with different oligonucleotides can have dramatic differences in biological responses, a finding with important implications to the therapeutic potential of miRNA blockers.

MicroRNA-21 has been shown to block apoptosis in a number of cell types, and several targets have been identified as directly regulated by miR-21 to mediate its anti-apoptotic activity (Frankel et al., 2008; Hu et al., 2008; Meng et al., 2006; Papagiannakopoulos et al., 2008; Thum et al., 2008; Zhu et al., 2007). Tropomyosin 1 (TPM1) was the first identified direct miR-21 target transcript (Zhu et al., 2007). Post-transcriptional regulation of TPM1 by miR-21 caused a 2-fold increase in TPM1 protein in breast cancer cells following miR-21 knockdown, no effect on TPM1 mRNA expression was detected (Zhu et al., 2007). TPM1 an actin-binding protein promotes the specialized type of apoptosis known as anoikis, by suppressing anchorage-independent growth (Raval et al., 2003). Phosphatase and tensin homolog (PTEN) is another pro-apoptotic protein, which inhibits the AKT and mTOR kinase

pathways to cause apoptosis (Tamura et al., 1998). Knockdown of miR-21 in human hepatocytes caused a 2 to 3-fold increase in PTEN protein, but no change on PTEN mRNA (Meng et al., 2006). We observed a slight increase in PTEN protein levels at 4 h post-transfection, however, specific tests of PTEN regulation by blockade of PTEN expression, or masking potential miR-21 sites failed to prevent apoptosis. Programmed cell death 4 (PDCD4) inhibits global cell translation by inhibiting the translation initiation factor 4a (Yang et al., 2003). Knockdown of miR-21 in human breast cancer cells causes an increase in PDCD4 mRNA and protein levels (Frankel et al., 2008). Recently, two factors involved in p53 tumor suppression, the p53-activator heterogeneous nuclear ribonucleoprotein K (HNRPK) and the p53 homologue Tap63, were identified as direct miR-21 targets in glioblastoma cells (Papagiannakopoulos et al., 2008). However, knockdown of these proteins in combination with miR-21 knockdown failed to rescue the apoptotic phenotype, suggesting that other miR-21 targets are involved in glioblastoma apoptosis (Papagiannakopoulos et al., 2008). Maspin, RECK, and Sprouty1 have also been indicated to play roles in apoptosis, and all have been identified as direct miR-21 targets (Thum et al., 2008; Zhu et al., 2007).

In this work we examined the protein levels of four of these known targets in response to miR-21 knockdown in murine granulosa cells, and none showed any change (Figure VII-7). Our studies were unable to demonstrate that any of the known miR-21 target transcripts were factors in mediating apoptosis in granulosa cells. To facilitate the identification of granulosa cell miR-21 target transcripts, we conducted a

microarray study. No significant changes in mRNA expression between LNA-NS and LNA-21 treated cells were detected; despite the drastic change in cell morphology as the cells underwent apoptosis. Nonetheless, pathway analysis of genes exhibiting a nonsignificant 1.5-fold or greater difference indicated that apoptotic genes were over represented. Failure to change mRNA expression indicates that miR-21 is mediating its effects primarily through translational regulation and not mRNA degradation. This is in agreement with a recent study in MCF-7 breast cancer cells (Yang et al., 2009b) where knockdown of miR-21 caused changes in protein levels, without corresponding changes in mRNA expression. Furthermore, Yang et al. (Yang et al., 2009b) identified 58 putative miR-21 targets in their proteomic analysis. Examination of genes expressed in murine granulosa cells at 0 and 1 h post-hCG (microarray analysis see reference (Carletti and Christenson, 2009), indicated that 22 of these 58 genes are expressed in periovulatory granulosa cells. Further analysis of these genes will be necessary to determine if they are miR-21 target transcripts in ovarian granulosa cells.

During the LH-induced transition of a granulosa cell to a luteal cell, apoptosis must be inhibited to allow the formation of a functional corpus luteum. The cleavage of caspase 3 irrevocably commits the cell to apoptosis, and thus measurement of cleaved caspase 3 levels provides a measurement of the amount of cell death occurring. We examined levels of cleaved caspase 3 in granulosa cells following LH and found that cleaved caspase 3 levels decrease within 6 h of hCG, concurrent with an increase in levels of miR-21. In caspase 3 knockout mice, atretic follicles fail to

undergo atresia (Matikainen et al., 2001). To examine the effect of *in vivo* effect of miR-21 in granulosa cells, murine ovaries were injected with LNA-21pt and apoptosis was examined. Apoptosis was significantly increased in LNA-21pt treated ovaries. Here, the knockdown of miR-21 may prevent the necessary decrease in cleaved caspase 3 levels, thus promoting atresia. During atresia, granulosa cells undergo apoptosis and apoptotic bodies appear in the antral space, and our TUNEL assay does show staining within the granulosa and antrum of antral follicles (Matikainen et al., 2001; Figure VII-9F). These follicles may be undergoing atresia due to miR-21 knockdown. Further studies are needed to examine the expression of miR-21 in healthy and atretic granulosa cells to determine its importance in atresia. Unexpectedly, there was a relatively low amount of TUNEL staining within corpus luteum of the LNA-21pt injected ovaries. This may be because the apoptotic cells have already been cleared, and non-apoptotic cells (perhaps those that were not effectively transfected) remain. Interestingly, granulosa cells from polycystic ovarian syndrome patients show a decrease in the amount of cleaved caspase 3 and an increase in anti-apoptotic proteins, perhaps due to high LH levels in these patients (Das et al., 2008; Diao et al., 2004). It would be interesting to examine the expression of miR-21 in PCOS and non-PCOS granulosa cells

Our in vivo expression profiling studies indicate that miR-21 gene expression in ovarian granulosa cells is regulated predominantly by increased transcription of the pri-miR-21 transcript. The miR-21 promoter is well conserved across a number of species and lies within the 10^{th} intron of a protein-coding gene, TMEM49 (Fujita et

al., 2008). While at least one study has suggested that TMEM49 and miR-21 are co-expressed (Hu et al., 2008), we show here, in agreement with others, that TMEM49 and miR-21 are expressed independently (Fujita et al., 2008; Wickramasinghe et al., 2009). Our results also indicate that in vivo, miR-21 is regulated by the endocrine hormones, FSH and LH, in the preovulatory and periovulatory follicular granulosa cells, respectively. Within the first 300 bases of the miR-21 transcription start site, a number of known transcription regulatory element binding sites are located (Fujita et al., 2008). Promoter analysis studies indicate that at least 5 regulatory elements (i.e., AP-1, Ets/IPU.1, NF1, Stat3, CCAAT/enhancer binding protein) impact miR-21 expression in HL-60 cells and multiple myeloma cells (Fujita et al., 2008; Loffler et al., 2007). Of these transcription factors, LH is known to upregulate the expression of 3 of these transcription factors in mural granulosa cells (Carletti and Christenson, 2009; Sharma and Richards, 2000; Sterneck et al., 1997). Indeed, both Fos and Jun, which interact with AP-1 response elements was shown to be upregulated within 1 h of LH/hCG administration (Carletti and Christenson, 2009; Sharma and Richards, 2000). Distinct AP-1 binding factors, different from those upregulated by LH, are induced in response to FSH (2-8 h post-FSH) and may account for FSH-induced upregulation of pri-miR-21 at 12 h post-eCG (Sharma and Richards, 2000). In contrast to the positive evidence for a possible AP-1/Fos/Jun regulatory loop, evidence for CCAAT/enhancer binding protein site playing a role in miR-21 expression is lacking, as granulosa cell expression of C/EBPβ is not increased prior to pri-miR-21 expression (Sterneck et al., 1997). Moreover, examination of miR-21

levels in C/EBPβ KO mice granulosa cells showed no difference in expression (Supplementary Figure VII-3). While expression of mature miR-21 followed that of the pri-miR-21 in vivo, our in vitro studies suggested a much more complex regulatory mechanism.

Quantitative RT-PCR analysis of mature miR-21 levels in cultured granulosa cells failed to show an increase in response to 8-Br-cAMP treatment. This was unexpected as the other two LH-induced miRNA, miR-132 and miR-212, exhibited increased expression in cultured granulosa cells following 8-Br-cAMP treatment (Fiedler et al., 2008). Further examination of *in vitro* granulosa cell pri-miR-21 and mature miR-21 expression indicated that mature levels increased in the absence of a change in transcription (i.e., pri-miR-21). Recently, a similar observation in human smooth muscle cells was observed (Davis et al., 2008b). In these cells, members of the transforming growth factor superfamily (BMP4, BMP2, and TGFβ) increased expression of the precursor and mature miR-21 transcripts, without affecting pri-miR-21 expression (Davis et al., 2008b). The mechanism of action, indicated that the ligands activate the signal transducer SMAD, which in turn recruits a RNA helicase p68 subunit of the Drosha microprocessor complex to the pri-miR-21, thus stimulating the processing of the abundantly present pri-miR-21 transcripts (Davis et al., 2008b). The disconnection of LH activity and granulosa cell function in cultured granulosa cells is well known. A large body of literature in rodents from the early 1970s describes a phenomena known as "spontaneous luteinization" whereby granulosa cells when removed from their follicular environment take on the attributes

of luteal cells over a few days in culture (Channing, 1970; Channing and Ledwitz-Rigby, 1975). Interestingly, plating of the granulosa cells initiates some of the LH-mediated molecular events occurring in the follicle after LH, such as increases in transcription factors involved in cell survival and cell signaling (Gonzalez-Robayna et al., 1999; Murphy, 2000). The phenomena of spontaneous luteinization has never fully been explained but is thought to result from a withdrawal of inhibitory signaling molecules once cells are dispersed and placed into culture. In these studies we show that levels of mature miR-21 increase in the absence of increased transcription. It is interesting to speculate that miR-21 levels may be one of several factors that contribute to the phenotypic changes occurring in luteinizing granulosa cells. The fact that most cancer cell lines and tumors also exhibit increased levels of miR-21 may be indicative of their disconnection with factors that normally prevent excessive miR-21 accumulation, which is then able to block apoptosis and promote oncogenesis.

In conclusion, this study has demonstrated that miR-21 is transcriptionally upregulated *in vivo* in response to LH, and we show that this upregulation occurs synchronously with a decrease in cleaved caspase 3 levels. In addition, we have provided evidence that both *in vitro* and *in vivo* miR-21 knockdown causes an increase in granulosa cell apoptosis. These results suggest that miR-21 plays an important role in post-transcriptionally regulating transcripts that are involved in preventing apoptosis in LH-induced terminally differentiating granulosa cells.

6. Supplemental Data.

Methods

PTEN Knockdown: Cultured granulosa cells were transfected with PTEN siRNA (20nM, Ambion) or control siRNA (20nM, Ambion) in Lipofectamine 2000 (Invitrogen)48 h after culture. 24 h after siRNA transfection, cells were transfected with LNA-NS or LNA-21 as above. Cells were collected after 24 h for apoptosis analysis (Annexin V, live/dead staining) as above, or collected in cell lysis buffer for Western blot analysis.

PTEN Blockers: LNA oligonucleotides were designed to span miR-21 binding sites in the PTEN 3'UTR and prevent miR-21 binding (see Klein…Goodman 2007). Two blockers were designed to two different miR-21 binding sites; blocker 1: 5'-cagcccatcctttgttgaaagc-3', blocker 2: 5'-cagccaatctctcggatgtcca-3'; and a control blocker designed to a site within the 3'UTR to which no known miRNA is predicted to bind (TargetScan): control blocker: 5'-ctctagtgggtcctatgcaatc-3'. Blockers (10nM) were transfected into cultured granulosa cells 48 h after culture. Cells were collected after 24 h for apoptosis analysis (Annexin V, live/dead staining) as above.

FIGURE VII-S1. *PTEN siRNA or PTEN "blockers" do not affect cell viability*. (A) Apoptosis assay (Annexin and Live/Dead stain) of granulosa cells (n=3) transfected with control or PTEN siRNA alone, or co-transfected with control or PTEN siRNA and LNA-NS or LNA-21. (B) Apoptosis assay (Annexin and Live/Dead stain) of granulosa cells (n=3) transfected with a control oligonucleotide or an oligonucleotide that blocks either one of the two miR-21 binding sites in the PTEN 3'UTR (blocker 1 or blocker 2), or co-transfected with both blockers (blocker 1+2). One-way ANOVA was used to determine statistical significance; ^{a,b}means ± SEM with different superscripts are different ($p<0.05$).

Figure VII-S1

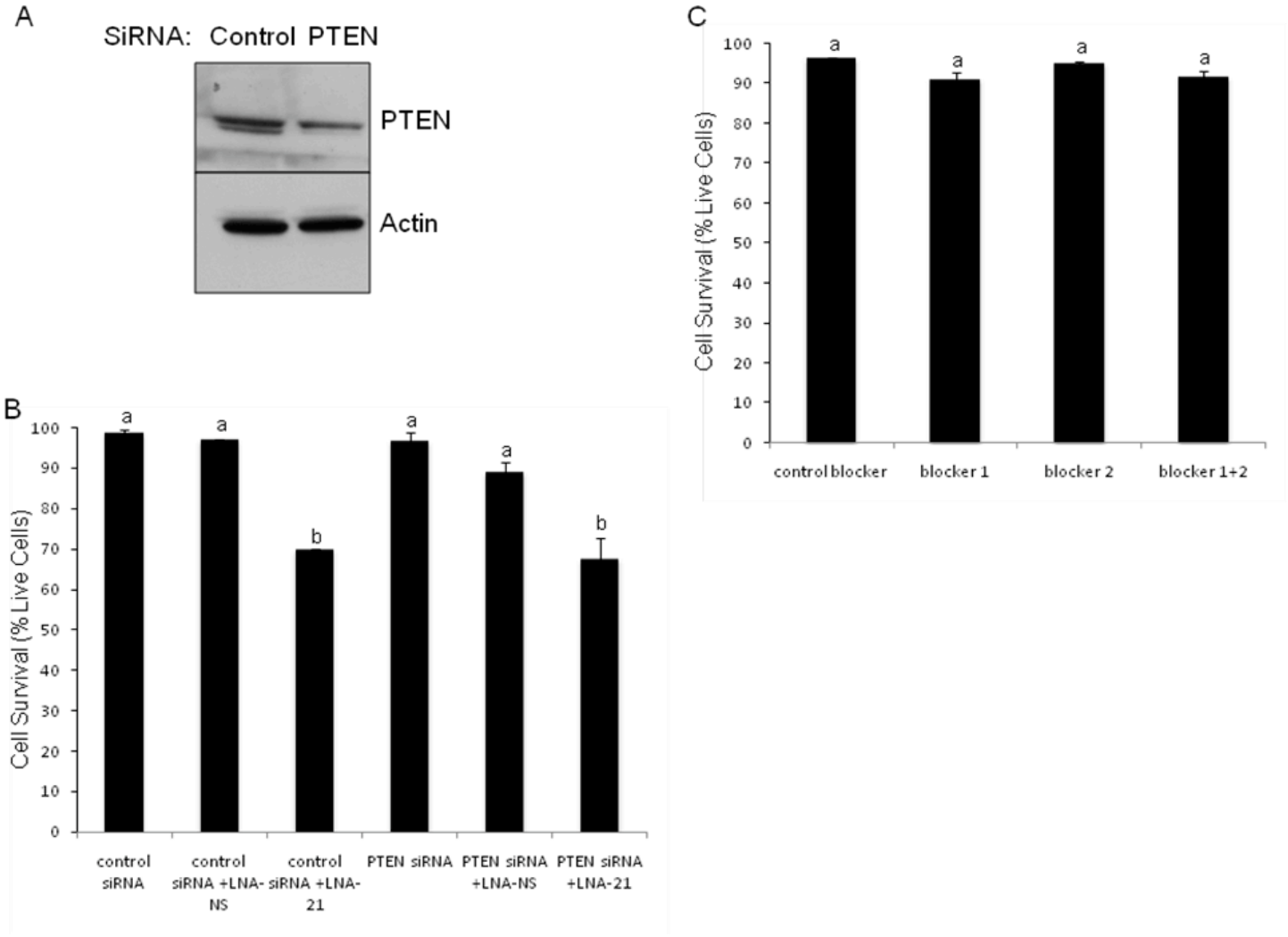


FIGURE VII-S2. *Fluorescence-tagged LNA-NSpt allows visualization of transfection efficiency in bursal-injected ovaries.* Representative fluorescence image (n=3) of a mouse ovary treated with fluorescence-tagged LNA-NSpt (A) stained with the DNA-binding dye Hoechst. Negative control TUNEL assay (B; non-working enzyme) in LNA-NSpt treated mouse ovary. Panel C is a 10X magnification of B. Neighboring section (D) of the LNA-NSpt treated ovary from A stained with the DNA-binding dye Hoechst. TUNEL assay to detect apoptotic cells in LNA-NS treated mouse ovary (E). Panel F is a 10X magnification of E. Bar = 500um.

Figure VII-S2

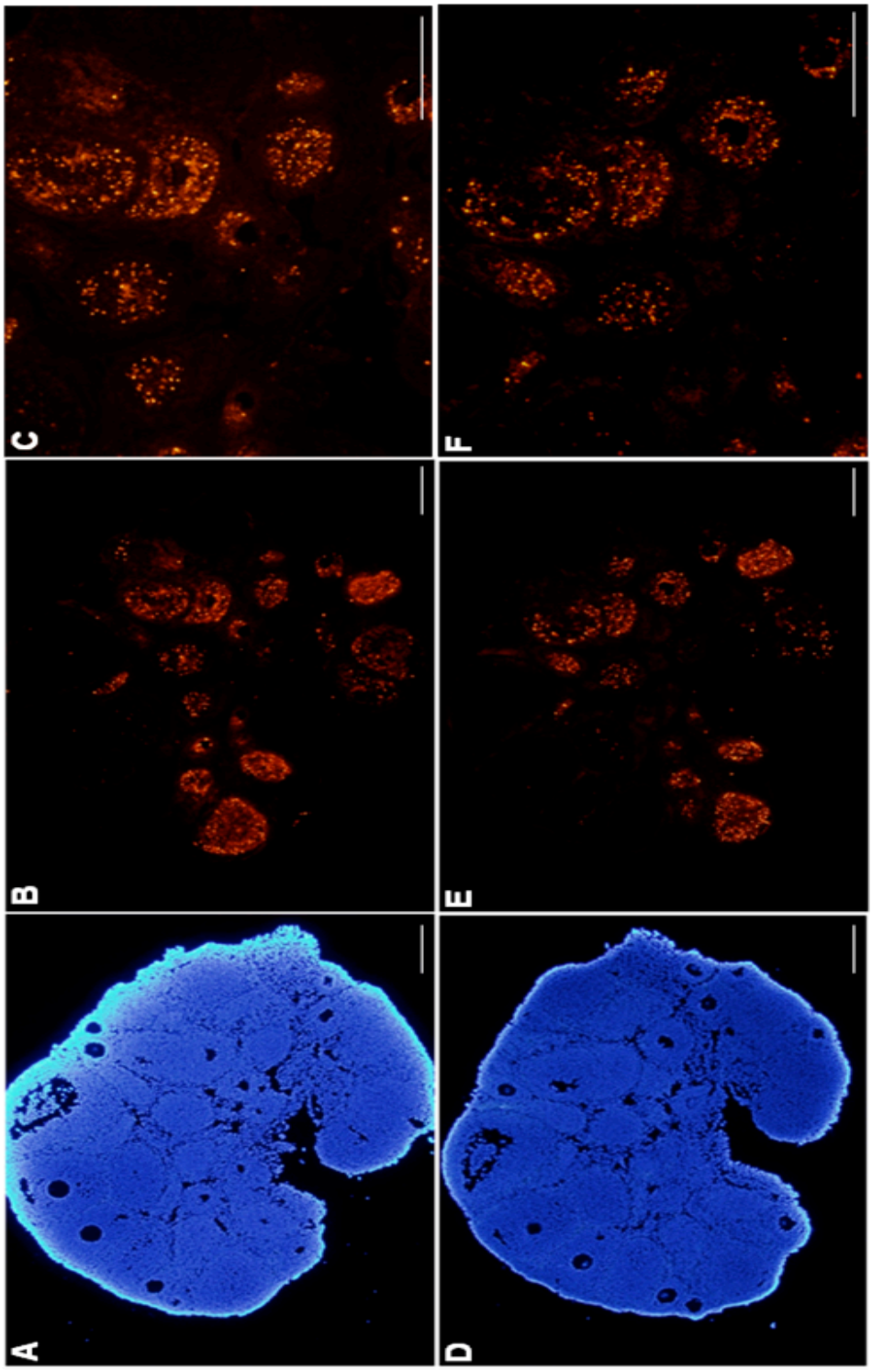
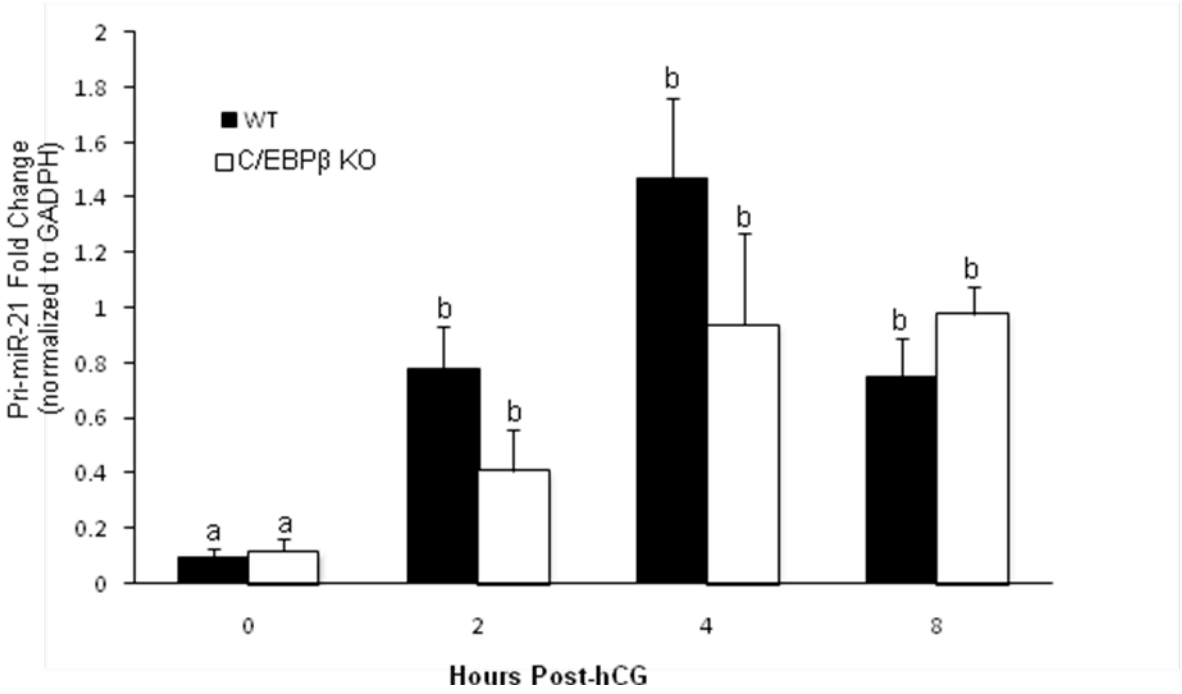


FIGURE VII-S3. *Pri-miR-21 levels increase following hCG in C/EBPβ wildtype and knockout mice.* Quantitative RT-PCR of pri-miR-21 in murine granulosa cells with eCG alone (0h) or 2, 4, or 8 h following hCG injection in C/EBPβ wildtype (WT; black bars) or knockout (KO; white bars) mice. All qRT-PCR data (n=4) were normalized to GAPDH; Two-way ANOVA (C) was used to determine statistical significance; ^{a,b}means ± SEM with different superscripts are different ($p<0.05$).

Figure VII-S3



VIII. Chapter Four:

MicroRNA-21 Enhances Global Translation

1. Abstract

Ovarian follicular granulosa cells respond to the ovulatory surge of luteinizing hormone with hypertrophy and neovascularization to form the corpus luteum. The rate of growth of the newly formed luteal tissue exceeds that of the most aggressive growing tumors, and many of the cellular/molecular events that occur during this transition are characteristic of transformed cells. We recently identified the "oncomiR" microRNA-21 (miR-21) as upregulated in mouse granulosa cells during luteinization. To determine the function of miR-21, granulosa cells were cultured in the presence of blocking 2'-O-methyl (anti-21) and locked nucleic acid (LNA-21) oligonucleotides. Both anti-21 and LNA-21 increased elongation factor 2 (EF2) phosphorylation. Phosphorylation of EF2 is associated with decreased protein synthesis, and ^{35}S incorporation into granulosa cell protein was 3-fold ($p<0.05$) lower in the absence of miR-21. Additionally, treatment with pre-miR-21 increased global translation 2.5-fold ($p<0.05$) compared to controls. Since EF2 phosphorylation cannot be directly affected by miR-21, Akt/mTOR (upstream modulators of EF2) were examined and also shown to be differentially phosphorylated by both knockdown and overexpression of miR-21. Furthermore, phosphorylated EF2 levels decreased *in vivo* following hCG. These results suggest that miRNA-21 plays a role in promoting global translation in granulosa cells following LH. This increase in global translation could play an important role in mediating the hypertrophy that occurs during the granulosa to luteal cell transition.

2. Introduction

Evidence for microRNA (miRNA) involvement in ovarian function has recently been described in mice lacking Dicer (Hong et al., 2008; Nagaraja et al., 2008; Otsuka et al., 2008). Conditional deletion of Dicer in murine granulosa cells using *the* anti-Mullerian hormone receptor-2 Cre recombinase caused a pronounced reduction in ovulation rates, increased incidence of trapped oocytes within luteinized follicles, and increased granulosa cell apoptosis (Hong et al., 2008; Nagaraja et al., 2008). Additionally, female Dicer hypomorph mice, which have ~20% of normal Dicer levels and a global reduction in miRNA expression, are infertile as a result of ovarian dysfunction (Otsuka et al., 2008). Otsuka et al. go on to show that loss of two specific miRNA involved in angiogenesis, miR-17-5p and let-7b, contributed to the defect in ovarian function, and that replacement was able to partially correct the angiogenic defect, but not fertility (Otsuka et al., 2008). These studies clearly implicate Dicer and its enzymatic byproducts, miRNA, within somatic cells of the ovary as playing a key role in ovarian function.

In order to identify the potential miRNA involved in modulating ovarian function, our laboratory compared miRNA gene expression using microarrays of granulosa cells collected before and 4 h after an ovulatory surge of LH/hCG (Fiedler et al., 2008). We identified three highly upregulated miRNA, miR-21, miR-132 and miR-212, and demonstrated in the case of miR-132 and miR-212 that expression was inducible by cyclic AMP, the primary transducer of LH action (Fiedler et al., 2008). While *in vitro* knockdown of miR-132 and miR-212 did impact the expression of c-

terminal binding protein -1 a previously reported target (Vo et al., 2005) it failed to dramatically modulate granulosa cell function and/or viability (Fiedler et al., 2008). Examination of miR-21 expression in granulosa cells demonstrated a unique expression pattern, as *in vivo* miR-21 expression was clearly regulated at the transcriptional level with primary miR-21 levels preceding changes in mature miR-21 levels (Carletti, 2009), while *in vitro*, mature miR-21 levels increased in the absence of preceding changes in transcription, and did not show a further increase upon cAMP stimulation (Carletti, 2009). Also in contrast to miR-132 and miR-212, knockdown of miR-21 in granulosa cells had a dramatic effect on cell viability as evidenced by the rapid induction of apoptosis both *in vitro* and *in vivo* following treatment with locked nucleic acid oligonucleotides complementary to miR-21 (Carletti, 2009).

Interestingly, comparison of antisense 2'O-methyl (anti-21) and antisense locked nucleic acid (LNA-21) oligonucleotides demonstrated different responses, likely due to the level of knockdown achieved with the two approaches (Carletti, 2009). Treatment of granulosa cells with anti-21 decreased miR-21 levels 9-fold, while the more potent LNA-21 reduced miR-21 levels 27-fold and elicited the increase in granulosa cell apoptosis (Carletti, 2009). Knockdown of miR-21 increases apoptosis in several cancer cell lines, including glioblastoma cell lines (Chan et al., 2005), breast cancer MCF-7 cells (Si et al., 2007), gastric cancer cell lines (Zhang et al., 2008b), and hepatocellular carcinoma cell lines (Connolly et al., 2008). We showed for the first time that miR-21 plays an anti-apoptotic role in a physiological system. Several targets have been identified for miR-21 that explain its

anti-apoptotic role, including the pro-apoptotic phosphatase and tensin homologue (PTEN), programmed cell death 4 (PDCD4), tropomyosin 1 (TPM1), Sprouty, reversion inducing-cystein-rich protein with kazal motifs (RECK), heterogenous nuclear ribonucleoprotein K (HNRPK), and tumor protein p63 (Tap63) (Chen et al., 2008b; Meng et al., 2006; Papagiannakopoulos et al., 2008; Thum et al., 2008; Zhu et al., 2007). Because of the anti-apoptotic role of miR-21, as well as reported stimulatory roles on proliferation, hypertrophy, differentiation, and a drastic upregulation of mir-21 in virtually every cancer/tumor examined, miR-21 is suggested to play an important role in the initiation and progression of cancer.

In our previous study, we examined protein expression of known miR-21 targets in LNA-21 treated cells (PTEN, PDCD4, TPM1, Sprouty (Chen et al., 2008b; Meng et al., 2006; Papagiannakopoulos et al., 2008; Thum et al., 2008; Zhu et al., 2007)) and did not demonstrate any to be significantly changed upon miR-21 knockdown, indicating that these are not miR-21 targets in granulosa cells. In attempt to identify novel targets of miR-21 that may be responsible for its anti-apoptotic role, we performed gene expression analysis on cultured granulosa cells treated with either a scrambled LNA (LNA-NS) or with LNA-21. However, there were no significant differences in levels of any mRNA (Carletti, 2009). Recently Yang et al. reported that miR-21 appears to act primarily through mRNA translational repression, rather than through mRNA degradation, and thus miR-21 knockdown does not have a significant effect on mRNA levels of its targets (Yang et al., 2009b). In fact, mRNA levels for the miR-21 targets PTEN, TPM1, and PDCD4 did not

change in LNA-21 treated cells, while protein levels increased (Asangani et al., 2008; Meng et al., 2007; Zhu et al., 2007). Thus, proteomics analysis is necessary to fully understand the effect miR-21 is having on protein expression in the cell.

Therefore, while our previous studies illustrate that miR-21 is playing an essential role in granulosa cell survival, the targets transcripts via which miR-21 acts on to prevent apoptosis remains to be determined. In the study, we have chosen to use a direct proteomic approach (i e., two-dimensional gel electrophoresis) to elucidate the potential miR-21 target proteins (Zhu et al., 2007). Interestingly, we are able to further our understanding of the function of miR-21 in ovarian granulosa cells, and show that miR-21 not only inhibits granulosa cell apoptosis, but also regulates global translation within the granulosa cell.

3. Experimental Procedures

In Vitro Granulosa Cell Culture. Granulosa cells were cultured as previously described (Fiedler et al., 2008). Briefly, ovaries from 26-day old CF-1 mice were isolated and incubated for 15 min in M-199 media containing 0.5M sucrose. Ovarian follicles were punctured into M199 media to release granulosa cells. Granulosa cells were seeded at $2.5x10^4$ cells per well into 6-well culture plates or $1.5x10^6$ cells per 10 cm culture dish. Wells and plates were coated with fibronectin before use. Cells were cultured in DMEM/F12 (10%FBS, 1% gentamicin) at 37°C with 5% CO_2.

Oligonucleotide Transfection. Transfections were performed as previously described (Fiedler et al., 2008). Briefly, control (i.e., nonspecific scrambled; anti-NS) and anti-miR-21 (anti-21) 2'-O-methyl RNA oligonucleotides were purchased from Ambion (Houston, TX). Control (nonspecific; pre-NS) and pre-miR-21 (pre-21) were also purchased from Ambion (Houston, TX). Locked nucleic acid (LNA) antisense molecules were synthesized as mixed LNA/DNA oligonucleotides with the following sequences: LNA-21, 5'-tcagtctgataagcta-3', and control LNA-NS, 5'-cgtcagtatgcgaatc-3' (underlined bases denote the LNA nucleotides) (IDT, Coralville, IA). Oligonucleotides were individually complexed with Lipofectamine 2000 reagent (Invitrogen) and added to the isolated granulosa cells 48 h after culture at a final concentration of 42 nM. Cells were transfected for 4, 8, or 24 h for protein analysis, or transfected for 8 h for [^{35}S] incorporation analysis.

Two-Dimensional Gel Electrophoresis. Following two days of culture, cells were plated and transfected as above. Transfected cells were collected after either 8 h (for LNA-NS and LNA-21 treated cells) or 24 h (for anti-NS and anti-21 treated cells) into 150 uL cold lysis buffer (Cell Signaling) containing 1mM PMSF, 40uM phenylarsine oxide (Sigma), and 10uM calyculin A (Calbiochem). Protein concentration was determined using Bio-Rad protein assay (Hercules, CA). Protein extracts (300ug/treatment) were sent directly to Applied Biomics (Hayward, CA) for 2-D DIGE and mass spectrometry analysis. Briefly, protein extract from control cells (anti-NS or LNA-NS) was covalently linked to Cy3 and protein extract from treated cells (anti-21 or LNA-21) was covalently linked to Cy5. Labeled proteins were

separated on a single 2-dimensional gel, with first dimension isoelectric focusing and second dimension SDS-PAGE. An image of the gel was taken at excitation wavelengths of 550nm (Cy3) and 650nm (Cy5) and images were overlayed using DeCyder software to find differentially regulated proteins. Differentially regulated proteins were picked and identified by mass spectrometry (MALDI/TOF/TOF).

Immunoblotting. Granulosa cell protein was collected into lysis buffer as above. Protein concentration of granulosa cell lysates was quantified using the Bio-Rad protein assay (Hercules, CA). Five to fifteen ug of each sample was denatured by diluting the sample 1:2 with sample buffer (2.8mL distilled water; 1.0mL 0.5M Tris-HCl, pH6.8; 0.8mL glycerol; 1.6mL 10% SDS; 0.4mL 2-β-mercaptoethanol; 0.4mL 0.05% (w/v) bromophenol blue) and heating for 5 minutes at 95°C. The proteins were separated on 12% SDS-PAGE gels in 5X electrode running buffer, pH 8.3 (25mM Tris base; 192mM glycine; 0.1% (w/v) SDS), and transferred to PVDF membranes (Millipore, cat# IPVH20200) in transfer buffer (12mM Tris-HCl; 96mM glycine; 20% (v/v) MeOH). Blots were incubated for 1 h at RT in a 5% milk solution to block nonspecific binding. Blots were then incubated overnight at 4°C with one of the following antibodies: EF2 (Cell Signaling #2332), phospho-EF2 (Thr56; Cell Signaling #2331), Akt (Cell Signaling #9272), phospho-Akt (Ser473; Cell Signaling #9271), TSC1 (Cell Signaling #4906), TSC2 (Cell Signaling #3612), phospho-TSC2 (Thr1462; Cell Signaling #3611), mTOR (Cell Signaling #2983), phospho-mTOR (Ser2448; Cell Signaling #2971), p70S6k (Cell Signaling #9202), phospho-p70S6k

(Thr389; Cell Signaling #9206), 4EBP1 (Cell Signaling #9452), phospho-4EBP1 (Thr37/46; Cell Signaling #9459), PPP2CA (Novus Biologicals NB100-844), and actin (Santa Cruz). After washing and incubation with the appropriate secondary antibody, protein-antibody complexes were visualized using West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's protocol.

Analysis of protein synthesis by [^{35}S] incorporation. Granulosa cells were cultured as above and transfected for 8 h with LNA-NS or LNA-21. Cells were then washed and incubated for 2 h with methionine-free DMEM. Then, 1mL of the methionine-free DMEM was added together with 5 μCi of [^{35}S] methionine (PerkinElmer, Boston) and cells incubated for 1 h at 37°C. Cells were lysed in 1mL lysis buffer, and then trichloroacetic acid (TCA) was added to a final concentration of 10% and cells were put on ice for 10 min. Precipitated protein was pelleted by spinning at 12000 x g for 10 minutes. TCA was extracted by addition of ether, and then 1M Tris base (pH 8.0) was added dropwise. The solution was placed on the scintillation counter and radiolabel was quantified to give the amount of "incorporated" label.

4. Results and Figures

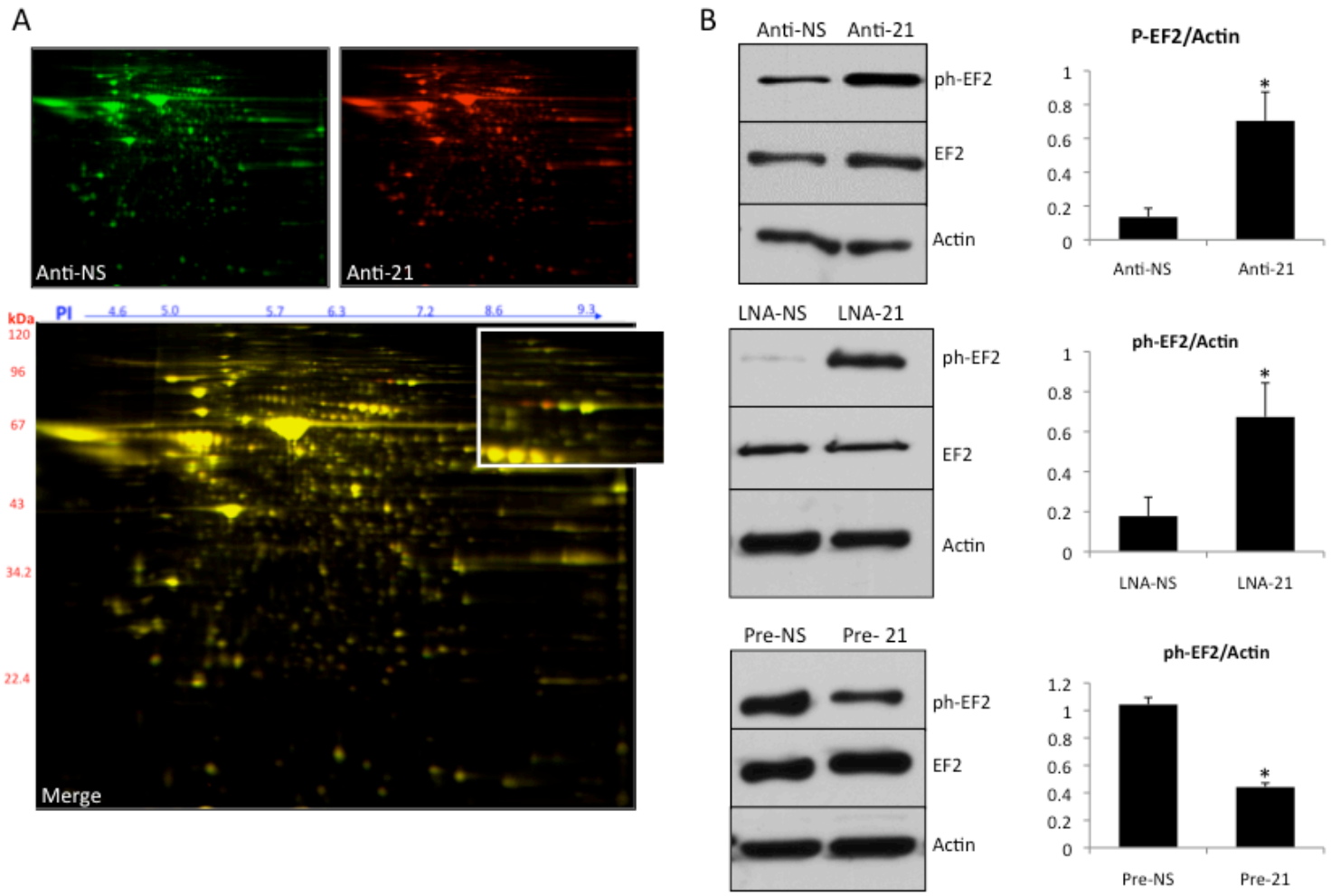
Phosphorylation of Elongation Factor 2 is Increased Following Knockdown of MicroRNA-21 in Cultured Murine Granulosa Cells. To identify targets of miR-21, protein was collected from cultured granulosa cells transfected with anti-NS or anti-21 for 24 h. Isolated protein was labeled with Cy3 (anti-NS) or Cy5 (anti-21) and subjected to two-dimensional gel electrophoresis. Changed proteins were visualized

as red or green, indicating upregulated or downregulated proteins, respectively (Figure VIII-1). Three spots were detected as changed between LNA-NS and LNA-21, and all three spots exhibited the same molecular weight. Mass spectrometry identified all three spots as elongation factor 2. (Figure VIII-1A inset and VIII-1B). Elongation factor 2 has two primary phosphorylation sites (Nairn et al., 2001), and the two spots upregulated in the anti-21 treated cells (red, Figure VIII-1) correspond to an increase in EF2 phosphorylation at these two spots. One EF2 spot was downregulated (green, Figure VIII-1A), the unphosphorylated form of EF2. Western blot analysis confirmed the upregulation in phospho-EF2 in anti-21 cells (Figure VIII-1B).

Because microRNA cannot directly affect protein phosphorylation. a second 2-D gel/tandem mass spectrometry analysis at an earlier timepoint (8 h) was completed using a more potent (LNA-21) miR-21 inhibitor. In this analysis a number of proteins were identified (Supplemental Table VIII-1) including elongation factor 2. Ingenuity pathway analysis (IPA) was used to determine if any of these proteins were linked to regulation of EF-2 and apoptosis and, with the exception of

FIGURE VIII-1. (A) Two-dimensional gel electrophoresis of fluorescent labeled protein from anti-NA (control, Cy3) or anti-21 (Cy5) treated granulosa cells. Isoelectric focusing was carried out in pH3-10 and second dimensional separation was carried out on 8-14% SDS-PAGE. Inset is three spots that represent elongation factor 2 (EF2). (B) Top, Western blotting confirming the upregulation of phospho-EF2 following Anti-21 treatment or, middle, LNA-21 treatment. Bottom, Western blotting indicating that phospho-EF2 is decreased following Pre-21 treatment.

Figure VIII-1



Annexin A5, none of the proteins linked to either of these pathways. Furthermore, none of the proteins had recognizable miR-21 target sites in their 3'UTR using 4 different miRNA prediction algorithms (Supplemental Table VIII-1). Additionally, changes in Annexin A5 could not be validated by Western blot analysis. Increased EF-2 phosphorylation was confirmed following LNA-21 treatment (Figure VIII-1C). The differential response was greater in LNA-21 versus antimir-21 treated cells and this was correlated with the 3-fold greater reduction in miR-21 levels in the LNA-21 treated cells (Carletti, 2009).

Exogenous administration of pre-miR-miR-21 to cultured granulosa cells increased mature miR-21 levels 95-fold above basal levels within 24 h after transfection (Supplemental Figure VIII-2). Levels of phospho-EF2 significantly decreased upon pre-miR-21 treatment compared to control (pre-NS) transfected cells, while total EF2 levels remained unchanged (Figure VIII-1B).

In a further attempt to identify targets specific to induction of apoptosis, proteins from granulosa cells treated with LNA-21 were compared to antimir-21 treated cells. Two-dimensional gel electrophoresis identified more than 40 spots, 14 of these were upregulated in LNA-21 treated cells, and thus would be direct pro-apoptotic targets of miR-21. Mass spectrometry identified these proteins (Supplemental Table VIII-2), but none of these have been previously linked to apoptosis by Ingenuity Pathway Analysis. Elongation factor 2 did not appear on this 2-dimensional gel as expected, since both treatments increase its phosphorylation status.

Global translation decreases following knock down of miR-21. Elongation factor 2 is a necessary for translational elongation, and phosphorylation of EF2 inhibits its activity by preventing its ability to associate with ribosomes (Carlberg et al., 1990). Cultured granulosa cells transfected with LNA-21 for 8 h exhibited a 3-fold decrease in the amount of incorporated ^{35}S-methionine compared to the LNA-NS control (Figure VIII-2A). Transfection with pre-21 caused a 2.5-fold increase in incorporated ^{35}S-methionine compared to Pre-NS control (Figure VIII-2B).

MicroRNA-21 knockdown caused deactivation of the Akt/mTOR pathway. Elongation factor 2 is downstream of the Akt and mTOR pathways (Connolly et al., 2006), and therefore we explored the activation of these pathways following miR-21 knockdown. Both mTOR and Akt are deactivated when dephosphorylated, resulting in deactivation (i.e., phosphorylation) of EF2 (Connolly et al., 2006). Both total and phosphorylated Akt levels decreased following miR-21 knockdown (Figure VIII-3). TSC2, a direct target of Akt, showed a slight decrease in phosphorylated levels, while total levels remained unchanged (Figure VIII-3). The structural component of the TSC complex, TSC1, also was unchanged. When dephosphorylated, the TSC1/2 complex inhibits mTOR phosphorylation and activity. mTOR phosphorylation decreased following miR-21 knockdown, while total levels were unchanged (Figure VIII-3). 4EBP1 and p70S6kinase are both direct targets of mTOR, and both showed

FIGURE VIII-2. Granulosa cells transfected with LNA-21 (A) or pre-21 (B) were pulse labeled with ^{35}S methionine for 1 h and incorporated label was detected in TCA precipitated protein pellets. *Means ± SEM are different ($p<0.05$).

Figure VIII-2

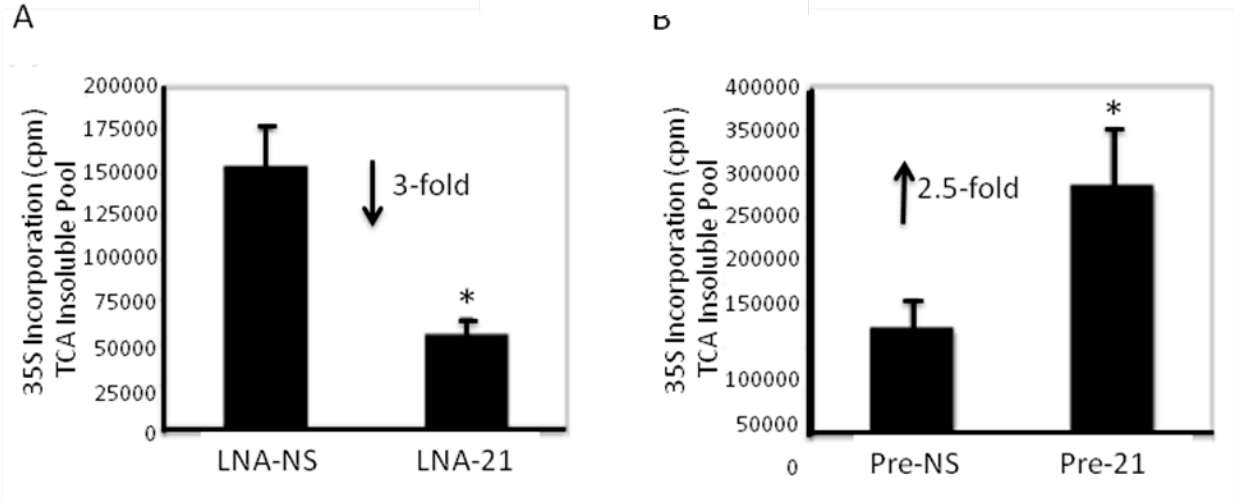
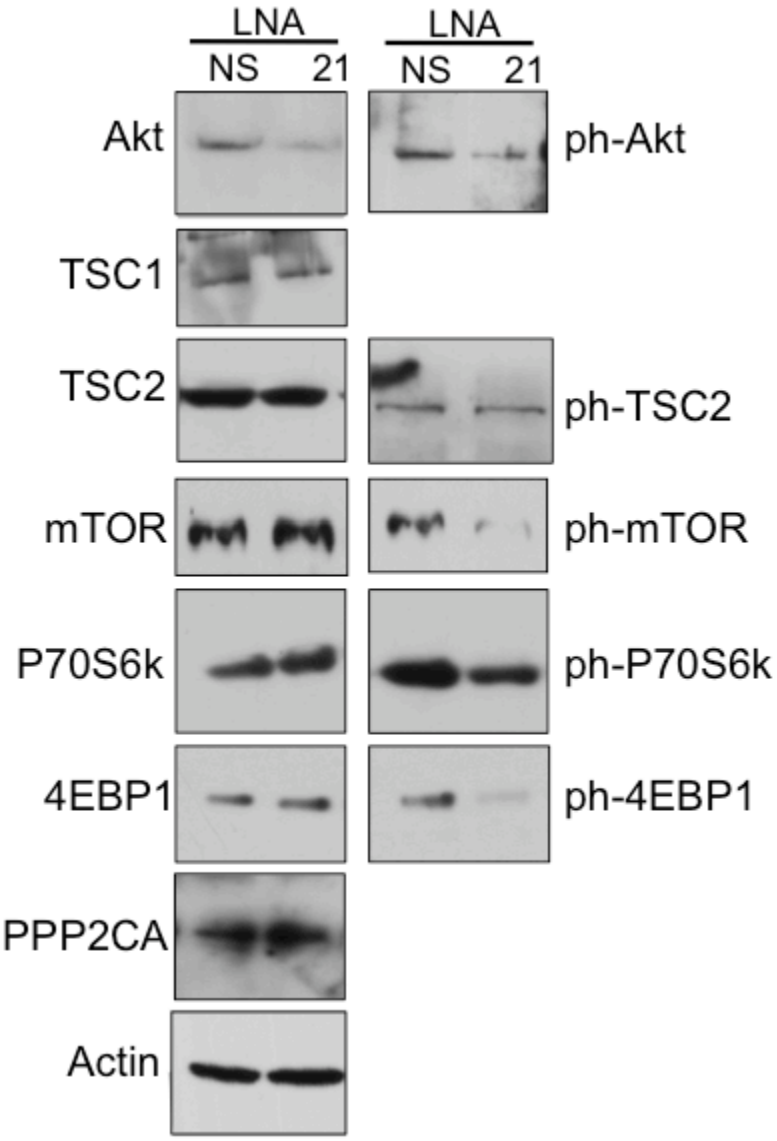


FIGURE VIII-3. Representative western blots (n=3) of proteins (total, left panel, or phosphorylated forms, right panel) upstream of EF2 following miR-21 knockdown.

Figure VIII-3



a decrease in phosphorylation following miR-21 knockdown. Levels of the direct activator of EF2, PPP2CA, were not changed (Figure VIII-3).

Total EF2 levels increase following hCG, and phospho-EF2 levels decrease. Previously, we have shown that miR-21 increases within 4 h post-hCG. Examination of phospho-EF2 levels following hCG showed that total EF2 levels increase within 1 h post-hCG and phospho-EF2 levels decrease by 4 h post-hCG (Figure VIII-4).

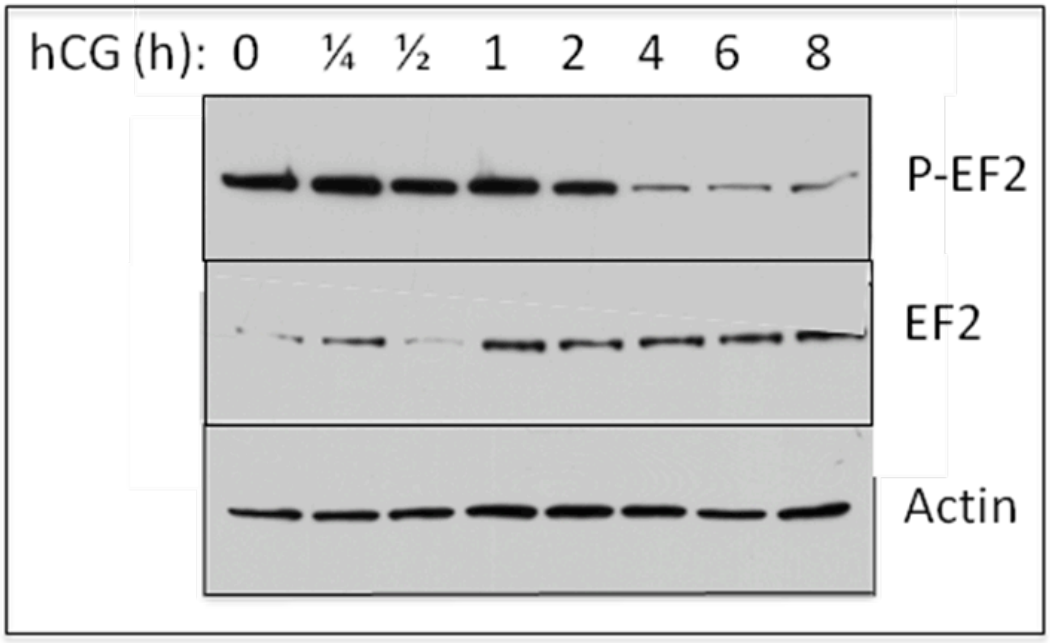
5. Discussion

Previously we have shown that miR-21 is upregulated following LH and, in agreement with others, has anti-apoptotic function. Here we show for the first time a role for miR-21 in promoting global translation.

MicroRNA-21 is upregulated in a variety of cancers and tumors (for review, Krichevsky and Gabriely, 2009). MicroRNA-21 has been implicated in the inhibition of apoptosis and the promotion of cell growth and cell proliferation, and is upregulated during ES cell and T cell differentiation (Singh et al., 2008; Wu et al., 2007). MicroRNA-21 levels have also been shown to increase during cardiac cell hypertrophy, however a mechanism has not been identified (Cheng et al., 2007). Bon a fide targets have been identified for miR-21, including the pro-apoptotic PTEN, PDCD4, TPM1, Sprouty, HNRPK, and Tap63 (Chen et al., 2008b; Meng et al., 2006; Papagiannakopoulos et al., 2008; Thum et al., 2008; Zhu et al., 2007). In addition, miR-21 expression has been shown to influence several cell signaling pathways,

FIGURE VIII-4. Representative western blots (n=3) of phosphorylated EF2, EF2, and control actin after 0, ¼, ½, 1, 2, 4, 6, and 8 h after hCG administration.

Figure VIII-4



including the ERK-MAP kinase pathway through inhibition of Sprouty (Thum et al., 2008), the Akt pathway, perhaps through inhibition of PTEN (Zhang et al., 2009), and

Elongation factor 2 promotes the translocation of the newly formed mRNA – tRNA complex from the A site in the ribosome to the P site (Carlberg et al., 1990; Nairn and Palfrey, 1987; Ryazanov et al., 1988). Phosphorylation of EF2 by EF2 kinase prevents the ability of EF2 to bind to the ribosome, which almost completely halts protein synthesis (Carlberg et al., 1990; Nairn and Palfrey, 1987; Ryazanov et al., 1988). EF2 kinase is activated by mammalian target of rapamycin (mTOR) (Browne and Proud, 2004), a large, multidomain kinase that plays a key role in cell growth and proliferation, apoptosis, differentiation, and oncogenesis (Hedhli et al., 2005; Meric-Bernstam and Gonzalez-Angulo, 2009; Schmelzle and Hall, 2000; Tyler et al., 2009). Besides EF2, mTOR also stimulates the translation initiator p70S6 kinase (p70S6K) and inhibits the translation suppressor 4EBP1 (Ma and Blenis, 2009). mTOR is activated by the phosphoinositide 3-kinase (PI3K) – Akt pathway (Sekulic et al., 2000), which inhibits an inhibitor of mTOR, the tumor suppressor complex TSC1/2 (Potter et al., 2002). Here we show that miR-21 regulates the activation of the Akt/mTOR pathway, as all members of the pathway examined demonstrate a change in phosphorylation/activity following miR-21 knockdown (Figure VIII-5).

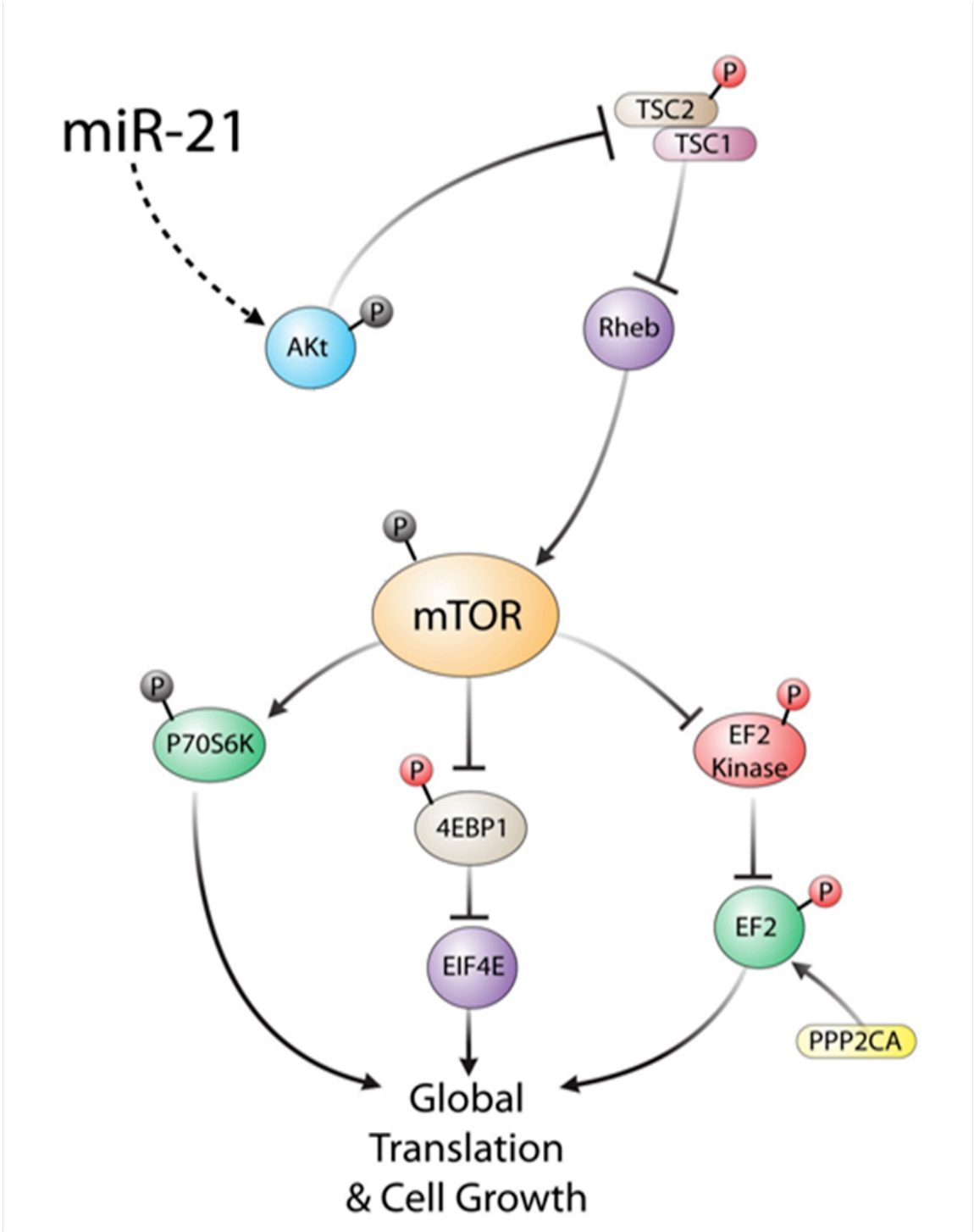
Interestingly, while only the phosphorylation statuses of most of the proteins in the Akt/mTOR pathway were affected by miR-21 knockdown, and not their total levels. However, total levels of Akt were reduced. Several possibilities can explain

this decrease. First, Akt mRNA could be a direct target of miR-21. However, bioinformatics analysis of the Akt 3'UTR does not indicate any miR-21 binding sites. In addition, the decline in Akt levels following miR-21 knockdown would suggest that miR-21 is acting by enhancing Akt levels, and while this phenomenon has been described, it does not appear to be a common miRNA mechanism. It is possible, however, that miR-21 targets and down regulates a transcriptional repressor of Akt. In fact, a predicted transcriptional repressor of Akt, estrogen receptor alpha, has fairly conserved miR-21 binding sites in its 3'UTR (Miranda et al., 2006). Another possibility is that Akt levels are reduced due to the suppression of global translation caused by miR-21 knockdown. Akt has a short half life (~180 minutes; (Baker et al., 2005)), and thus the change in translation could have a more rapid effect on Akt protein levels compared to the protein levels of proteins with longer half lives, such as mTOR, which has a half life of 18 h (Polak and Hall, 2009). Studies are ongoing to examine the protein expression levels of other short-half life proteins.

Previously we have shown that miR-21 knockdown induces apoptosis in periovulatory granulosa cells through a novel mechanism (Carletti, 2009). Akt activity is necessary for cell survival (Datta et al., 1999), and thus the downregulation of Akt induced by miR-21 knockdown could cause the observed increase in apoptosis.

FIGURE VIII-5. Model of miR-21 action on the Akt/mTOR pathway. Akt, mTOR, and p70S6k are activated by phosphorylation (gray circles), and the others are inactivated by phosphorylation (red circles).

Figure VII-5.


miR-21
TSC2
P
TSC1
AKt
P
Rheb
mTOR
P
P70S6K
P
4EBP1
P
EIF4E
EF2
Kinase
P
EF2
P
PPP2CA
Global
Translation
& Cell Growth

6. Supplemental Data

TABLE VIII-S1. Proteins Changed between LNA-NS and LNA-21 transfected cells (8 h) as determined by two-dimensional gel electrophoresis and mass spectrometry. The 3'UTR for changed proteins was analyzed using four different bioinformatic tools for predicted miR-21 binding sites.

Table VIII-S1

Tandem MSMS Predicted Protein	Fold Change	miR-21 sites in 3'UTR			
		PicTar	MiRanda	TargetScan	RNA22
EF2	1.94	no	no	no	no
Proliferation-Associated 2G4	1.79	no	no	no	no
Sorcin	1.77	no	no	no	no
Protein Disulfide Isomerase-Related Protein	1.68	no	no		no
COMM Domain Containing 3	1.68	no	no	no	no
Triosephosphate Isomerase 1	1.66	no	no	no	no
Cai Protein	1.62	no	no	no	no
Annexin A5	1.61	no	no	no	no
Heat Shock Protein 1 (chaperonin)	1.6	no	no	no	yes - 1
Chain A, Structure of Recombinant Mouse L Chain Ferritin	1.58	no	no	no	no
ATP Synthase, H+ Transporting Mitochondrial F1 Complex	1.57	no	no	no	no
Transaldolase 1	1.56	no	no	no	no
Annexin A1	1.52	no	no	no	no
Putative Beta-Actin	1.5	no	no	no	no
Lamin Isoform A	1.41	no	no	no	yes - 1
Septin 2	1.39	no	no	no	no
Ran Protein	1.26	no	no	no	no
Prolyl 4-hydroxylase, beta polypeptide	1.16	no	no	no	no
Vimentin	-1.75	no	no	no	no

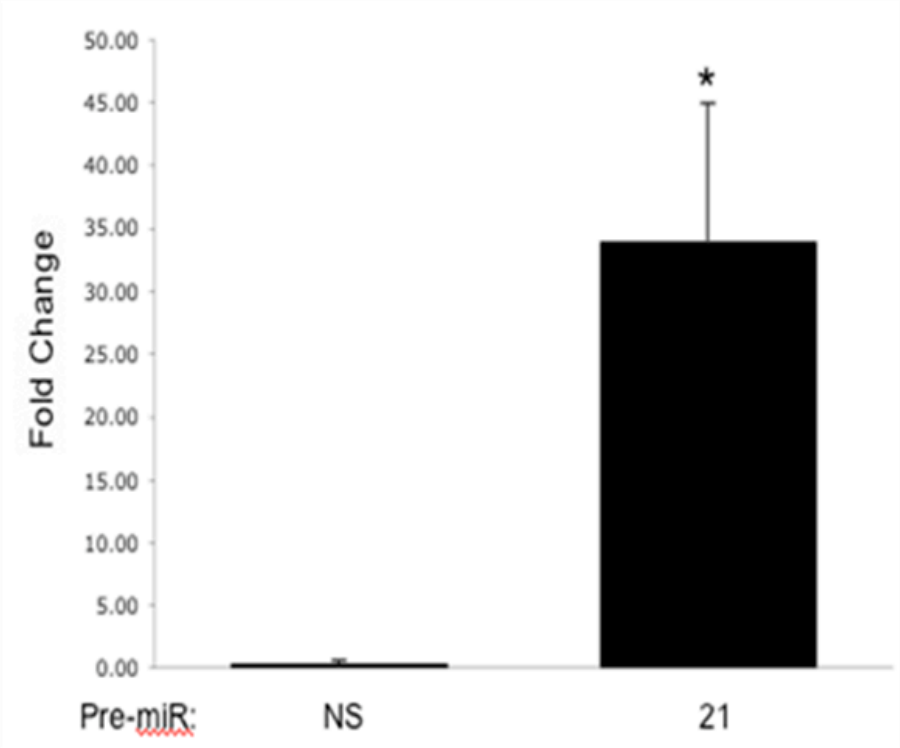
TABLE VIII-S2. Proteins Changed between LNA-21 and ANTI-21 transfected cells (8 h) as determined by two-dimensional gel electrophoresis and mass spectrometry.

TABLE VIII-S2

Tandem MSMS Predicted Protein	Fold Change (Anti-21/LNA-21)
Stathmin 1	1.39
Destrin	1.25
Ferritin Light Chain 1	-1.86
Alpha Isoform of Regulatory Subunit A, Protein Phosphatase 2	-1.95
Guanosine Disphosphate (GDP) Dissociation Inhibitor 1	-2.17
Apolipoprotein A-1	-2.34

FIGURE VIII-S1. Addition of exogenous pre-miR-21 increases mature miR-21 levels 95-fold. *Means ± SEM are different ($p<0.05$).

Figure VIII-S1



IX. Chapter Five:

MicroRNA in the Ovary and Female Reproductive Tract

1. Introduction

The ability to enhance reproductive efficiency or inhibit reproductive activity is dependent upon a solid understanding of reproductive physiology. In the female, reproductive success relies on the coordinated actions of the hypothalamic- pituitary-gonadal axis and its regulation of the cervical, uterine, and oviductal tissues. The reproductive tract responds to the cyclical changes in pituitary and ovarian hormones to provide an optimal environment for gamete transport and development, a suitable site for implantation and pregnancy, and protection against pathogens. Whereas much is known about the effect the pituitary and gonadal hormones have on reproductive function, there still remain many unanswered questions concerning the molecular mechanisms related to how these hormones elicit their action(s). The past forty years have shed considerable light on transcriptional gene regulation and cellular signaling pathways within reproductive tissues. However, much less is known about post-transcriptional gene regulation in these tissues, even though it plays an essential role in the synthesis of all proteins and is known to be important in the general regulation of cell differentiation and proliferation. Post-transcriptional gene regulation includes the splicing, editing, transport, storage, turnover, and translation of mRNA, and can result in a difference in expression levels between the transcriptome (mRNA) and the proteome (proteins) of the cell (Glisovic et al., 2008; Gygi et al., 1999; Ideker et al., 2001). All mRNA transcripts pass through some type of post-transcriptional processing, and although some post-transcriptional gene regulation may be considered almost constitutive in manner, other forms are

dynamically regulated (Hammarskjold, 2001; Medina et al., 2008). Therefore, it is easy to envision that post-transcriptional gene regulation may be important for coordinating the changes in gene expression that are necessary for the rapid phenotypic changes that occur in the reproductive tract of cycling and pregnant females.

In the female reproductive system several examples of post-transcriptional gene regulation have been shown to be involved in gonadal and reproductive tract function. In ovarian granulosa cells, degradation of the LH receptor transcript is increased after the LH surge, and this is mediated by mevalonate kinase, an enzyme in the cholesterol biosynthetic pathway, that also acts as an RNA binding protein (Wang and Menon, 2005). Connexin43, a major component of granulosa cell and oocyte gap junctions, is also translationally repressed in granulosa cells after the LH surge, although the mechanism(s) that mediates this repression has not been identified (Kalma et al., 2004). Additionally, in the ovine uterus, estrogen treatment caused the RNA-binding protein AUF1p45 to stabilize the expression of estrogen receptor α (Ing et al., 2008). Because of the difficulties in examining post-transcriptional gene regulation, few examples exist in reproductive tissues; however, the large numbers of expressed RNA-binding proteins and now microRNA (miRNA) suggests this form of gene regulation may play an important role in reproductive tissues (Berezikov et al., 2006; Keene, 2001).

The recent identification of miRNA as important post-transcriptional gene regulators has led to an explosion in our knowledge of the role post-transcriptional

gene regulation plays in organ function. MicroRNA are ~21nt RNA molecules that bind to the 3'UTR of target mRNA to affect their translation. It is predicted that 1-5% of genes encode for miRNA, and they regulate the expression of as many as 30% of mRNA (Berezikov et al., 2005; Lewis et al., 2005). Thousands of miRNA have now been identified in a multitude of organisms (microRNA.sanger.ac.uk/). However, the functional importance of individual miRNA and the identity of their specific mRNA targets are just now beginning to be elucidated in rodents and human cell lines. It remains to be determined if the function of miRNA is conserved across species in the same manner as protein-encoding genes. MicroRNA have been shown to be important in many biological processes, including cell proliferation, differentiation, and apoptosis (Asangani et al., 2008; Cloonan et al., 2008; Silber et al., 2008). Because miRNA have been demonstrated to be important in other developmental and differentiation systems, we propose that miRNA also play a vital role in the development and function of reproductive tissues. In this review we will discuss the current findings that support this hypothesis for the uterus, oviduct, ovary, and oocyte/early embryo and how the dysregulation of miRNA may lead to reproductive disease.

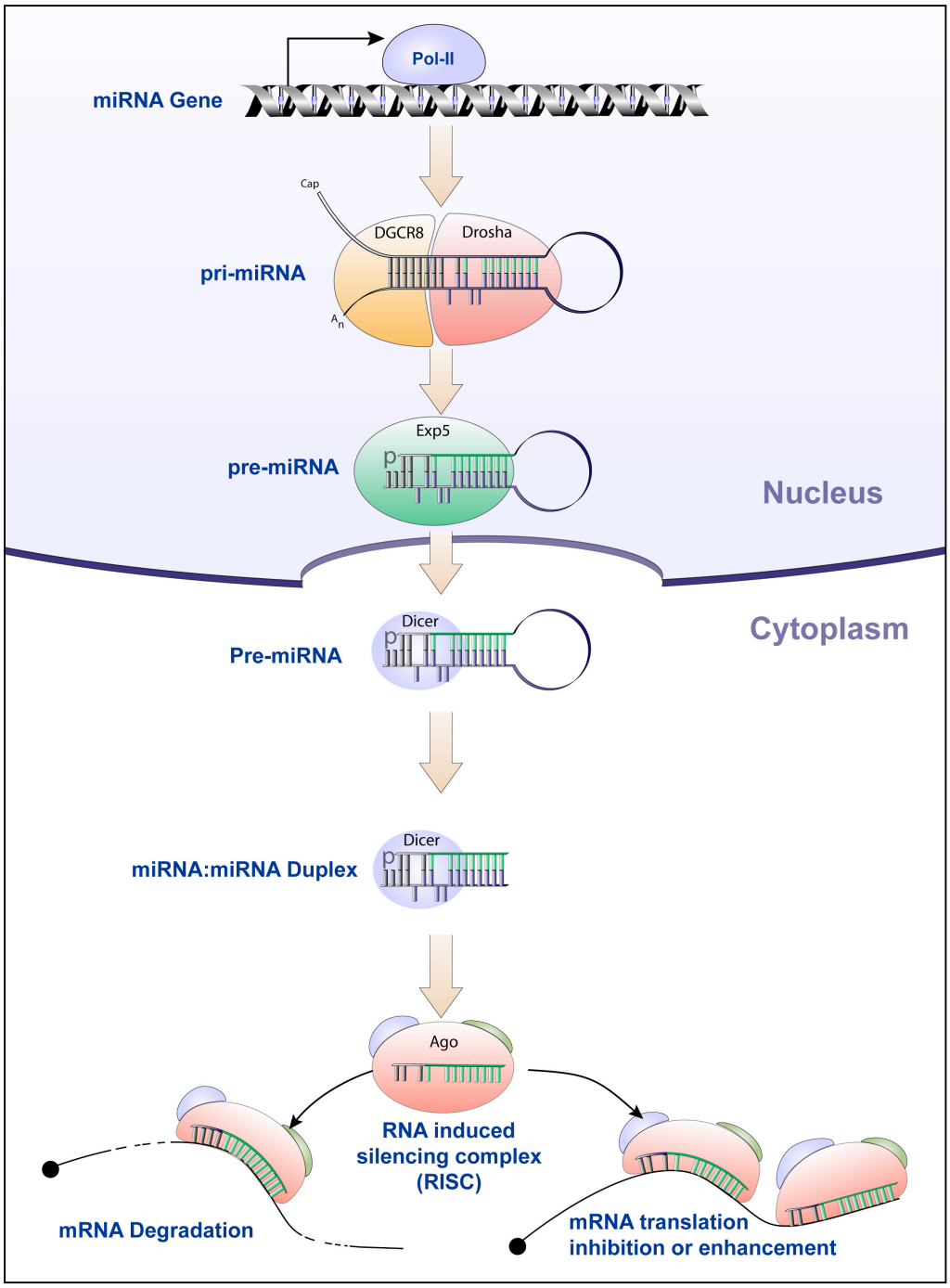
2. MicroRNA Biogenesis and Function

MicroRNA genes reside between genes (i.e., intergenic) and within introns of genes, and in a few cases reside within exons or makeup the entire intron (i.e., mirtrons; Berezikov et al., 2007; Saini et al., 2007). As a result miRNA can be

transcribed either independently (intergenic and intronic) or in succession with a mRNA if they reside within an intron or exon. The initial long primary RNA transcript (pri-miRNA; Figure IX-1) is capped (methyl-7-GpppG) and polyadenylated, and thus transcription most likely is mediated by polymerase II or III (Cai et al., 2004). Within the nucleus, the pri-miRNA is bound by the RNA-binding protein DGCR8 (DiGeorge syndrome critical region 8) and cleaved by the RNase III enzyme Drosha to form an ~70nt stem-loop pre-miRNA (Figure 1). Following export from the nucleus by exportin 5, the pre-miRNA is further cleaved by a second RNase III enzyme, Dicer, to form a transient ~21nt RNA duplex. One strand of the duplex is preferentially loaded onto the RNA-induced silencing complex (RISC), a large protein complex composed of Dicer, TRBP (TAR RNA-binding protein), and one of several different Argonaute proteins (Hutvagner and Simard, 2008). Within RISC the miRNA binds to the 3' untranslated region (UTR) of the target mRNA transcript with almost exact target specificity between the 5' end of the miRNA and the mRNA, a 7-8 nucleotide region called the 'seed sequence'. Usually, this binding blocks translation through RISC interaction with eIF6, which prevents assembly of 80S ribosomes (Chendrimada et al., 2007), or through inhibition of translation after initiation (Jackson and Standart, 2007). However, recent reports have also indicated that a change in the particular Argonaute protein within RISC can cause a shift from translational inhibition to translational enhancement (Vasudevan et al., 2007). Other reports have also suggested that miRNA, with or without perfect sequence complementarity, can cause an increase in mRNA degradation by endonucleolytic

FIGURE IX-1. Working model of miRNA biogenesis and function. Within the nucleus, microRNA (miRNA) are transcribed by RNA polymerase II (Pol-II) into single strand RNA which folds into a double stranded primary miRNA (pri-miRNA). Pri-miRNA are subsequently bound in the cell nucleus by DiGeorge syndrome critical region 8 (DGCR8) and then cleaved by Drosha yielding the precursor pre-miRNA. Pre-miRNA are then transported from the nucleus via exportin-5 (Exp5) to the cytoplasm where they are further cleaved by the RNAse III endonuclease Dicer into a double-stranded miRNA duplex. The double stranded miRNA separates into two single strands with one being degraded and the other strand forming a complex with the Argonaute proteins (Ago), forming the RNA-inducing silencing complex (RISC). The RISC then binds to target mRNA leading to either translational repression, translational enhancement, or transcript degradation.

Figure IX-1


Pol-II
miRNA Gene
Cap
DGCR8
Drosha
pri-miRNA
An
Exp5
pre-miRNA
Nucleus
Cytoplasm
Dicer
Pre-miRNA
Dicer
miRNA:miRNA Duplex
Ago
RNA induced
silencing complex
(RISC)
mRNA Degradation
mRNA translation
inhibition or enhancement

cleavage or deadenylation, respectively (Jackson and Standart, 2007). Therefore, miRNA have been shown to affect post-transcriptional gene regulation in one of three ways: translational inhibition, translational enhancement, and mRNA degradation. Reproductive tissues may use all of these mechanisms or just one; future experiments will enhance our understanding of miRNA mediated post-transcriptional regulation in these tissues.

Presently, the role of miRNA in reproductive tissues is primarily investigated using four different methods: 1) knocking down global miRNA expression by creating tissue specific conditional *Dicer1* knockout mice [complete deletion of *Dicer1* is embryonic lethal (Bernstein et al., 2003)]; 2) identifying miRNA expression profiles within cells and tissues using cloning and microarray analyses; 3) investigating specific miRNA function through the use of complementary 2'O-methyl or locked nucleic acid (LNA) oligonucleotide inhibitors; and 4) identifying specific individual miRNA using a candidate gene approach, in which genes are individually analyzed for regulation by miRNA. In this review we will discuss the reproductive tissue miRNA profiles that have been completed and what has been discovered about the role miRNA play in reproductive tissues through the use of conditional Dicer mutants and candidate gene approaches.

3. MicroRNA in the Ovary

Recruitment of growing follicles, atresia, ovulation, luteal tissue formation and regression are dynamically regulated events that reoccur on a cyclical basis

within the ovary. Few tissues exhibit the ephemeral lifecycle seen in follicular and luteal tissues within the adult, making this system very unique. These events involve dramatic changes in cellular growth, angiogenesis, steroidogenesis, cell cycle status, and apoptosis, and are tightly regulated at the endocrine and tissue level. Defects in regulatory control can lead to ovarian failure due to disruption of folliculogenesis, block of ovulation, and corpus luteum insufficiency, etc. Although the transcriptional regulation of ovulation and luteinization has been well studied, it stands to reason that post-transcriptional gene regulation may be critical for reproductive tissue function. Understanding the molecular events, including the role of miRNA in post-transcriptional gene regulation, that occur during these transitory periods might provide insight into how we might be able to enhance reproductive efficiencies and alleviate deficiencies.

Recently, Otsuka and coworkers, demonstrated that the Dicer1 hypomorph (*Dicer1*$^{d/d}$; ~75% loss of Dicer1 mRNA levels) mouse, developed using a gene trap method, exhibited female infertility (Otsuka et al., 2007; Otsuka et al., 2008). Transplantation of wild type ovaries into *Dicer1*$^{d/d}$ females resulted in offspring, but wild type females transplanted with *Dicer1*$^{d/d}$ ovaries failed to establish pregnancies, indicating that the fertility defect was inherent to the ovary. Further characterization of *Dicer1*$^{d/d}$ mice indicated that ovulation occurred normally and that the ovulated eggs were fertilized and underwent the first embryonic cell division. In contrast, serum progesterone levels and expression of LH receptor, cytochrome p450 11a1 (Cyp11a1), and prolactin receptor, genes necessary for progesterone production and

corpus luteum formation/maintenance, were all decreased in *Dicer1*$^{d/d}$ mice. Proper corpus luteum function requires a dramatic increase in vasculature, and miRNA have previously been shown to be important for angiogenesis in embryonic development (Yang et al., 2005). Therefore, Otsuka et al. (2008) examined vasculature in these *Dicer1*$^{d/d}$ mice and found a decrease in both the number and the length of the blood vessels in the corpus luteum and correlated this with the upregulation of anti-angiogenic factors, Timp1 and platelet factor 4, in *Dicer1*$^{d/d}$ mice. MicroRNA-17-5p and let-7p were found to regulate Timp1 expression and loss of these in *Dicer1*$^{d/d}$ mice were predicted to cause the reduction in angiogenesis. Knockdown of these two miRNA in wild type mice impaired corpus luteum angiogenesis and decreased serum progesterone levels. Furthermore, injection of miR-17-5p and let-7p into the ovarian bursa of *Dicer1*$^{d/d}$ mice restored the vasculature within the corpora lutea and increased progesterone levels, but failed to maintain pregnancy, indicating that other miRNA might still be involved (Yang et al., 2005).

Our laboratory mated the anti-Mullerian hormone receptor, type 2 (Amhr2)-Cre mouse (Jamin et al., 2002) with a mouse homozygous for locus of crossover in P1 (loxP) insertions surrounding the second RNase III domain of *Dicer1* (Harfe et al., 2005). This selectively knocked out Dicer1 expression in Mullerian duct derivatives (the oviduct, uterus, and cervix) and in the granulosa cells of secondary and small antral follicles (Hong et al., 2008). The female *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice were infertile as evidenced by failure to deliver offspring over a 5 month period, but the male mice were able to sire offspring (Hong et al., 2008). *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$

female mice mated normally and had normal estrous cycles, but exhibited a reduced ovulation rate (10.7 ± 0.9 and 7.0 ± 1.1, for the wild type and *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice, respectively). This effect was not due to a disruption in the secretion of hormones, as the reduced ovulation rate was also observed in gonadotropin-stimulated immature mice (16.2 ± 1.4 and 3.7 ± 1.5, for the wild type and *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice, respectively). Moreover, these *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice had reduced ovarian weights compared to wild type controls (Hong et al., 2008). Ovulated oocytes from *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mice collected at d1 post-coitus underwent *in vitro* embryonic development that mirrored that observed in the wild type mice (Hong et al., 2008). These results suggest the loss of Dicer1 expression impacts ovarian function, however, since these mice were still able to ovulate competent oocytes, the ovarian loss of Dicer1/miRNA does not explain the infertility seen in these mice.

To examine the importance of miRNA within the ovary, four studies have examined ovarian miRNA expression using several different methodologies and experimental paradigms (Choi et al., 2007; Fiedler et al., 2008; Kim et al., 2006; Ro et al., 2007). Choi et al. (2007) examined the expression of miRNA in the newborn mouse ovary, and further examined the effect that knocking down newborn ovary of homeobox gene (Nobox), a transcription factor necessary for oocyte differentiation and survival, had on miRNA expression (Choi et al., 2007). They identified 177 miRNA in the newborn ovary and found that 4 were decreased ~2-fold in the Nobox$^{-/-}$ ovaries (Choi et al., 2007). In 2006, a computational analysis of the pig genome

identified 58 miRNA, and northern blot analysis confirmed the expression of two of these miRNA (i.e., miR-31 and miR-92) within the porcine ovary (Kim et al., 2006). Ro et al used a cloning technique to identify miRNA expressed in the ovaries of 2-wk-old and adult mice (Ro et al., 2007). Combined, they identified 122 miRNA from whole ovaries of the immature and mature mice. Because whole ovarian tissue was used and the stage of the estrous cycle of the adult ovaries was unknown, the usefulness of this data set is limited.

To examine somatic cell miRNA expression and test whether LH can regulate miRNA expression, we performed a microRNA microarray on mouse granulosa cells before (0h) and 4h after hCG (Fiedler et al., 2008). We identified 196 and 206 miRNA as detectable before and 4h after hCG respectively, with 31 miRNA showing high abundance, 64 intermediate abundance, and 117 low abundance. Of the total 212 miRNA detected, only thirteen miRNA were differentially expressed between 0h and 4h post-hCG, with 3 upregulated and 10 downregulated ($p<0.05$; Fiedler et al., 2008). Among the upregulated miRNA were miR-132 and miR-212. MicroRNA-132 has previously been shown to be transcriptionally regulated by cAMP in neuronal cells (Vo et al., 2005), and to post-transcriptionally regulate the expression of several different genes (Klein et al., 2007). Included among the miR-132 targets was the transcriptional co-repressor C-terminal binding protein (CtBP1; Klein et al., 2007). Interestingly, CtBP1 in conjunction with steroidogenic factor-1, was recently shown to regulate adrenal steroidogenesis (Dammer and Sewer, 2008). We demonstrated that cultured granulosa cells respond to cAMP with an increase in miR-132 and miR-

212 levels, and that LNA oligonucleotide knockdown of miR-132 and miR-212 caused an increase in CtBP1 protein levels, suggesting that these miRNA post-transcriptionally regulate CtBP1 expression in granulosa cells (Fiedler et al., 2008). These early experiments demonstrate a role for miRNA in ovarian function and, furthermore, several miRNA have been identified and their role in ovarian function is currently under investigation.

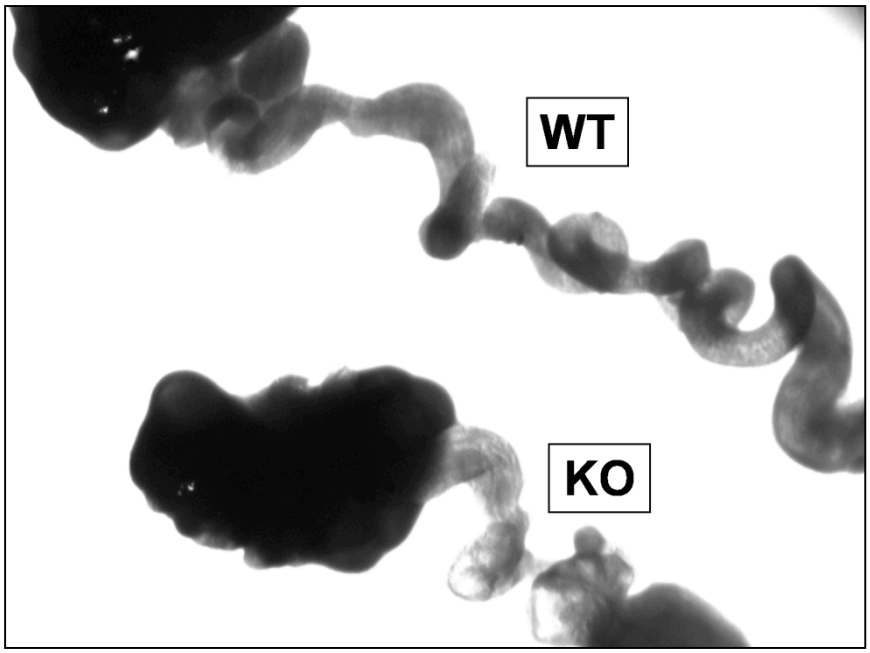
4. MicroRNA in the Oviduct

The oviduct is responsible for the transport and protection of the oocyte and embryo during fertilization and early development, respectively. The cells surrounding the oviductal lumen are either secretory cells, which secrete proteins to maintain the embryo in a healthy environment, or ciliated cells, which shuttle the oocyte/embryo towards the uterus. High levels of estrogen increase the activity of both of these cells, and the subsequent high levels of progesterone decrease their activity. Therefore, like the ovary, the oviduct undergoes cyclic phenotypic changes, and thus miRNA may be important for these changes as well.

Presently, expression of miRNA within the oviduct has not been reported. However, our gross morphological analysis of the *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mouse oviduct demonstrated that it was less than half the normal length and diameter of wild type females (Hong et al., 2008; Figure IX-2). Histological examination indicated a reduced or absent smooth muscle layer in the isthmus portion of the oviduct, which was likely associated with the transparent and fragile nature of this tissue (Hong et al.,

FIGURE IX-2. Wild type (top) and *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ (bottom) oviducts and ovaries and from immature age-matched mice. The *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ oviducts are approximately one-third the length of the wild type and note the transparency of this tissue compared to the wild type particularly in the isthmus region due to a reduced/absent smooth muscle layer.

Figure IX-2



2008). Additionally, embryos maintained within the oviduct of the *Dicer*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ mouse through day 3 (d3) and d4 of pregnancy, exhibited delayed development and increased fragmentation and degeneration. Furthermore, on d4 of pregnancy, when embryos within wild type females had all traversed through the utero-tubal junction to reside in the uterus, none of the embryos in the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ mice had (Hong et al., 2008). Indeed, it appears that the failure of oviductal support and transport is the primary cause of infertility in these mice (Hong et al., 2008).

The truncated oviductal phenotype has similarities with changes seen in the β-catenin and wingless-type MMTV integration site family 7a (Wnt7a) knockout mice (Arango et al., 2005; Deutscher and Hung-Chang Yao, 2007; Miller and Sassoon, 1998). Interestingly, β-catenin protein levels were reduced in the oviducts of *Dicer*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ mice (Hong et al., 2008), suggesting that Dicer1 and the resulting miRNA may be involved in the expression of members of the Wnt signaling pathway.

5. MicroRNA in the Uterus

Like the ovary and oviduct, the uterus undergoes drastic changes throughout the menstrual/estrous cycle. The uterus transitions from a highly proliferative state that is nonreceptive to blastocyst implantation when estrogen is high, to a highly secretory state that is receptive to blastocyst implantation when progesterone is high. A global analysis of miRNA expressed both in the uterus and ovary suggests that the miRNA profiles of the two are very similar, indicating that similar mechanisms may

be in place to regulate these cyclic changes (Shingara et al., 2005). The identification of miRNA expressed in the uterus, and the functional analysis of individual expressed miRNA, has shed light onto the physiological changes that occur in the uterus in response to steroids and pregnancy.

Similar to the effects deletion of Dicer1 had on the oviduct, we observed that the *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ mouse uterus was less than half the normal length and diameter of wild type females. In addition, the *Dicer1*$^{fl/fl}$*;Amhr2*$^{Cre/+}$ uterus was one-third the weight of wild type uteri (Hong et al., 2008). Histology indicated a reduced smooth muscle layer and reduced presence of uterine glands (Hong et al., 2008). Presently, it is unknown whether the uteri of these mice are capable of establishing and maintaining a pregnancy.

To examine whether estrogen affects uterine miRNA expression, Nothnick treated ovariectomized mice with estradiol 17-β or vehicle (control), and performed miRNA microarrays on whole uterine tissue (Nothnick, 2008). One-hundred and twenty-three miRNA were expressed within the uterus, with 30 miRNA upregulated and 19 miRNA downregulated after estradiol treatment. One of the downregulated miRNA, miR-705, was predicted to target matrix metalloproteinase-9 (MMP-9) mRNA. Prior to estrogen treatment, MMP-9 protein expression was low but its mRNA expression was high, and following estradiol treatment these expression profiles were inversed. Therefore, a model was proposed in which prior to estradiol treatment miR-705 is highly expressed, resulting in the translational repression of MMP-9, and following estrogen treatment miR-705 levels fall so MMP-9 can be

translated (Nothnick, 2008).

Two studies have examined the expression of miRNA in the uterus in response to embryo implantation. In the first study, a miRNA microarray was performed on uteri from d1 pregnant mice, during which the uterus is pre-receptive to embryo implantation because estrogen is high, versus uteri from d4 pregnant mice, during which the uterus is receptive to embryo implantation because progesterone is high (Chakrabarty et al., 2007). Two miRNA upregulated in the receptive stage, miR-101a and miR-199a*, are regulators of prostaglandin-endoperoxide synthase 2 (Ptgs2). The expression of Ptgs2 in the uterine luminal epithelium is necessary for embryo implantation. Thus, miR-101a and miR-199a* may play a role in tightly regulating Ptgs2 expression at the site of embryo implantation, thereby preventing unnecessary growth elsewhere in the uterus. Furthermore, Ptgs2 expression is necessary for decidualization of the uterine stromal cells, and the expression profiles of miR-101a and Ptgs2 are inversely correlated during decidualization. Therefore, miR-101a and miR-199a are likely important regulators of Ptgs2 during embryo implantation and the concurrent decidualization (Chakrabarty et al., 2007).

The second study directly examined the expression of miRNA at implantation sites by performing miRNA microarrays on implantation sites versus non-implantation sites in the mouse uterus (Hu et al., 2008). Thirteen miRNA were identified as upregulated in the implantation sites, and two downregulated. Interestingly, Northern blot analysis indicated that only the precursor forms of two miRNA, miR-290-5p and miR-292-5p, were upregulated, even though the mature

forms were not changed. Recent evidence suggests that miRNA themselves can be post-transcriptionally regulated (Davis et al., 2008), and these two transcripts may be being stored for later use during placenta development. Additionally, a miRNA previously shown to be involved in cellular proliferation and differentiation, miR-21, was upregulated in the implantation sites, with high expression in the sub-luminal stroma around the blastocyst (Hu et al., 2008). MicroRNA-21 was not expressed on d1-4 of pregnancy, but was upregulated on d5 of pregnancy only at the implantation site, and then was high at d6-8 in the decidua. MicroRNA-21 was not expressed during pseudopregnancy, so its expression was dependent on the presence of the embryo. Within these implantation sites, miR-21 was shown to target the Reck transcript for degradation. Reck inhibits MMP-2 and MMP-9 secretion and/or activity (Nothnick, 2008). In the inter-implantation sties, when miR-21 was low, Reck was high; and in implantation sites, when miR-21 was high, Reck was low, thus allowing for active MMP. The authors speculate that this allows for the breakdown of the extracellular matrix and the angiogenesis necessary for placenta development. Interestingly, miR-21 has binding sites for signal transducer and activator of transcription 3 (STAT3) in its promoter that have been shown to enhance miR-21 transcription (Loffler et al., 2007). Leukemia inhibitor factor (Lif) is necessary for embryo implantation, and Lif causes STAT3 to be activated and translocated to the nucleus in mouse uterine epithelium (Cheng et al., 2001). As the authors point out, future studies may reveal that the expression of miR-21 in the implantation sites is mediated by Lif expression. Future studies may also identify other potential targets

of miR-21 during embryo implantation and placental development.

6. MicroRNA in the Oocyte and Fertilized Egg

When the oocyte is growing and arrested in prophase I of meiosis, it is transcriptionally active and accumulates and stores a number of transcripts for later use. However, after the LH surge as oocyte maturation is initiated, transcriptional activity decreases to barely detectable levels (Kinloch et al., 1993; Paynton et al., 1988). Therefore, the precisely timed events of oocyte maturation and early embryogenesis rely on post-transcriptional and post-translational regulation of maternal RNA transcripts and proteins. Stored RNA transcripts are recruited for translation in response to specific physiological cues (Eichenlaub-Ritter and Peschke, 2002). For example, the mRNA of maturation promoting factor is recruited to the polysome in response to LH, and plays an initiating and necessary role in the process of meiotic maturation (Dekel, 2005). Recent evidence suggests that miRNA may play a role in translationally repressing these stored mRNA until they are needed.

Dicer1 was shown to be highly expressed in oocytes, with the highest expression in the transcriptionally repressed germinal vesicle oocyte (Cui et al., 2007; Murchison et al., 2007). Dicer1 expression progressively decreased during oocyte maturation and fertilization, with the largest decrease at the two-cell stage when maternal transcripts are globally degraded (Cui et al., 2007; Murchison et al., 2007). The importance of miRNA to the oocyte has been demonstrated through the use of oocyte-specific Dicer1 knockout mice (Murchison et al., 2007; Tang et al., 2007).

These *Dicer1*$^{-/-}$ mice are infertile, although their ovaries are histologically normal and responsive to gonadotropins. The germinal vesicle oocytes are also normal, so oocyte-expressed Dicer1 does not appear necessary for oocyte development and ovarian function. However, further examination revealed that in 90% of the *Dicer1*$^{-/-}$ oocytes the spindles were misaligned and disorganized, resulting in a loss of meiotic maturation and polar body production (Murchison et al., 2007; Tang et al., 2007). Microarray analysis of these *Dicer1*$^{-/-}$ oocytes indicated that 861 mRNA were upregulated >2-fold, and 173 of these were previously shown to be degraded during meiotic maturation (Murchison et al., 2007). Therefore, the maintenance of these transcripts could be responsible for the defects seen in chromosomal segregation and meiotic maturation.

Oocyte-sperm fusion causes oocyte activation, which results in a further decrease in transcription (Ram and Schultz, 1993). Therefore, the resumption of meiosis and the cytoplasmic events that occur during the activation are based on post-transcriptional regulation. In the mouse, transcription does not resume until the late one-cell stage, 12 h later (Ram and Schultz, 1993). The first indication that miRNA were important during the fertilization and early embryogenesis events came in zebrafish, with the discovery that miR-430 binds to over 300 maternal transcripts to cause their down regulation directly prior to the start of embryonic transcription (Giraldez et al., 2006). In the human, sperm were shown to transfer their RNA to the oocyte (Ostermeier et al., 2004), and an appealing hypothesis is that sperm-borne miRNA are responsible for the downregulation of maternal transcripts, this

hypothesis was tested in the mouse (Amanai et al., 2006). Since only the nuclear and perinuclear structures of the sperm enter the oocyte, these structures were subjected to miRNA microarray analysis, and 54 miRNA were consistently found to be present. This miRNA profile was compared to that of recently fertilized eggs and unfertilized eggs. All 54 of the sperm miRNA were present in the unfertilized egg, suggesting that the sperm does not make a meaningful contribution to the oocyte. To further show that sperm borne miRNA are not important to the process, they treated sperm with 2'O-methyl oligonucleotide inhibitors complementary to each of the five miRNA and saw no effect on fertilization or early embryogenesis (Amanai et al., 2006). However, the microarray used in this study only had 238 probes, corresponding to 238 miRNA, so more recently identified miRNA may still play an important role in the process.

Finally, two studies have examined the expression of miRNA in the early embryo, one in the murine embryo and one in the bovine embryo. Tang et al. used a real-time PCR-based miRNA expression profile method to identify the miRNA expression profiles of the murine oocyte, one-cell zygote, two-cell embryo, four-cell embryo, and eight-cell embryo (Tang et al., 2007). Interestingly, between the one-cell and two-cell stage there was a 60% decrease in miRNA expression levels, indicating that miRNA may be degraded as the maternal mRNA are. However, between the two-cell and four-cell stage, as embryonic transcription proceeds, miRNA-expression increases ~2.2-fold. Among the first miRNA to be transcribed are those from the miR-290 cluster, which have previously been shown to play a role

on DNA methylation and telomere-length homestasis (Benetti et al., 2008; Tang et al., 2007). Coutinho et al. examined miRNA expression in d30 bovine embryos (Coutinho et al., 2007). Included among miRNA specifically expressed in the embryo (compared to the thymus, small intestine, mesenteric lymph node, and abomasums lymph node) were miR-122a and miR-199a* (Coutinho et al., 2007). MicroRNA-122a has previously been shown to target cyclin G1 (Gramantieri et al., 2007), and miR-199a* has previously been shown to be pro-apoptotic by targeting the Met proto-oncogene, resulting in decreased extracellular signal-regulated kinase 2 (ERK2) levels (Kim et al., 2008). Due to the remarkable events occurring during early embryonic development, it is likely that miRNA mediated post-transcriptional gene regulation may play a greater role in gene expression in those developing tissues than that seen in adult tissues (Blakaj and Lin, 2008).

7. MicroRNA in Reproductive Disease

Since miRNA play such a vital role in cell differentiation events, it is easy to envision how the dysregulation of miRNA expression could lead to a disease state. MicroRNA have been demonstrated to be important in multiple types of cancer including ovarian, endometrial, and cervical; as well as non-malignant pathologies, such as uterine fibroids and endometriosis. In fact, miRNA have arguably been more thoroughly studied in the disease state of reproductive tissues than the normal state, and here we will briefly touch on some recent findings in respect to miRNA and their dysregulation in diseased reproductive tissues of women.

Ovarian cancer is the sixth most common cancer in women, and miRNA expression has been studied in several types of ovarian cancer and a variety of cancer cell lines (Corney et al., 2007; Dahiya et al., 2008; Iorio et al., 2007; Nam et al., 2008; Yang et al., 2008; Zhang et al., 2008a). To date, the predominant work focuses on elucidation of miRNA signatures that can be used for diagnosis. These studies compare cancerous tissue to either normal tissue or to immortalized cancer cell lines. Interestingly, a set of common miRNA associated with ovarian cancer has not been generated, which may be due to the nature of the controls or reference samples used to compare the cancer tissues.

In the uterus, endometrial carcinogenesis affects almost 40,000 women per year. Boren et al. examined the expression of miRNA in endometrial adenocarcinomas versus normal endometrial tissue and identified thirteen miRNA as differentially expressed (Boren et al., 2008). They also identified mRNA differences between the two tissues and found that 9% of the differentially expressed mRNA were predicted targets of the changed miRNA.

Uterine leiomyomas (i.e., fibroids) are a benign uterine pathology that affect 30 to 50% of women. Several studies have examined the expression of miRNA in uterine leiomyomas. In two studies, miRNA microarrays were performed to examine the differential miRNA profiles of uterine leiomyomas and normal myometrium. Marsh et al. identified 81 miRNA as differentially expressed, including increased expression of an anti-apoptotic miRNA, miR-21, and decreased expression of a miRNA previously shown to be decreased in pancreatic adenocarcinoma, miR-139

(Marsh et al., 2008). Wang et al identified 45 miRNA as differentially expressed and further grouped the miRNA expression profiles from the uterine leiomyomas based on tumor size and demographics (Wang et al., 2007). Members of the let-7 miRNA family showed high expression in small leiomyomas, but low expression in large leiomyomas (Wang et al., 2007). These miRNA target high-mobility group A2 (HMGA2), a transcription factor highly expressed in large leiomyomas that positively regulates cell growth and proliferation and negatively regulates apoptosis (Peng et al., 2008). Members of the let-7 miRNA family were shown *in vitro* to prevent HMGA2 expression, and therefore high levels of let-7 miRNA can prevent HMGA2 expression and thus tumor growth.

Endometriosis, a disease characterized by the presence of endometrial tissue outside the uterine cavity, causes infertility in 30-40% of patients with the condition. Pan et al. performed a miRNA microarray on human samples of normal endometrium, eutopic endometrium, and ectopic endometrium (Pan et al., 2008). Forty-eight miRNA were identified as differentially expressed. MiR-18a, miR-181a and miR-142-5p were shown to have predicted target transcripts that were already implicated in endometriosis including estrogen receptor alpha, estrogen receptor beta, and progesterone receptor, respectively. Moreover, the expression of several miRNA (miR-20a, miR-21, and miR-26a) was regulated by treatment with the ovarian steroids estrogen or progesterone (medroxyprogesterone acetate), as well as the steroid receptor antagonists ICI-182780 and RU486 (Pan et al., 2007).

8. Summary and Conclusions

The role of miRNA in reproductive tissue function and development are presently being elucidated. To date much of the work has focused on miRNA profiling of the reproductive organs, but the expression, regulation, and function of miRNA within specific tissues and cells still needs to be determined. To help establish functional connections, conditional Dicer knockout mice have been used to show the consequences that the lack of miRNA have on ovarian, oviductal, uterine, and oocyte/embryo function and development. Much less is known about the specific miRNA and their targets that cause these phenotypic effects, but this area of research is rapidly moving forward. Within the next several years it is expected that a wealth of information regarding miRNA-mediated post-transcriptional gene regulation in reproductive tissues will be known. Because of the importance miRNA have in a variety of tissues and in diseases, understanding the role of miRNA in reproductive tissues will provide insight into how we can better enhance reproductive efficiency or inhibit reproductive activity.

X. Chapter Six:

Concluding Statements

Gaining a better understanding of the molecular events occurring in reproductive tissues is important in order to identify molecular networks and targets that can be perturbed to either enhance or inhibit fertility. Because of the central role of LH in inducing ovulation and luteinization, we have focused on understanding the molecular events that occur in periovulatory granulosa cells in response to LH. Interestingly, while much work has gone into studying the transcriptional changes that occur in periovulatory granulosa cells in response to LH, little research has been dedicated to studying the post-transcriptional changes that occur, even though these changes are just as important in establishing the final proteome and thus phenotype of the cell. In this dissertation we extend current knowledge on the transcriptional events initiated by LH, and, furthermore, elucidate a role of a specific post-transcriptional regulator, miR-21, in periovulatory granulosa cells.

1. LH rapidly effects gene expression in granulosa cells.

Previous studies examining LH regulation on gene expression following LH were performed at late timepoints following LH (4-12 h), and thus may miss transiently expressed genes that play just as important a role in the granulosa cell response to LH. Here we identified a large group of genes that are rapidly (within 1 h) changed following LH. Many of these genes are transcription factors that may be upregulating the expression of genes that play a direct role in ovulation and luteinization. Future studies will identify the direct targets of these transcription factors. In addition, we performed temporal expression analysis of identified genes,

which may allow for functional connections between genes to be identified. Our data therefore provides a list of LH-regulated genes that can be further analyzed to establish functions and connections of the molecular pathways identified as changed. In addition, we identify a novel protein, epigen, as rapidly upregulated following LH, and show that it stimulates cumulus cell expansion.

2. MicroRNA-21 transcription is upregulated in murine granulosa cells following LH administration.

This study was originally undertaken to confirm a microRNA expression assay that indicated miR-21 upregulation following LH. We validated this result, and further showed that primary miR-21 levels preceded mature miR-21 levels, and thus transcription of miR-21 is increased following LH. Future studies are needed to identify the molecular mechanism behind this increase. In the above study, we identify a number of transcription factors as upregulated preceding miR-21 upregulation, and an investigation of these genes may indicate that one is responsible for the induction of miR-21. Interestingly, the miR-21 promoter has sites for several of these upregulated genes, including Jun/Fos, and has sites for other genes previously shown to be upregulated following LH, such as C/EBPβ. However, miR-21 levels appear to be only marginally changed in C/EBPβ knockout mice, and thus it is doubtful that C/EBPβ is the primary transcriptional regulator of miR-21.

Future studies should also be directed towards identifying other miRNA that are changed in response to LH and thus may play a role in ovarian function. It would

be especially interesting to identify miRNA that are different between cumulus and granulosa cell populations, especially because the subtle changes in gene/protein expression that is mediated by miRNA can explain the differences in function and responses of two closely related populations of cells.

3. MicroRNA-21 has an anti-apoptotic role in granulosa cells.

Knockdown of miR-21 both *in vitro* and *in vivo* caused an increase in granulosa cell apoptosis. This is in agreement with multiple papers that suggest an anti-apoptotic role for miR-21 in cancer cell lines. Here we show, for the first time, that miR-21 also inhibits apoptosis in a physiological situation. It is well established that LH acts as a pro-survival factor in granulosa cells, and here we show that LH-induced upregulation of miR-21 helps mediate the pro-survival effect. Interestingly, an examination of known miR-21 targets that have been implicated in its anti-apoptotic role in cancer cell lines indicates that none of these are miR-21 targets in granulosa cells. It is important to note that the molecular networks that are present within each cell type can be highly variable, and thus can dramatically impact the effect of a single molecule on the cell. Therefore, the recent identification of cell type-specific RNA-binding proteins that have the ability to counteract miRNA activity may explain this discrepancy. Furthermore, the presence of multiple polyA signals within the 3'UTR of a mRNA transcript can cause 3'UTRs to be of varying lengths, and this can differ between cell types. Therefore, miRNA sites can be lost if an earlier polyA site is used within the 3'UTR.

We show here that miR-21 plays an antiapoptotic role in granulosa cells in periovulatory follicles, and it would be interesting to further this examination and investigate the role of miR-21 in granulosa cells of other follicles. For example, atresia of antral follicles is initiated by granulosa cell death (as opposed to atresia of early follicles, which is initiated by oocyte death). Furthermore, cleaved caspase 3 is required for follicular atresia (Matikainen et al., 2001). Therefore, it would be interesting to examine the role of miR-21 in atretic follicles. For example, it may be that atretic follicles have decreased expression of miR-21, resulting in an increase in cleaved caspase 3 levels and thus leading to granulosa cell death and subsequent follicle atresia.

From a clinical standpoint, granulosa cells from polycystic ovarian syndrome (PCOS) patients have decreased granulosa cell apoptosis and decreased cleaved caspase 3 levels (Das et al., 2008). In addition, anti-apoptotic factors are upregulated in PCOS granulosa cells (Das et al., 2008). Since we have shown here that miR-21 is an anti-apopototic factor, it would be interesting to examine the expression of miR-21 in these PCOS granulosa cells.

4. MicroRNA-21 promotes global translation in granulosa cells.

To identify miR-21 targets that are responsible for the anti-apoptotic action of miR-21 in granulosa cells, we used a proteomic approach to identify proteins that are changed upon miR-21 knockdown. Serendipitously, we found a change in the activity of elongation factor 2 (EF2), a regulator of translation elongation.

MicroRNA-21 knockdown resulted in an increase in phosphorylation of EF2, or a decrease in activity. This change corresponded to a decrease in global translation of the cell. Therefore, miR-21 indirectly promotes global translation within granulosa cells. The change in phosphorylated EF2 levels was confirmed *in vivo*, as total EF2 levels increased following LH while phosphorylated EF2 levels decreased.

Following LH stimulation, granulosa cells undergo massive hypertrophy as they increase mitochondria and endoplasmic reticulum in preparation for a rise in progesterone synthesis. These changes are mediated by an increase in global protein synthesis within the cell, and here we show a role for miR-21 in mediating this increase. Therefore, it would be expected that the loss of miR-21 could cause a loss in proper luteal growth and function, and we are currently exploring this possibility by knocking down miR-21 function *in vivo* and examining corpus luteum structure and steroidogenic ability.

5. MicroRNA-21 activates the Akt/mTOR pathway.

The Akt/mTOR pathway is a major cell signaling pathway in the cell. Here we show that miR-21 knockdown causes a decrease in Akt/mTOR signaling pathway. Interestingly, the effect of miR-21 on this pathway could explain both the apoptosis role of miR-21 as well as the translation role of miR-21. Ongoing studies are examining further upstream modulators of these pathways in order to identify the direct miR-21 target responsible for these changes. The Akt/mTOR pathway is

activated by a number of other cell signaling pathways, many of which have a well described increase in activity following LH.

Collectively, these studies demonstrate that LH induces a number of molecular changes, both transcriptional and post-transcriptional, within the granulosa cell to induce ovulation and luteinization. We provide evidence that miR-21 plays an important and necessary role to ovarian function. This is the first study to examine the role of a specific miRNA to granulosa cell function, and the first to examine the role of a specific miRNA in the periovulatory follicle.

XI. Chapter Seven:

References

Abdollahi, A., K. A. Lord, B. Hoffman-Liebermann, and D. A. Liebermann. 1991. Sequence and expression of a cdna encoding myd118: A novel myeloid differentiation primary response gene induced by multiple cytokines. Oncogene 6: 165-167.
Abelson, J. F. et al. 2005. Sequence variants in slitrk1 are associated with tourette's syndrome. Science 310: 317-320.
Adashi, E. Y. 1994. Endocrinology of the ovary. Hum Reprod 9: 815-827.
Ademokun, A., and M. Turner. 2008. Regulation of b-cell differentiation by micrornas and rna-binding proteins. Biochem Soc Trans 36: 1191-1193.
Albarracin, C. T., and G. Gibori. 1991. Prolactin action on luteal protein expression in the corpus luteum. Endocrinology 129: 1821-1830.
Albarracin, C. T., H. C. Palfrey, W. R. Duan, M. C. Rao, and G. Gibori. 1994. Prolactin regulation of the calmodulin-dependent protein kinase iii elongation factor-2 system in the rat corpus luteum. J Biol Chem 269: 7772-7776.
Albers, C. 1999. Sociology of families: Readings. Pine Forge Press, Thousand Oaks, CA.
Albertini, D. F., and E. Anderson. 1974. The appearance and structure of intercellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions. J Cell Biol 63: 234-250.
Allen, W. M., and O. Wintersteiner. 1934. Crystalline progestin. Science 80: 190-191.
Amanai, M., M. Brahmajosyula, and A. C. Perry. 2006. A restricted role for sperm-borne micrornas in mammalian fertilization. Biol Reprod 75: 877-884.
Amsterdam, A., S. Rotmensch, A. Furman, E. A. Venter, and I. Vlodavsky. 1989. Synergistic effect of human chorionic gonadotropin and extracellular matrix on in vitro differentiation of human granulosa cells: Progesterone production and gap junction formation. Endocrinology 124: 1956-1964.
Anderson, E., and D. F. Albertini. 1976. Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. J Cell Biol 71: 680-686.
Anderson, P., and N. Kedersha. 2006. Rna granules. J Cell Biol 172: 803-808.
Armstrong, D. T., and M. A. Opavsky. 1988. Superovulation of immature rats by continuous infusion of follicle-stimulating hormone. Biol Reprod 39: 511-518.
Asangani, I. A. et al. 2008. Microrna-21 (mir-21) post-transcriptionally downregulates tumor suppressor pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27: 2128-2136.
Austin, C. R. 1961. The mammalian egg. Thomas, Springfield, IL.
Avnit-Sagi, T., L. Kantorovich, S. Kredo-Russo, E. Hornstein, and M. D. Walker. 2009. The promoter of the pri-mir-375 gene directs expression selectively to the endocrine pancreas. PLoS ONE 4: e5033.

Babu, P. S., H. Krishnamurthy, P. J. Chedrese, and M. R. Sairam. 2000. Activation of extracellular-regulated kinase pathways in ovarian granulosa cells by the novel growth factor type 1 follicle-stimulating hormone receptor. Role in hormone signaling and cell proliferation. J Biol Chem 275: 27615-27626.
Baccarini, I. M. 1971. A light and electron microscopic study of the perifollicular region of the rat ovary during the estrous cycle. J Reprod Med 7: 93-98.
Bachelot, A., and N. Binart. 2005. Corpus luteum development: Lessons from genetic models in mice. Curr Top Dev Biol 68: 49-84.
Baker, A. F. et al. 2005. Stability of phosphoprotein as a biological marker of tumor signaling. Clin Cancer Res 11: 4338-4340.
Beers, W. H. 1975. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell 6: 379-386.
Benetti, R. et al. 2008. A mammalian microrna cluster controls DNA methylation and telomere recombination via rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol 15: 998.
Berezikov, E., W. J. Chung, J. Willis, E. Cuppen, and E. C. Lai. 2007. Mammalian mirtron genes. Mol Cell 28: 328-336.
Berezikov, E. et al. 2005. Phylogenetic shadowing and computational identification of human microrna genes. Cell 120: 21-24.
Berezikov, E. et al. 2006. Many novel mammalian microrna candidates identified by extensive cloning and rake analysis. Genome Res 16: 1289-1298.
Bjersing, L., and S. Cajander. 1975. Ovulation and the role of the ovarian surface epithelium. Experientia 31: 605-608.
Blakaj, A., and H. Lin. 2008. Piecing together the mosaic of early mammalian development through micrornas. J Biol Chem 283: 9505-9508.
Bleil, J. D., and P. M. Wassarman. 1980. Structure and function of the zona pellucida: Identification and characterization of the proteins of the mouse oocyte's zona pellucida. Dev Biol 76: 185-202.
Bohnsack, M. T., K. Czaplinski, and D. Gorlich. 2004. Exportin 5 is a rangtp-dependent dsrna-binding protein that mediates nuclear export of pre-mirnas. RNA 10: 185-191.
Borchert, G. M., W. Lanier, and B. L. Davidson. 2006. Rna polymerase iii transcribes human micrornas. Nat Struct Mol Biol 13: 1097-1101.
Boren, T. et al. 2008. Micrornas and their target messenger rnas associated with endometrial carcinogenesis. Gynecol Oncol 110: 206-215.
Borenfreund, E., E. Fitt, and A. Bendich. 1961. Isolation and properties of deoxyribonucleic acid from mammalian sperm. Nature 191: 1375-1377.
Brennecke, J., A. Stark, R. B. Russell, and S. M. Cohen. 2005. Principles of microrna-target recognition. PLoS Biol 3: e85.
Brown, S. E., L. 1995. The best intentions: Unintended pregnancy and the well-being of children and families. National Academy Press, Washington DC.

Browne, G. J., and C. G. Proud. 2004. A novel mtor-regulated phosphorylation site in elongation factor 2 kinase modulates the activity of the kinase and its binding to calmodulin. Mol Cell Biol 24: 2986-2997.
Buccione, R., A. C. Schroeder, and J. J. Eppig. 1990. Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol Reprod 43: 543-547.
Bueno, M. J., I. P. de Castro, and M. Malumbres. 2008. Control of cell proliferation pathways by microrna s. Cell Cycle 7: 3143-3148.
Cai, X., C. H. Hagedorn, and B. R. Cullen. 2004. Human microrna s are processed from capped, polyadenylated transcripts that can also function as mrnas. RNA 10: 1957-1966.
Calarco, P. G., R. P. Donahue, and D. Szollosi. 1972. Germinal vesicle breakdown in the mouse oocyte. J Cell Sci 10: 369-385.
Cameron, M. R., J. S. Foster, A. Bukovsky, and J. Wimalasena. 1996. Activation of mitogen-activated protein kinases by gonadotropins and cyclic adenosine 5'-monophosphates in porcine granulosa cells. Biol Reprod 55: 111-119.
Campbell, K. L. 1979. Ovarian granulosa cells isolated with egta and hypertonic sucrose: Cellular integrity and function. Biol Reprod 21: 773-786.
Carabatsos, M. J., C. Sellitto, D. A. Goodenough, and D. F. Albertini. 2000. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. Dev Biol 226: 167-179.
Care, A. et al. 2007. Microrna-133 controls cardiac hypertrophy. Nat Med 13: 613-618.
Carlberg, U., A. Nilsson, and O. Nygard. 1990. Functional properties of phosphorylated elongation factor 2. Eur J Biochem 191: 639-645.
Carletti, M. Z., and L. K. Christenson. 2009. Rapid effects of lh on gene expression in the mural granulosa cells of mouse periovulatory follicles. Reproduction 137: 843-855.
Carletti, M. Z., S.D. Fiedler, M. Visvanathan, L.K. Christenson. 2009. Microrna-21 blocks apoptosis in periovulatory mouse granulosa cells. Submitted.
Cavazos, L. F. et al. 1969. Fine structure and progesterone levels in the corpus luteum of the pig during the estrous cycle. Biol Reprod 1: 83-106.
Cavender, J. L., and W. J. Murdoch. 1988. Morphological studies of the microcirculatory system of periovulatory ovine follicles. Biol Reprod 39: 989-997.
Chaffin, C. L., K. M. Schwinof, and R. L. Stouffer. 2001. Gonadotropin and steroid control of granulosa cell proliferation during the periovulatory interval in rhesus monkeys. Biol Reprod 65: 755-762.
Chaffin, C. L., and R. L. Stouffer. 2002. Local role of progesterone in the ovary during the periovulatory interval. Rev Endocr Metab Disord 3: 65-72.
Chakrabarty, A. et al. 2007. Microrna regulation of cyclooxygenase-2 during embryo implantation. Proc Natl Acad Sci U S A 104: 15144-15149.

Chalfie, M., H. R. Horvitz, and J. E. Sulston. 1981. Mutations that lead to reiterations in the cell lineages of c. Elegans. Cell 24: 59-69.
Chan, J. A., A. M. Krichevsky, and K. S. Kosik. 2005. Microrna-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 65: 6029-6033.
Chandra, A., and E. H. Stephen. 1998. Impaired fecundity in the united states: 1982-1995. Fam Plann Perspect 30: 34-42.
Chang, S. S. et al. 2008. Microrna alterations in head and neck squamous cell carcinoma. Int J Cancer 123: 2791-2797.
Channing, C. P. 1970. Influences of the in vivo and in vitro hormonal environment upon luteinization of granulosa cells in tissue culture. Recent Prog Horm Res 26: 589-622.
Channing, C. P., and F. Ledwitz-Rigby. 1975. Methods for assessing hormone-mediated differentiation of ovarian cells in culture and in short-term incubations. Methods Enzymol 39: 183-230.
Chen, A. et al. 2008a. Complementary analysis of microrna and mrna expression during phorbol 12-myristate 13-acetate (tpa)-induced differentiation of hl-60 cells. Biotechnol Lett 30: 2045-2052.
Chen, L., P. T. Russell, and W. J. Larsen. 1993. Functional significance of cumulus expansion in the mouse: Roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Mol Reprod Dev 34: 87-93.
Chen, Y. et al. 2008b. Microrna-21 down-regulates the expression of tumor suppressor pdcd4 in human glioblastoma cell t98g. Cancer Lett 272: 197-205.
Chen, Z., J. I. Torrens, A. Anand, B. M. Spiegelman, and J. M. Friedman. 2005. Krox20 stimulates adipogenesis via c/ebpbeta-dependent and -independent mechanisms. Cell Metab 1: 93-106.
Chendrimada, T. P. et al. 2007. Microrna silencing through risc recruitment of eif6. Nature 447: 823-828.
Chendrimada, T. P. et al. 2005. Trbp recruits the dicer complex to ago2 for microrna processing and gene silencing. Nature 436: 740-744.
Cheng, Y. et al. 2007. Micrornas are aberrantly expressed in hypertrophic heart: Do they play a role in cardiac hypertrophy? Am J Pathol 170: 1831-1840.
Chivukula, R. R., and J. T. Mendell. 2008. Circular reasoning: Micrornas and cell-cycle control. Trends Biochem Sci 33: 474-481.
Cho, W. C. 2007. Oncomirs: The discovery and progress of micrornas in cancers. Mol Cancer 6: 60.
Cho, W. K., S. Stern, and J. D. Biggers. 1974. Inhibitory effect of dibutyryl camp on mouse oocyte maturation in vitro. J Exp Zool 187: 383-386.
Choi, Y. et al. 2007. Microarray analyses of newborn mouse ovaries lacking nobox. Biol Reprod 77: 312-319.
Christenson, L. K., P. F. Johnson, J. M. McAllister, and J. F. Strauss, 3rd. 1999. Ccaat/enhancer-binding proteins regulate expression of the human

steroidogenic acute regulatory protein (star) gene. J Biol Chem 274: 26591-26598.
Christenson, L. K., R. L. Stouffer, and J. F. Strauss, 3rd. 2001. Quantitative analysis of the hormone-induced hyperacetylation of histone h3 associated with the steroidogenic acute regulatory protein gene promoter. J Biol Chem 276: 27392-27399.
Chun, S. Y. et al. 2001. Expression of messenger ribonucleic acid for the antiapoptosis gene p11 in the rat ovary: Gonadotropin stimulation in granulosa cells of preovulatory follicles. Endocrinology 142: 2311-2317.
Clark, B. J., and D. M. Stocco. 1995. Expression of the steroidogenic acute regulatory (star) protein: A novel lh-induced mitochondrial protein required for the acute regulation of steroidogenesis in mouse leydig tumor cells. Endocr Res 21: 243-257.
Cloonan, N. et al. 2008. The mir-17-5p microrna is a key regulator of the g1/s phase cell cycle transition. Genome Biol 9: R127.
Cole, H. H., Hart, G. 1930. The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. American Journal of Physiology 93: 57-68.
Colegrove-Otero, L. J., N. Minshall, and N. Standart. 2005. Rna-binding proteins in early development. Crit Rev Biochem Mol Biol 40: 21-73.
Connolly, E., S. Braunstein, S. Formenti, and R. J. Schneider. 2006. Hypoxia inhibits protein synthesis through a 4e-bp1 and elongation factor 2 kinase pathway controlled by mtor and uncoupled in breast cancer cells. Mol Cell Biol 26: 3955-3965.
Connolly, E. et al. 2008. Elevated expression of the mir-17-92 polycistron and mir-21 in hepadnavirus-associated hepatocellular carcinoma contributes to the malignant phenotype. Am J Pathol 173: 856-864.
Conti, M. et al. 2002. Role of cyclic nucleotide signaling in oocyte maturation. Mol Cell Endocrinol 187: 153-159.
Conti, M., M. Hsieh, J. Y. Park, and Y. Q. Su. 2006. Role of the epidermal growth factor network in ovarian follicles. Mol Endocrinol 20: 715-723.
Corcoran, D. L. et al. 2009. Features of mammalian microrna promoters emerge from polymerase ii chromatin immunoprecipitation data. PLoS ONE 4: e5279.
Corner, G. W., Allen, W.M. 1929. Physiology of the corpus lutem. Ii. Production of a special uterine reaction (progestational proliferation) by extracts of the corpus luteum. American Journal of Physiology 88: 326-339.
Corney, D. C., A. Flesken-Nikitin, A. K. Godwin, W. Wang, and A. Y. Nikitin. 2007. Microrna-34b and microrna-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res 67: 8433-8438.
Couet, J. et al. 1990. Changes in 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase messenger ribonucleic acid, activity and protein levels

during the estrous cycle in the bovine ovary. Endocrinology 127: 2141-2148.
Coutinho, L. L. et al. 2007. Discovery and profiling of bovine micrornas from immune-related and embryonic tissues. Physiol Genomics 29: 35-43.
Crisp, T. M., D. A. Dessouky, and F. R. Denys. 1970. The fine structure of the human corpus luteum of early pregnancy and during the progestational phase of the mestrual cycle. Am J Anat 127: 37-69.
Cui, X. S., X. H. Shen, and N. H. Kim. 2007. Dicer1 expression in preimplantation mouse embryos: Involvement of oct3/4 transcription at the blastocyst stage. Biochem Biophys Res Commun 352: 231-236.
Curry, T. E., Jr., and M. F. Smith. 2006. Impact of extracellular matrix remodeling on ovulation and the folliculo-luteal transition. Semin Reprod Med 24: 228-241.
Dahiya, N. et al. 2008. Microrna expression and identification of putative mirna targets in ovarian cancer. PLoS ONE 3: e2436.
Dammer, E. B., and M. B. Sewer. 2008. Phosphorylation of ctbp1 by camp-dependent protein kinase modulates induction of cyp17 by stimulating partnering of ctbp1 and 2. J Biol Chem 283: 6925-6934.
Das, M. et al. 2008. Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. J Clin Endocrinol Metab 93: 881-887.
Datta, S. R., A. Brunet, and M. E. Greenberg. 1999. Cellular survival: A play in three akts. Genes Dev 13: 2905-2927.
Davis, B. J. et al. 1999. Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin e2 and interleukin-1beta. Endocrinology 140: 2685-2695.
Davis, B. N., A. C. Hilyard, G. Lagna, and A. Hata. 2008a. Smad proteins control drosha-mediated microrna maturation. Nature 454: 56-61.
Davis, B. N., A. C. Hilyard, G. Lagna, and A. Hata. 2008b. Smad proteins control drosha-mediated microrna maturation. Nature.
Davis, J. S. 1994. Mechanisms of hormone action: Luteinizing hormone receptors and second-messenger pathways. Curr Opin Obstet Gynecol 6: 254-261.
Dekel, N. 2005. Cellular, biochemical and molecular mechanisms regulating oocyte maturation. Mol Cell Endocrinol 234: 19-25.
Dennis, G., Jr. et al. 2003. David: Database for annotation, visualization, and integrated discovery. Genome Biol 4: P3.
Diao, F. Y. et al. 2004. The molecular characteristics of polycystic ovary syndrome (pcos) ovary defined by human ovary cdna microarray. J Mol Endocrinol 33: 59-72.
Dillhoff, M., J. Liu, W. Frankel, C. Croce, and M. Bloomston. 2008. Microrna-21 is overexpressed in pancreatic cancer and a potential predictor of survival. J Gastrointest Surg 12: 2171-2176.

Dineva, J. et al. 2007. Expression of atrial natriuretic peptide, progesterone, apoptosis-related proteins and caspase-3 in in vitro luteinized and leptin-treated porcine granulosa cells. Endocr Regul 41: 11-18.
Dissen, G. A., C. Romero, A. N. Hirshfield, and S. R. Ojeda. 2001. Nerve growth factor is required for early follicular development in the mammalian ovary. Endocrinology 142: 2078-2086.
Doneda, L., F. G. Klinger, L. Larizza, and M. De Felici. 2002. Kl/kit co-expression in mouse fetal oocytes. Int J Dev Biol 46: 1015-1021.
Dong, J. et al. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383: 531-535.
Dragovic, R. A. et al. 2005. Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. Endocrinology 146: 2798-2806.
Duggavathi, R., and B. D. Murphy. 2009. Development. Ovulation signals. Science 324: 890-891.
Earnshaw, W. C., L. M. Martins, and S. H. Kaufmann. 1999. Mammalian caspases: Structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68: 383-424.
Eichenlaub-Ritter, U., and M. Peschke. 2002. Expression in in-vivo and in-vitro growing and maturing oocytes: Focus on regulation of expression at the translational level. Hum Reprod Update 8: 21-41.
Eisenbach, M., and I. Tur-Kaspa. 1999. Do human eggs attract spermatozoa? Bioessays 21: 203-210.
Enders, A. C. 1973. Cytology of the corpus luteum. Biol Reprod 8: 158-182.
Eppig, J. J. 1980. Regulation of cumulus oophorus expansion by gonadotropins in vivo and in vitro. Biol Reprod 23: 545-552.
Eppig, J. J. 1982. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev Biol 89: 268-272.
Eppig, J. J., Viveiros, M.M., Marin-Bivens, C., De La Fuente, R. 2003. Regulation of mammalian oocyte maturation. In: P. Long, Adashi, E.Y. (ed.) The ovary. Elsevier, Science, San Diego.
Eppig, J. J., K. Wigglesworth, F. Pendola, and Y. Hirao. 1997. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biol Reprod 56: 976-984.
Erickson, G. F., V. G. Garzo, and D. A. Magoffin. 1991. Progesterone production by human granulosa cells cultured in serum free medium: Effects of gonadotrophins and insulin-like growth factor i (igf-i). Hum Reprod 6: 1074-1081.
Erickson, G. F., D. A. Magoffin, C. A. Dyer, and C. Hofeditz. 1985. The ovarian androgen producing cells: A review of structure/function relationships. Endocr Rev 6: 371-399.

Espey, L. L., and J. S. Richards. 2002. Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. Biol Reprod 67: 1662-1670.
Espey, L. L. et al. 2000. Induction of early growth response protein-1 gene expression in the rat ovary in response to an ovulatory dose of human chorionic gonadotropin. Endocrinology 141: 2385-2391.
Esquela-Kerscher, A., and F. J. Slack. 2006. Oncomirs - micrornas with a role in cancer. Nat Rev Cancer 6: 259-269.
Falcieri, E., P. Gobbi, L. Zamai, and M. Vitale. 1994. Ultrastructural features of apoptosis. Scanning Microsc 8: 653-665; discussion 665-656.
Fan, H. Y. et al. 2009. Mapk3/1 (erk1/2) in ovarian granulosa cells are essential for female fertility. Science 324: 938-941.
Fawcett, D. W., J. A. Long, and A. L. Jones. 1969. The ultrastructure of endocrine glands. Recent Prog Horm Res 25: 315-380.
Feber, A. et al. 2008. Microrna expression profiles of esophageal cancer. J Thorac Cardiovasc Surg 135: 255-260; discussion 260.
Fei, J., F. Lan, M. Guo, Y. Li, and Y. Liu. 2008. Inhibitory effects of anti-mirna oligonucleotides (amos) on a549 cell growth. J Drug Target 16: 688-693.
Ferby, I. et al. 2006. Mig6 is a negative regulator of egf receptor-mediated skin morphogenesis and tumor formation. Nat Med 12: 568-573.
Fiedler, S. D., M. Z. Carletti, X. Hong, and L. K. Christenson. 2008. Hormonal regulation of microrna expression in periovulatory mouse mural granulosa cells. Biol Reprod 79: 1030-1037.
Fiedler, S. D., M.Z. Carletti, L.K. Christenson. 2009. Quantitative rt-pcr methods for mature microrna expression analysis. In: K. Nicola (ed.) Rt-pcr protocols, edition 2: Methods in molecular biology.
Fischer, A., N. Schumacher, M. Maier, M. Sendtner, and M. Gessler. 2004. The notch target genes hey1 and hey2 are required for embryonic vascular development. Genes Dev 18: 901-911.
Fontana, L., A. Sorrentino, G. Condorelli, and C. Peschle. 2008. Role of micrornas in haemopoiesis, heart hypertrophy and cancer. Biochem Soc Trans 36: 1206-1210.
Frankel, L. B. et al. 2008. Programmed cell death 4 (pdcd4) is an important functional target of the microrna mir-21 in breast cancer cells. J Biol Chem 283: 1026-1033.
Fujita, S. et al. 2008. Mir-21 gene expression triggered by ap-1 is sustained through a double-negative feedback mechanism. J Mol Biol 378: 492-504.
Fukuda, T. et al. 2007. Dead-box rna helicase subunits of the drosha complex are required for processing of rrna and a subset of micrornas. Nat Cell Biol 9: 604-611.
Fulop, C. et al. 2003. Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. Development 130: 2253-2261.

Gandolfi, T. A., and F. Gandolfi. 2001. The maternal legacy to the embryo: Cytoplasmic components and their effects on early development. Theriogenology 55: 1255-1276.
Gangaraju, V. K., and H. Lin. 2009. Micrornas: Key regulators of stem cells. Nat Rev Mol Cell Biol 10: 116-125.
Giraldez, A. J. et al. 2006. Zebrafish mir-430 promotes deadenylation and clearance of maternal mrnas. Science 312: 75-79.
Glisovic, T., J. L. Bachorik, J. Yong, and G. Dreyfuss. 2008. Rna-binding proteins and post-transcriptional gene regulation. FEBS Lett 582: 1977-1986.
Goldring, N. B. et al. 1987. Cholesterol side-chain cleavage p450 messenger ribonucleic acid: Evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. Endocrinology 120: 1942-1950.
Gonzalez, G., and R. R. Behringer. 2009. Dicer is required for female reproductive tract development and fertility in the mouse. Mol Reprod Dev 76: 678-688.
Gonzalez-Robayna, I. J., T. N. Alliston, P. Buse, G. L. Firestone, and J. S. Richards. 1999. Functional and subcellular changes in the a-kinase-signaling pathway: Relation to aromatase and sgk expression during the transition of granulosa cells to luteal cells. Mol Endocrinol 13: 1318-1337.
Gramantieri, L. et al. 2007. Cyclin g1 is a target of mir-122a, a microrna frequently down-regulated in human hepatocellular carcinoma. Cancer Res 67: 6092-6099.
Griffiths-Jones, S., R. J. Grocock, S. van Dongen, A. Bateman, and A. J. Enright. 2006. Mirbase: Microrna sequences, targets and gene nomenclature. Nucleic Acids Res 34: D140-144.
Gross, P. R., L. I. Malkin, and W. A. Moyer. 1964. Templates for the first proteins of embryonic development. Proc Natl Acad Sci U S A 51: 407-414.
Gross, V. S., G. Wessel, H. M. Florman, and T. Ducibella. 2000. A monoclonal antibody that recognizes mammalian cortical granules and a 32-kilodalton protein in mouse eggs. Biol Reprod 63: 575-581.
Grunweller, A. et al. 2003. Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2'-o-methyl rna, phosphorothioates and small interfering rna. Nucleic Acids Res 31: 3185-3193.
Gueripel, X., V. Brun, and A. Gougeon. 2006. Oocyte bone morphogenetic protein 15, but not growth differentiation factor 9, is increased during gonadotropin-induced follicular development in the immature mouse and is associated with cumulus oophorus expansion. Biol Reprod 75: 836-843.
Gygi, S. P., Y. Rochon, B. R. Franza, and R. Aebersold. 1999. Correlation between protein and mrna abundance in yeast. Mol Cell Biol 19: 1720-1730.

Hamilton, G. S., and D. T. Armstrong. 1991. The superovulation of synchronous adult rats using follicle-stimulating hormone delivered by continuous infusion. Biol Reprod 44: 851-856.
Hampl, A., J. Pachernik, and P. Dvorak. 2000. Levels and interactions of p27, cyclin d3, and cdk4 during the formation and maintenance of the corpus luteum in mice. Biol Reprod 62: 1393-1401.
Han, J. et al. 2006. Molecular basis for the recognition of primary microrna by the drosha-dgcr8 complex. Cell 125: 887-901.
Harfe, B. D., M. T. McManus, J. H. Mansfield, E. Hornstein, and C. J. Tabin. 2005. The rnaseiii enzyme dicer is required for morphogenesis but not patterning of the vertebrate limb. Proc Natl Acad Sci U S A 102: 10898-10903.
Hashimi, S. T. et al. 2009. Microrna profiling identifies mir-34a and mir-21 and their target genes jag1 and wnt1 in the co-ordinate regulation of dendritic cell differentiation. Blood.
Havelock, J. C. et al. 2005. The ngfi-b family of transcription factors regulates expression of 3beta-hydroxysteroid dehydrogenase type 2 in the human ovary. Mol Hum Reprod 11: 79-85.
He, X., L. He, and G. J. Hannon. 2007. The guardian's little helper: Microrna in the p53 tumor suppressor network. Cancer Res 67: 11099-11101.
Hedhli, N., M. Pelat, and C. Depre. 2005. Protein turnover in cardiac cell growth and survival. Cardiovasc Res 68: 186-196.
Hedin, L., R. J. Rodgers, E. R. Simpson, and J. S. Richards. 1987. Changes in content of cytochrome p450(17)alpha, cytochrome p450scc, and 3-hydroxy-3-methylglutaryl coa reductase in developing rat ovarian follicles and corpora lutea: Correlation with theca cell steroidogenesis. Biol Reprod 37: 211-223.
Hennebold, J. D., M. Tanaka, J. Saito, B. R. Hanson, and E. Y. Adashi. 2000. Ovary-selective genes i: The generation and characterization of an ovary-selective complementary deoxyribonucleic acid library. Endocrinology 141: 2725-2734.
Henshaw, S. K. 1998. Unintended pregnancy in the united states. Fam Plann Perspect 30: 24-29, 46.
Hickey, G. J. et al. 1988. Hormonal regulation, tissue distribution, and content of aromatase cytochrome p450 messenger ribonucleic acid and enzyme in rat ovarian follicles and corpora lutea: Relationship to estradiol biosynthesis. Endocrinology 122: 1426-1436.
Hickey, G. J., J. S. Krasnow, W. G. Beattie, and J. S. Richards. 1990. Aromatase cytochrome p450 in rat ovarian granulosa cells before and after luteinization: Adenosine 3',5'-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cdna and 5' genomic DNA. Mol Endocrinol 4: 3-12.

Hillebrand, J., S. A. Barbee, and M. Ramaswami. 2007. P-body components, microrna regulation, and synaptic plasticity. ScientificWorldJournal 7: 178-190.
Hirshfield, A. N. 1991. Development of follicles in the mammalian ovary. Int Rev Cytol 124: 43-101.
Hong, S. J. et al. 2004. Establishment of a capillary-cumulus model to study the selection of sperm for fertilization by the cumulus oophorus. Hum Reprod 19: 1562-1569.
Hong, X., L. J. Luense, L. K. McGinnis, W. B. Nothnick, and L. K. Christenson. 2008. Dicer1 is essential for female fertility and normal development of the female reproductive system. Endocrinology 149: 6207-6212.
Hoodbhoy, T., E. J. Carroll, Jr., and P. Talbot. 2000. Relationship between p62 and p56, two proteins of the mammalian cortical granule envelope, and hyalin, the major component of the echinoderm hyaline layer, in hamsters. Biol Reprod 62: 979-987.
Hoodbhoy, T., P. Dandekar, P. Calarco, and P. Talbot. 2001. P62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplantation development. Mol Reprod Dev 59: 78-89.
Horie, K. et al. 1991. The expression of c-kit protein during oogenesis and early embryonic development. Biol Reprod 45: 547-552.
Horton, E. W., and N. L. Poyser. 1976. Uterine luteolytic hormone: A physiological role for prostaglandin f2alpha. Physiol Rev 56: 595-651.
Houbaviy, H. B., M. F. Murray, and P. A. Sharp. 2003. Embryonic stem cell-specific micrornas. Dev Cell 5: 351-358.
Hsieh, M. et al. 2007. Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation. Mol Cell Biol 27: 1914-1924.
Hu, C. L., R. G. Cowan, R. M. Harman, and S. M. Quirk. 2004. Cell cycle progression and activation of akt kinase are required for insulin-like growth factor i-mediated suppression of apoptosis in granulosa cells. Mol Endocrinol 18: 326-338.
Hu, S. J. et al. 2008. Microrna expression and regulation in mouse uterus during embryo implantation. J Biol Chem 283: 23473-23484.
Huang, E. J. et al. 1993. The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. Dev Biol 157: 100-109.
Huslig, R. L., A. Malik, and M. R. Clark. 1987. Human chorionic gonadotropin stimulation of immunoreactive prostaglandin synthase in the rat ovary. Mol Cell Endocrinol 50: 237-246.
Hutvagner, G. et al. 2001. A cellular function for the rna-interference enzyme dicer in the maturation of the let-7 small temporal rna. Science 293: 834-838.

Hutvagner, G., and M. J. Simard. 2008. Argonaute proteins: Key players in rna silencing. Nat Rev Mol Cell Biol 9: 22-32.
Ideker, T. et al. 2001. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science 292: 929-934.
Ing, N. H., D. A. Massuto, and L. A. Jaeger. 2008. Estradiol up-regulates auf1p45 binding to stabilizing regions within the 3'-untranslated region of estrogen receptor alpha mrna. J Biol Chem 283: 1764-1772.
Iorio, M. V. et al. 2007. Microrna signatures in human ovarian cancer. Cancer Res 67: 8699-8707.
Irizarry, R. A. et al. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4: 249-264.
Ismail, R. S., Y. Okawara, J. N. Fryer, and B. C. Vanderhyden. 1996. Hormonal regulation of the ligand for c-kit in the rat ovary and its effects on spontaneous oocyte meiotic maturation. Mol Reprod Dev 43: 458-469.
Jabara, S. et al. 2003. Stromal cells of the human postmenopausal ovary display a distinctive biochemical and molecular phenotype. J Clin Endocrinol Metab 88: 484-492.
Jackson, R. J., and N. Standart. 2007. How do microrna regulate gene expression? Sci STKE 2007: re1.
Jacquin, T. D. et al. 1996. Reorganization of pontine rhythmogenic neuronal networks in krox-20 knockout mice. Neuron 17: 747-758.
Jamin, S. P., N. A. Arango, Y. Mishina, M. C. Hanks, and R. R. Behringer. 2002. Requirement of bmpr1a for mullerian duct regression during male sexual development. Nat Genet 32: 408-410.
Ji, R. et al. 2007. Microrna expression signature and antisense-mediated depletion reveal an essential role of microrna in vascular neointimal lesion formation. Circ Res 100: 1579-1588.
Jovanovic, M., and M. O. Hengartner. 2006. Mirnas and apoptosis: Rnas to die for. Oncogene 25: 6176-6187.
Kalma, Y., I. Granot, D. Galiani, A. Barash, and N. Dekel. 2004. Luteinizing hormone-induced connexin 43 down-regulation: Inhibition of translation. Endocrinology 145: 1617-1624.
Kamel, F., J. A. Balz, C. L. Kubajak, and V. A. Schneider. 1987. Gonadal steroids modulate pulsatile luteinizing hormone secretion by perifused rat anterior pituitary cells. Endocrinology 120: 1651-1657.
Kaneko, T. et al. 2000. Hyaluronic acid inhibits apoptosis in granulosa cells via cd44. J Assist Reprod Genet 17: 162-167.
Kanellopoulou, C. et al. 2005. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev 19: 489-501.
Keene, J. D. 2001. Ribonucleoprotein infrastructure regulating the flow of genetic information between the genome and the proteome. Proc Natl Acad Sci U S A 98: 7018-7024.

Keene, J. D., and S. A. Tenenbaum. 2002. Eukaryotic mrnps may represent posttranscriptional operons. Mol Cell 9: 1161-1167.
Keller, D. M. et al. 2007. Characterization of pancreatic transcription factor pdx-1 binding sites using promoter microarray and serial analysis of chromatin occupancy. J Biol Chem 282: 32084-32092.
Keshet, E. et al. 1991. Embryonic rna expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. Embo J 10: 2425-2435.
Ketting, R. F. 2009. Semiconserved regulation of mesendoderm differentiation by micrornas. Dev Cell 16: 487-488.
Khvorova, A., A. Reynolds, and S. D. Jayasena. 2003. Functional sirnas and mirnas exhibit strand bias. Cell 115: 209-216.
Kim, H. J., X. S. Cui, E. J. Kim, W. J. Kim, and N. H. Kim. 2006. New porcine microrna genes found by homology search. Genome 49: 1283-1286.
Kim, S. et al. 2008. Microrna mir-199a* regulates the met proto-oncogene and the downstream extracellular signal-regulated kinase 2 (erk2). J Biol Chem 283: 18158-18166.
Kim, V. N. 2005. Microrna biogenesis: Coordinated cropping and dicing. Nat Rev Mol Cell Biol 6: 376-385.
Kim, Y. K., and V. N. Kim. 2007. Processing of intronic micrornas. EMBO J 26: 775-783.
Kinloch, R. A., R. J. Roller, and P. M. Wassarman. 1993. Quantitative analysis of specific messenger rnas by ribonuclease protection. Methods Enzymol 225: 294-303.
Kiriakidou, M., J. M. McAllister, T. Sugawara, and J. F. Strauss, 3rd. 1996. Expression of steroidogenic acute regulatory protein (star) in the human ovary. J Clin Endocrinol Metab 81: 4122-4128.
Klein, M. E. et al. 2007. Homeostatic regulation of mecp2 expression by a creb-induced microrna. Nat Neurosci 10: 1513-1514.
Kline, D., and J. T. Kline. 1992. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. Dev Biol 149: 80-89.
Kochupurakkal, B. S. et al. 2005. Epigen, the last ligand of erbb receptors, reveals intricate relationships between affinity and mitogenicity. J Biol Chem 280: 8503-8512.
Krichevsky, A. M., and G. Gabriely. 2009. Mir-21: A small multi-faceted rna. J Cell Mol Med 13: 39-53.
Kuehn, H., Liberzon, A., Reich, M., Mesirov, J.P. 2008. Using genepattern for gene expression analysis. Broad Institute of MIT and Harvard, Cambridge, MA.
Kumar, T. R., Y. Wang, N. Lu, and M. M. Matzuk. 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15: 201-204.

Ladman, A. J. 1964. Determination of length of biological life of pregnant mare's serum gonadotrophin in immature mice. Proc Soc Exp Biol Med 117: 498-499.
Lagos-Quintana, M., R. Rauhut, W. Lendeckel, and T. Tuschl. 2001. Identification of novel genes coding for small expressed rnas. Science 294: 853-858.
Lagos-Quintana, M. et al. 2002. Identification of tissue-specific microrнas from mouse. Curr Biol 12: 735-739.
Lai, E. C. 2002. Micro rnas are complementary to 3' utr sequence motifs that mediate negative post-transcriptional regulation. Nat Genet 30: 363-364.
Lamond, D. R. 1960. Induction of ovulation in mice with placental gonadotrophins. J Endocrinol 20: 277-287.
Landgraf, P. et al. 2007. A mammalian microrna expression atlas based on small rna library sequencing. Cell 129: 1401-1414.
Lau, N. C., L. P. Lim, E. G. Weinstein, and D. P. Bartel. 2001. An abundant class of tiny rnas with probable regulatory roles in caenorhabditis elegans. Science 294: 858-862.
Leblanc, S. E. et al. 2005. Regulation of cholesterol/lipid biosynthetic genes by egr2/krox20 during peripheral nerve myelination. J Neurochem 93: 737-748.
Lee, D. et al. 2004a. Epiregulin is not essential for development of intestinal tumors but is required for protection from intestinal damage. Mol Cell Biol 24: 8907-8916.
Lee, J. et al. 1999. Gonadotropin stimulation of pituitary adenylate cyclase-activating polypeptide (pacap) messenger ribonucleic acid in the rat ovary and the role of pacap as a follicle survival factor. Endocrinology 140: 818-826.
Lee, R. C., and V. Ambros. 2001. An extensive class of small rnas in caenorhabditis elegans. Science 294: 862-864.
Lee, R. C., R. L. Feinbaum, and V. Ambros. 1993. The c. Elegans heterochronic gene lin-4 encodes small rnas with antisense complementarity to lin-14. Cell 75: 843-854.
Lee, Y. et al. 2003. The nuclear rnase iii drosha initiates microrna processing. Nature 425: 415-419.
Lee, Y. et al. 2004b. Microrna genes are transcribed by rna polymerase ii. EMBO J 23: 4051-4060.
Legan, S. J., G. A. Coon, and F. J. Karsch. 1975. Role of estrogen as initiator of daily lh surges in the ovariectomized rat. Endocrinology 96: 50-56.
LeMaire, W. J., N. S. Yang, H. H. Behrman, and J. M. Marsh. 1973. Preovulatory changes in the concentration of prostaglandins in rabbit graafian follicles. Prostaglandins 3: 367-376.
Leo, C. P., S. Y. Hsu, S. Y. Chun, H. W. Bae, and A. J. Hsueh. 1999. Characterization of the antiapoptotic bcl-2 family member myeloid cell leukemia-1 (mcl-1)

and the stimulation of its message by gonadotropins in the rat ovary. Endocrinology 140: 5469-5477.
Leo, C. P., M. D. Pisarska, and A. J. Hsueh. 2001. DNA array analysis of changes in preovulatory gene expression in the rat ovary. Biol Reprod 65: 269-276.
Lewis, B. P., C. B. Burge, and D. P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microrna targets. Cell 120: 15-20.
Li, Q. X., N. Ke, R. Sundaram, and F. Wong-Staal. 2006. Nr4a1, 2, 3--an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. Histol Histopathol 21: 533-540.
Li, W., and K. Ruan. 2009. Microrna detection by microarray. Anal Bioanal Chem 394: 1117-1124.
Lim, H. et al. 1997. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91: 197-208.
Lincoln, A. J. et al. 2002. Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation. Nat Genet 30: 446-449.
Liu, J. et al. 2004. Argonaute2 is the catalytic engine of mammalian rnai. Science 305: 1437-1441.
Liu, Z. et al. 2009. Il6: An autocrine regulator of the mouse cumulus cell-oocyte complex expansion process. Endocrinology.
Loffler, D. et al. 2007. Interleukin-6 dependent survival of multiple myeloma cells involves the stat3-mediated induction of microrna-21 through a highly conserved enhancer. Blood 110: 1330-1333.
Luck, M. R., and Y. Zhao. 1993. Identification and measurement of collagen in the bovine corpus luteum and its relationship with ascorbic acid and tissue development. J Reprod Fertil 99: 647-652.
Luetteke, N. C. et al. 1999. Targeted inactivation of the egf and amphiregulin genes reveals distinct roles for egf receptor ligands in mouse mammary gland development. Development 126: 2739-2750.
Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg, and U. Kutay. 2004. Nuclear export of microrna precursors. Science 303: 95-98.
Ma, X. M., and J. Blenis. 2009. Molecular mechanisms of mtor-mediated translational control. Nat Rev Mol Cell Biol 10: 307-318.
Magarelli, P. C., R. J. Zachow, and D. A. Magoffin. 1996. Developmental and hormonal regulation of rat theca-cell differentiation factor secretion in ovarian follicles. Biol Reprod 55: 416-420.
Markosyan, N., and D. M. Duffy. 2009. Prostaglandin e2 acts via multiple receptors to regulate plasminogen-dependent proteolysis in the primate periovulatory follicle. Endocrinology 150: 435-444.
Maroney, P. A., Y. Yu, J. Fisher, and T. W. Nilsen. 2006. Evidence that microrna are associated with translating messenger rnas in human cells. Nat Struct Mol Biol 13: 1102-1107.

Marquez, R. T., and A. P. McCaffrey. 2008. Advances in microrna: Implications for gene therapists. Hum Gene Ther 19: 27-38.
Marsh, E. E. et al. 2008. Differential expression of microrna species in human uterine leiomyoma versus normal myometrium. Fertil Steril 89: 1771-1776.
Marsh, J. M., R. W. Butcher, K. Savard, and E. W. Sutherland. 1966. The stimulatory effect of luteinizing hormone on adenosine 3',5'-monophosphate accumulation in corpus luteum slices. J Biol Chem 241: 5436-5440.
Matikainen, T. et al. 2001. Caspase-3 gene knockout defines cell lineage specificity for programmed cell death signaling in the ovary. Endocrinology 142: 2468-2480.
Matsubara, H. et al. 2000. Gonadotropins and cytokines affect luteal function through control of apoptosis in human luteinized granulosa cells. J Clin Endocrinol Metab 85: 1620-1626.
Mattioli, M. et al. 2001. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. Biol Reprod 65: 1014-1019.
McClellan, M. C., M. A. Dieckman, J. H. Abel, Jr., and G. D. Niswender. 1975. Luteinizing hormone, progesterone and the morphological development of normal and superovulated corpora lutea in sheep. Cell Tissue Res 164: 291-307.
McGee, E. A., and A. J. Hsueh. 2000. Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21: 200-214.
McRae, R. S., H. M. Johnston, M. Mihm, and P. J. O'Shaughnessy. 2005. Changes in mouse granulosa cell gene expression during early luteinization. Endocrinology 146: 309-317.
Mehlmann, L. M. 2005. Oocyte-specific expression of gpr3 is required for the maintenance of meiotic arrest in mouse oocytes. Dev Biol 288: 397-404.
Mehlmann, L. M., T. L. Jones, and L. A. Jaffe. 2002. Meiotic arrest in the mouse follicle maintained by a gs protein in the oocyte. Science 297: 1343-1345.
Mehlmann, L. M., and D. Kline. 1994. Regulation of intracellular calcium in the mouse egg: Calcium release in response to sperm or inositol trisphosphate is enhanced after meiotic maturation. Biol Reprod 51: 1088-1098.
Mehlmann, L. M. et al. 2004. The gs-linked receptor gpr3 maintains meiotic arrest in mammalian oocytes. Science 306: 1947-1950.
Mehlmann, L. M., M. Terasaki, L. A. Jaffe, and D. Kline. 1995. Reorganization of the endoplasmic reticulum during meiotic maturation of the mouse oocyte. Dev Biol 170: 607-615.
Memili, E., T. Dominko, and N. L. First. 1998. Onset of transcription in bovine oocytes and preimplantation embryos. Mol Reprod Dev 51: 36-41.

Meng, F. et al. 2006. Involvement of human micro-rna in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology 130: 2113-2129.
Meng, F. et al. 2007. Microrna-21 regulates expression of the pten tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133: 647-658.
Meric-Bernstam, F., and A. M. Gonzalez-Angulo. 2009. Targeting the mtor signaling network for cancer therapy. J Clin Oncol 27: 2278-2287.
Miller, D. J., X. Gong, G. Decker, and B. D. Shur. 1993. Egg cortical granule n-acetylglucosaminidase is required for the mouse zona block to polyspermy. J Cell Biol 123: 1431-1440.
Miranda, K. C. et al. 2006. A pattern-based method for the identification of microrna binding sites and their corresponding heteroduplexes. Cell 126: 1203-1217.
Miyazaki, S., H. Shirakawa, K. Nakada, and Y. Honda. 1993. Essential role of the inositol 1,4,5-trisphosphate receptor/ca2+ release channel in ca2+ waves and ca2+ oscillations at fertilization of mammalian eggs. Dev Biol 158: 62-78.
Montagnoli, A., D. Guardavaccaro, G. Starace, and F. Tirone. 1996. Overexpression of the nerve growth factor-inducible pc3 immediate early gene is associated with growth inhibition. Cell Growth Differ 7: 1327-1336.
Morohashi, K., S. Honda, Y. Inomata, H. Handa, and T. Omura. 1992. A common trans-acting factor, ad4-binding protein, to the promoters of steroidogenic p-450s. J Biol Chem 267: 17913-17919.
Muinos-Gimeno, M. et al. 2009. Allele variants in functional microrna target sites of the neurotrophin-3 receptor gene (ntrk3) as susceptibility factors for anxiety disorders. Hum Mutat.
Murchison, E. P. et al. 2007. Critical roles for dicer in the female germline. Genes Dev 21: 682-693.
Murphy, B. D. 2000. Models of luteinization. Biol Reprod 63: 2-11.
Nachtigall, R. D. 2006. International disparities in access to infertility services. Fertil Steril 85: 871-875.
Nagaraja, A. K. et al. 2008. Deletion of dicer in somatic cells of the female reproductive tract causes sterility. Mol Endocrinol 22: 2336-2352.
Nairn, A. C. et al. 2001. Elongation factor-2 phosphorylation and the regulation of protein synthesis by calcium. Prog Mol Subcell Biol 27: 91-129.
Nairn, A. C., and H. C. Palfrey. 1987. Identification of the major mr 100,000 substrate for calmodulin-dependent protein kinase iii in mammalian cells as elongation factor-2. J Biol Chem 262: 17299-17303.
Nam, E. J. et al. 2008. Microrna expression profiles in serous ovarian carcinoma. Clin Cancer Res 14: 2690-2695.

Nilsson, L., and G. Selstam. 1975. Effects of lh and hcg on the metabolism of the prepubertal rat ovary: Stimulation of amino acid transport, protein synthesis, and glycolysis. Acta Endocrinol (Copenh) 78: 122-134.
Nothnick, W. B. 2008. Regulation of uterine matrix metalloproteinase-9 and the role of micrornas. Semin Reprod Med 26: 494-499.
Nottrott, S., M. J. Simard, and J. D. Richter. 2006. Human let-7a mirna blocks protein production on actively translating polyribosomes. Nat Struct Mol Biol 13: 1108-1114.
Nuovo, G. J. et al. 2009. A methodology for the combined in situ analyses of the precursor and mature forms of micrornas and correlation with their putative targets. Nat Protoc 4: 107-115.
Obernosterer, G., P. J. Leuschner, M. Alenius, and J. Martinez. 2006. Post-transcriptional regulation of microrna expression. RNA 12: 1161-1167.
Ochsner, S. A. et al. 2003. Disrupted function of tumor necrosis factor-alpha-stimulated gene 6 blocks cumulus cell-oocyte complex expansion. Endocrinology 144: 4376-4384.
Ohta, N. et al. 1999. Expression of cd44 in human cumulus and mural granulosa cells of individual patients in in-vitro fertilization programmes. Mol Hum Reprod 5: 22-28.
Oonk, R. B., J. S. Krasnow, W. G. Beattie, and J. S. Richards. 1989. Cyclic amp-dependent and -independent regulation of cholesterol side chain cleavage cytochrome p-450 (p-450scc) in rat ovarian granulosa cells and corpora lutea. Cdna and deduced amino acid sequence of rat p-450scc. J Biol Chem 264: 21934-21942.
Orczyk, G. P., and H. R. Behrman. 1972. Ovulation blockade by aspirin or indomethacin--in vivo evidence for a role of prostaglandin in gonadotrophin secretion. Prostaglandins 1: 3-20.
Orom, U. A., S. Kauppinen, and A. H. Lund. 2006. Lna-modified oligonucleotides mediate specific inhibition of microrna function. Gene 372: 137-141.
Ostermeier, G. C., D. Miller, J. D. Huntriss, M. P. Diamond, and S. A. Krawetz. 2004. Reproductive biology: Delivering spermatozoan rna to the oocyte. Nature 429: 154.
Otsuka, M. et al. 2008. Impaired microrna processing causes corpus luteum insufficiency and infertility in mice. J Clin Invest 118: 1944-1954.
Pan, Q., X. Luo, and N. Chegini. 2008. Differential expression of micrornas in myometrium and leiomyomas and regulation by ovarian steroids. J Cell Mol Med 12: 227-240.
Panigone, S., M. Hsieh, M. Fu, L. Persani, and M. Conti. 2008. Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Mol Endocrinol 22: 924-936.
Papagiannakopoulos, T., A. Shapiro, and K. S. Kosik. 2008. Microrna-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. Cancer Res 68: 8164-8172.

Park, J. I. et al. 2007. Activation of protein kinase czeta mediates luteinizing hormone- or forskolin-induced ngfi-b expression in preovulatory granulosa cells of rat ovary. Mol Cell Endocrinol 270: 79-86.
Park, J. I., H. J. Park, Y. I. Lee, Y. M. Seo, and S. Y. Chun. 2003. Regulation of ngfi-b expression during the ovulatory process. Mol Cell Endocrinol 202: 25-29.
Park, J. Y. et al. 2004. Egf-like growth factors as mediators of lh action in the ovulatory follicle. Science 303: 682-684.
Park, O. K., and K. E. Mayo. 1991. Transient expression of progesterone receptor messenger rna in ovarian granulosa cells after the preovulatory luteinizing hormone surge. Mol Endocrinol 5: 967-978.
Parkes, A. S. 1929. The internal secretions of the ovary. Longmans, Green, & Co., London.
Parr, E. L. 1975. Rupture of ovarian follicles at ovulation. J Reprod Fertil Suppl: 1-22.
Pasquinelli, A. E. et al. 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory rna. Nature 408: 86-89.
Passeri, D. et al. 2006. Btg2 enhances retinoic acid-induced differentiation by modulating histone h4 methylation and acetylation. Mol Cell Biol 26: 5023-5032.
Pastorelli, L. M. et al. 2009. Genetic analyses reveal a requirement for dicer1 in the mouse urogenital tract. Mamm Genome 20: 140-151.
Paynton, B. V., R. Rempel, and R. Bachvarova. 1988. Changes in state of adenylation and time course of degradation of maternal mrnas during oocyte maturation and early embryonic development in the mouse. Dev Biol 129: 304-314.
Peng, X. R., A. J. Hsueh, P. S. LaPolt, L. Bjersing, and T. Ny. 1991. Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. Endocrinology 129: 3200-3207.
Peng, Y. et al. 2008. Antiproliferative effects by let-7 repression of high-mobility group a2 in uterine leiomyoma. Mol Cancer Res 6: 663-673.
Perreault, S. D., R. R. Barbee, and V. L. Slott. 1988. Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Dev Biol 125: 181-186.
Peters, H., A. G. Byskov, R. Himelstein-Braw, and M. Faber. 1975. Follicular growth: The basic event in the mouse and human ovary. J Reprod Fertil 45: 559-566.
Peters, L., and G. Meister. 2007. Argonaute proteins: Mediators of rna silencing. Mol Cell 26: 611-623.
Petersen, C. P., M. E. Bordeleau, J. Pelletier, and P. A. Sharp. 2006. Short rnas repress translation after initiation in mammalian cells. Mol Cell 21: 533-542.

Pincus, G., and E. V. Enzmann. 1934. Can mammalian eggs undergo normal development in vitro? Proc Natl Acad Sci U S A 20: 121-122.
Piontkewitz, Y., Dekel, N. 1993. Hetanol, an alkanol that blocks gap junctions, induces oocyte maturation. Endocrine Journal 1: 365-372.
Pirino, G., M. P. Wescott, and P. J. Donovan. 2009. Protein kinase a regulates resumption of meiosis by phosphorylation of cdc25b in mammalian oocytes. Cell Cycle 8: 665-670.
Polak, P., and M. N. Hall. 2009. Mtor and the control of whole body metabolism. Curr Opin Cell Biol 21: 209-218.
Potter, C. J., L. G. Pedraza, and T. Xu. 2002. Akt regulates growth by directly phosphorylating tsc2. Nat Cell Biol 4: 658-665.
Pratt, A. J., and I. J. Macrae. 2009. The rna induced silencing complex: A versatile gene-silencing machine. J Biol Chem.
Priedkalns, J., A. F. Weber, and R. Zemjanis. 1968. Qualitative and quantitative morphological studies of the cells of the membrame granulosa, theca interna and corpus luteum of the bovine ovary. Z Zellforsch Mikrosk Anat 85: 501-520.
Quirk, S. M., R. G. Cowan, and R. M. Harman. 2004. Progesterone receptor and the cell cycle modulate apoptosis in granulosa cells. Endocrinology 145: 5033-5043.
Racowsky, C., and K. V. Baldwin. 1989. In vitro and in vivo studies reveal that hamster oocyte meiotic arrest is maintained only transiently by follicular fluid, but persistently by membrana/cumulus granulosa cell contact. Dev Biol 134: 297-306.
Rajasekhar, V. K., and E. C. Holland. 2004. Postgenomic global analysis of translational control induced by oncogenic signaling. Oncogene 23: 3248-3264.
Ram, P. T., and R. M. Schultz. 1993. Reporter gene expression in g2 of the 1-cell mouse embryo. Dev Biol 156: 552-556.
Ramachandra, R. K., M. Salem, S. Gahr, C. E. Rexroad, 3rd, and J. Yao. 2008. Cloning and characterization of micrornas from rainbow trout (oncorhynchus mykiss): Their expression during early embryonic development. BMC Dev Biol 8: 41.
Rankin, T. L. et al. 2001. Defective zonae pellucidae in zp2-null mice disrupt folliculogenesis, fertility and development. Development 128: 1119-1126.
Rao, M. C., A. R. Midgley, Jr., and J. S. Richards. 1978. Hormonal regulation of ovarian cellular proliferation. Cell 14: 71-78.
Raval, G. N. et al. 2003. Loss of expression of tropomyosin-1, a novel class ii tumor suppressor that induces anoikis, in primary breast tumors. Oncogene 22: 6194-6203.
Raver-Shapira, N., and M. Oren. 2007. Tiny actors, great roles: Micrornas in p53's service. Cell Cycle 6: 2656-2661.

Recheis, B., A. Osanger, S. Haubenwallner, W. J. Schneider, and J. Nimpf. 2000. Chicken coagulation factor xiiia is produced by the theca externa and stabilizes the ovarian follicular wall. J Biol Chem 275: 35320-35327.
Rehwinkel, J., I. Behm-Ansmant, D. Gatfield, and E. Izaurralde. 2005. A crucial role for gw182 and the dcp1:Dcp2 decapping complex in mirna-mediated gene silencing. RNA 11: 1640-1647.
Reich, R., R. Miskin, and A. Tsafriri. 1986. Intrafollicular distribution of plasminogen activators and their hormonal regulation in vitro. Endocrinology 119: 1588-1593.
Reinhart, B. J. et al. 2000. The 21-nucleotide let-7 rna regulates developmental timing in caenorhabditis elegans. Nature 403: 901-906.
Reynaud, K., R. Cortvrindt, J. Smitz, and M. A. Driancourt. 2000. Effects of kit ligand and anti-kit antibody on growth of cultured mouse preantral follicles. Mol Reprod Dev 56: 483-494.
Richards, J. S., and A. I. Rolfes. 1980. Hormonal regulation of cyclic amp binding to specific receptor proteins in rat ovarian follicles. Characterization by photoaffinity labeling. J Biol Chem 255: 5481-5489.
Richards, J. S., D. L. Russell, S. Ochsner, and L. L. Espey. 2002. Ovulation: New dimensions and new regulators of the inflammatory-like response. Annu Rev Physiol 64: 69-92.
Richards, J. S., D. L. Russell, R. L. Robker, M. Dajee, and T. N. Alliston. 1998. Molecular mechanisms of ovulation and luteinization. Mol Cell Endocrinol 145: 47-54.
Ro, S., C. Park, K. M. Sanders, J. R. McCarrey, and W. Yan. 2007a. Cloning and expression profiling of testis-expressed microrna s. Dev Biol 311: 592-602.
Ro, S. et al. 2007b. Cloning and expression profiling of small rnas expressed in the mouse ovary. RNA 13: 2366-2380.
Robker, R. L., and J. S. Richards. 1998a. Hormonal control of the cell cycle in ovarian cells: Proliferation versus differentiation. Biol Reprod 59: 476-482.
Robker, R. L., and J. S. Richards. 1998b. Hormone-induced proliferation and differentiation of granulosa cells: A coordinated balance of the cell cycle regulators cyclin d2 and p27kip1. Mol Endocrinol 12: 924-940.
Robson, J. M. 1936. The action oe progesterone on the uterus of the rabbit and its antagonism by oestrone. J Physiol 88: 100-111.
Rodgers, R. J., H. F. Irving-Rodgers, I. L. van Wezel, M. Krupa, and T. C. Lavranos. 2001. Dynamics of the membrana granulosa during expansion of the ovarian follicular antrum. Mol Cell Endocrinol 171: 41-48.
Rodier, A. et al. 1999. Btg1: A triiodothyronine target involved in the myogenic influence of the hormone. Exp Cell Res 249: 337-348.

Rouault, J. P. et al. 1996. Identification of btg2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. Nat Genet 14: 482-486.
Rouault, J. P. et al. 1992. Btg1, a member of a new family of antiproliferative genes. EMBO J 11: 1663-1670.
Runner, M. N., and A. Gates. 1954. Conception in prepuberal mice following artificially induced ovulation and mating. Nature 174: 222-223.
Runner MN, P. J. 1953. Transplantation and survival of unfertilized ova of the mouse in relation to postovulatory age. Journal of Experimental Zoology 124: 303-316.
Russell, D. L., K. M. Doyle, I. Gonzales-Robayna, C. Pipaon, and J. S. Richards. 2003. Egr-1 induction in rat granulosa cells by follicle-stimulating hormone and luteinizing hormone: Combinatorial regulation by transcription factors cyclic adenosine 3',5'-monophosphate regulatory element binding protein, serum response factor, sp1, and early growth response factor-1. Mol Endocrinol 17: 520-533.
Russell, D. L., and A. Salustri. 2006. Extracellular matrix of the cumulus-oocyte complex. Semin Reprod Med 24: 217-227.
Ryazanov, A. G., E. A. Shestakova, and P. G. Natapov. 1988. Phosphorylation of elongation factor 2 by ef-2 kinase affects rate of translation. Nature 334: 170-173.
Sahmi, M., E. S. Nicola, and C. A. Price. 2006. Hormonal regulation of cytochrome p450 aromatase mrna stability in non-luteinizing bovine granulosa cells in vitro. J Endocrinol 190: 107-115.
Sahmi, M., E. S. Nicola, J. M. Silva, and C. A. Price. 2004. Expression of 17beta- and 3beta-hydroxysteroid dehydrogenases and steroidogenic acute regulatory protein in non-luteinizing bovine granulosa cells in vitro. Mol Cell Endocrinol 223: 43-54.
Saini, H. K., S. Griffiths-Jones, and A. J. Enright. 2007. Genomic analysis of human microrna transcripts. Proc Natl Acad Sci U S A 104: 17719-17724.
Sakamoto, S. et al. 2009. The nf90-nf45 complex functions as a negative regulator in the microrna processing pathway. Mol Cell Biol.
Sakurai, T., K. Tamura, S. Okamoto, T. Hara, and H. Kogo. 2003. Possible role of cyclooxygenase ii in the acquisition of ovarian luteal function in rodents. Biol Reprod 69: 835-842.
Salustri, A., M. Yanagishita, C. B. Underhill, T. C. Laurent, and V. C. Hascall. 1992. Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. Dev Biol 151: 541-551.
Sathananthan, A. H. et al. 1985. The origin and distribution of cortical granules in human oocytes with reference to golgi, nucleolar, and microfilament activity. Ann N Y Acad Sci 442: 251-264.
Schetter, A. J. et al. 2008. Microrna expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA 299: 425-436.

Schmelzle, T., and M. N. Hall. 2000. Tor, a central controller of cell growth. Cell 103: 253-262.
Schmidt, J., J. de Avila, and D. McLean. 2006. Regulation of protein tyrosine phosphatase 4a1, b-cell translocation gene 2, nuclear receptor subfamily 4a1 and diacylglycerol o-acyltransferase 1 by follicle stimulating hormone in the rat ovary. Reprod Fertil Dev 18: 757-765.
Schoenfelder, M., and R. Einspanier. 2003. Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. Biol Reprod 69: 269-277.
Schwarz, D. S. et al. 2003. Asymmetry in the assembly of the rnai enzyme complex. Cell 115: 199-208.
Sekulic, A. et al. 2000. A direct linkage between the phosphoinositide 3-kinase-akt signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res 60: 3504-3513.
Sethi, P., and W. J. Lukiw. 2009. Micro-rna abundance and stability in human brain: Specific alterations in alzheimer's disease temporal lobe neocortex. Neurosci Lett.
Shao, R., E. Rung, B. Weijdegard, and H. Billig. 2006. Induction of apoptosis increases sumo-1 protein expression and conjugation in mouse periovulatory granulosa cells in vitro. Mol Reprod Dev 73: 50-60.
Sharma, S. C., and J. S. Richards. 2000. Regulation of ap1 (jun/fos) factor expression and activation in ovarian granulosa cells. Relation of jund and fra2 to terminal differentiation. J Biol Chem 275: 33718-33728.
Sharov, A. A., Dudekula, D.B., Ko, M.S.H. 2005. Principal component and significance analysis of microarrays with nia array analysis tool. Bioinformatics Advance Access.
Shedlovsky, T., A. Rothen, R. Greep, V. A. N. D. HB, and B. F. Chow. 1940. The isolation in pure form of the interstitial cell-stimulating (luteinizing) hormone of the anterior lobe of the pituitary gland. Science 92: 178-180.
Shimada, H. et al. 2001. Hypocoagulable state of human preovulatory ovarian follicular fluid: Role of sulfated proteoglycan and tissue factor pathway inhibitor in the fluid. Biol Reprod 64: 1739-1745.
Shimada, M., I. Hernandez-Gonzalez, I. Gonzalez-Robayna, and J. S. Richards. 2006. Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: Key roles for prostaglandin synthase 2 and progesterone receptor. Mol Endocrinol 20: 1352-1365.
Shimoda, K., E. Sato, T. Tanaka, T. Toyoda. 1993. Morphological differentiation of the microvasculature during follicular development, ovulation, and luteinization of mouse ovaries. Dev Growth Differ. 35: 431-437.
Shingara, J. et al. 2005. An optimized isolation and labeling platform for accurate microrna expression profiling. RNA 11: 1461-1470.
Si, M. L. et al. 2007. Mir-21-mediated tumor growth. Oncogene 26: 2799-2803.

Sicinski, P. et al. 1996. Cyclin d2 is an fsh-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature 384: 470-474.
Silber, J. et al. 2008. Mir-124 and mir-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med 6: 14.
Singh, S. K., M. N. Kagalwala, J. Parker-Thornburg, H. Adams, and S. Majumder. 2008. Rest maintains self-renewal and pluripotency of embryonic stem cells. Nature 453: 223-227.
Sinkkonen, L. et al. 2008. Microrna control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol 15: 259-267.
Sirois, J., and J. S. Richards. 1993. Transcriptional regulation of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. Evidence for the role of a cis-acting c/ebp beta promoter element. J Biol Chem 268: 21931-21938.
Sirois, J., D. L. Simmons, and J. S. Richards. 1992. Hormonal regulation of messenger ribonucleic acid encoding a novel isoform of prostaglandin endoperoxide h synthase in rat preovulatory follicles. Induction in vivo and in vitro. J Biol Chem 267: 11586-11592.
Smith, M. F., E. W. McIntush, and G. W. Smith. 1994. Mechanisms associated with corpus luteum development. J Anim Sci 72: 1857-1872.
Sonenberg, N., and A. G. Hinnebusch. 2007. New modes of translational control in development, behavior, and disease. Mol Cell 28: 721-729.
Sonenberg, N., J.W.B. Hershey, M. Mathews. 2000. Translational control of gene expression. 2nd ed. CSHL Press, Cold Spring Harbor, NY.
Speroff, L., Fritz, M.A. 2004. Clinical gynecologic endocrinology and infertility. 7th ed. Lippincott, Williams, and Wilkins, Philadelphia.
Sriraman, V. et al. 2008a. Regulated expression of adam8 (a disintegrin and metalloprotease domain 8) in the mouse ovary: Evidence for a regulatory role of luteinizing hormone, progesterone receptor, and epidermal growth factor-like growth factors. Biol Reprod 78: 1038-1048.
Sriraman, V., S. R. Modi, Y. Bodenburg, L. A. Denner, and R. J. Urban. 2008b. Identification of erk and jnk as signaling mediators on protein kinase c activation in cultured granulosa cells. Mol Cell Endocrinol 294: 52-60.
Stephen, E. H., and A. Chandra. 1998. Updated projections of infertility in the united states: 1995-2025. Fertil Steril 70: 30-34.
Sterneck, E., L. Tessarollo, and P. F. Johnson. 1997. An essential role for c/ebpbeta in female reproduction. Genes Dev 11: 2153-2162.
Stocco, C. 2008. Aromatase expression in the ovary: Hormonal and molecular regulation. Steroids 73: 473-487.
Strachan, L. et al. 2001. Cloning and biological activity of epigen, a novel member of the epidermal growth factor superfamily. J Biol Chem 276: 18265-18271.

Strauss, J. F., 3rd, and M. Kafrissen. 2004. Waiting for the second coming. Nature 432: 43-45.
Sutovsky, P., and G. Schatten. 1997. Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. Biol Reprod 56: 1503-1512.
Tahbaz, N. et al. 2004. Characterization of the interactions between mammalian paz piwi domain proteins and dicer. EMBO Rep 5: 189-194.
Talbot, P., B. D. Shur, and D. G. Myles. 2003. Cell adhesion and fertilization: Steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. Biol Reprod 68: 1-9.
Talotta, F. et al. 2009. An autoregulatory loop mediated by mir-21 and pdcd4 controls the ap-1 activity in ras transformation. Oncogene 28: 73-84.
Tamura, M. et al. 1998. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor pten. Science 280: 1614-1617.
Tang, F. et al. 2007. Maternal micrornas are essential for mouse zygotic development. Genes Dev 21: 644-648.
Tatsuguchi, M. et al. 2007. Expression of micrornas is dynamically regulated during cardiomyocyte hypertrophy. J Mol Cell Cardiol 42: 1137-1141.
Taylor, C. T., Y. M. Lawrence, C. R. Kingsland, M. M. Biljan, and K. S. Cuthbertson. 1993. Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. Hum Reprod 8: 2174-2179.
Tesarik, J., L. Pilka, J. Drahorad, D. Cechova, and L. Veselsky. 1988. The role of cumulus cell-secreted proteins in the development of human sperm fertilizing ability: Implication in ivf. Hum Reprod 3: 129-132.
Thomas, F. H., and B. C. Vanderhyden. 2006. Oocyte-granulosa cell interactions during mouse follicular development: Regulation of kit ligand expression and its role in oocyte growth. Reprod Biol Endocrinol 4: 19.
Thomson, A. 1919. The ripe human graafian follicle, together with some suggestions as to its mode of rupture. J Anat 54: 1-40.
Thomson, J. M. et al. 2006. Extensive post-transcriptional regulation of micrornas and its implications for cancer. Genes Dev 20: 2202-2207.
Thum, T. et al. 2008. Microrna-21 contributes to myocardial disease by stimulating map kinase signalling in fibroblasts. Nature 456: 980-984.
Tomkins, G. M. et al. 1969. Control of specific gene expression in higher organisms. Expression of mammalian genes may be controlled by repressors acting on the translation of messenger rna. Science 166: 1474-1480.
Tong, A. W., and J. Nemunaitis. 2008. Modulation of mirna activity in human cancer: A new paradigm for cancer gene therapy? Cancer Gene Ther 15: 341-355.
Topilko, P. et al. 1998. Multiple pituitary and ovarian defects in krox-24 (ngfi-a, egr-1)-targeted mice. Mol Endocrinol 12: 107-122.

Trabucchi, M. et al. 2009. The rna-binding protein ksrp promotes the biogenesis of a subset of micrornas. Nature.
Tsafriri, A. 1995. Ovulation as a tissue remodelling process. Proteolysis and cumulus expansion. Adv Exp Med Biol 377: 121-140.
Tsafriri, A., Chun, S.-Y. 1996. Ovulation. . In: E. Y. Adashi, Rock, J.A., Rosenwaks, Z. (ed.) Reproductive endocrinology, surgery, and technology. p 235-249. Lippincott-Raven, Philadelphia.
Tsai, Y. H. et al. 2009. The m type k15 protein of kaposi's sarcoma-associated herpesvirus regulates microrna expression via its sh2-binding motif to induce cell migration and invasion. J Virol 83: 622-632.
Tyler, W. A. et al. 2009. Activation of the mammalian target of rapamycin (mtor) is essential for oligodendrocyte differentiation. J Neurosci 29: 6367-6378.
Vairapandi, M., A. G. Balliet, B. Hoffman, and D. A. Liebermann. 2002. Gadd45b and gadd45g are cdc2/cyclinb1 kinase inhibitors with a role in s and g2/m cell cycle checkpoints induced by genotoxic stress. J Cell Physiol 192: 327-338.
Van Blerkom, J., and P. Motta. 1978. A scanning electron microscopic study of the luteo-follicular complex. Iii. Repair of ovulated follicle and the formation of the corpus luteum. Cell Tissue Res 189: 131-153.
Van De Kerckhove, D. 1959. Content of deoxyribonucleic acid of the germinal vesicle of the primary oocyte in the rabbit. Nature 183: 329.
Van Soom, A., S. Tanghe, I. De Pauw, D. Maes, and A. de Kruif. 2002. Function of the cumulus oophorus before and during mammalian fertilization. Reprod Domest Anim 37: 144-151.
Varani, S. et al. 2002. Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. Mol Endocrinol 16: 1154-1167.
Vasudevan, S., Y. Tong, and J. A. Steitz. 2007. Switching from repression to activation: Micrornas can up-regulate translation. Science 318: 1931-1934.
Verde, F., J. C. Labbe, M. Doree, and E. Karsenti. 1990. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of xenopus eggs. Nature 343: 233-238.
Verghese, E. T., A. M. Hanby, V. Speirs, and T. A. Hughes. 2008. Small is beautiful: Micrornas and breast cancer-where are we now? J Pathol 215: 214-221.
Viswanathan, S. R., G. Q. Daley, and R. I. Gregory. 2008. Selective blockade of microrna processing by lin28. Science 320: 97-100.
Vo, N. et al. 2005. A camp-response element binding protein-induced microrna regulates neuronal morphogenesis. Proc Natl Acad Sci U S A 102: 16426-16431.
Wang, L., and K. M. Menon. 2005. Regulation of luteinizing hormone/chorionic gonadotropin receptor messenger ribonucleic acid expression in the rat

ovary: Relationship to cholesterol metabolism. Endocrinology 146: 423-431.
Wang, T. et al. 2007. A micro-rna signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. Genes Chromosomes Cancer 46: 336-347.
Wang, X., A. R. Kole, and G. S. Greenwald. 1995. In vitro and in vivo evidence on the site of neutralization of equine chorionic gonadotrophin (ecg) by an ecg antiserum. J Reprod Fertil 104: 237-241.
Wassarman, P. M. 2005. Contribution of mouse egg zona pellucida glycoproteins to gamete recognition during fertilization. J Cell Physiol 204: 388-391.
Webb, R. J., H. Bains, C. Cruttwell, and J. Carroll. 2002. Gap-junctional communication in mouse cumulus-oocyte complexes: Implications for the mechanism of meiotic maturation. Reproduction 123: 41-52.
Wek, R. C., H. Y. Jiang, and T. G. Anthony. 2006. Coping with stress: Eif2 kinases and translational control. Biochem Soc Trans 34: 7-11.
Wickramasinghe, N. S. et al. 2009. Estradiol downregulates mir-21 expression and increases mir-21 target gene expression in mcf-7 breast cancer cells. Nucleic Acids Res.
Woessner, J. F., Jr. et al. 1989. Connective tissue breakdown in ovulation. Steroids 54: 491-499.
Wu, H. et al. 2007. Mirna profiling of naive, effector and memory cd8 t cells. PLoS ONE 2: e1020.
Wu, Y. et al. 2005. The orphan nuclear receptors nurr1 and ngfi-b modulate aromatase gene expression in ovarian granulosa cells: A possible mechanism for repression of aromatase expression upon luteinizing hormone surge. Endocrinology 146: 237-246.
Yamakuchi, M., M. Ferlito, and C. J. Lowenstein. 2008. Mir-34a repression of sirt1 regulates apoptosis. Proc Natl Acad Sci U S A 105: 13421-13426.
Yamashita, Y. et al. 2007. Hormone-induced expression of tumor necrosis factor alpha-converting enzyme/a disintegrin and metalloprotease-17 impacts porcine cumulus cell oocyte complex expansion and meiotic maturation via ligand activation of the epidermal growth factor receptor. Endocrinology 148: 6164-6175.
Yang, G. H. et al. 2009a. Micrornas are involved in erythroid differentiation control. J Cell Biochem.
Yang, H. et al. 2008. Microrna expression profiling in human ovarian cancer: Mir-214 induces cell survival and cisplatin resistance by targeting pten. Cancer Res 68: 425-433.
Yang, H. S. et al. 2003. The transformation suppressor pdcd4 is a novel eukaryotic translation initiation factor 4a binding protein that inhibits translation. Mol Cell Biol 23: 26-37.
Yang, W. J. et al. 2005. Dicer is required for embryonic angiogenesis during mouse development. J Biol Chem 280: 9330-9335.

Yang, Y., R. Chaerkady, M. A. Beer, J. T. Mendell, and A. Pandey. 2009b. Identification of mir-21 targets in breast cancer cells using a quantitative proteomic approach. Proteomics 9: 1374-1384.
Yi, R., Y. Qin, I. G. Macara, and B. R. Cullen. 2003. Exportin-5 mediates the nuclear export of pre-microrna and short hairpin rnas. Genes Dev 17: 3011-3016.
Yokoo, M. et al. 2002. Identification of hyaluronic acid-binding proteins and their expressions in porcine cumulus-oocyte complexes during in vitro maturation. Biol Reprod 67: 1165-1171.
Yu, J., N. B. Hecht, and R. M. Schultz. 2002. Rna-binding properties and translation repression in vitro by germ cell-specific msy2 protein. Biol Reprod 67: 1093-1098.
Zachow, R. J., S. R. Weitsman, and D. A. Magoffin. 1997. Hepatocyte growth factor regulates ovarian theca-interstitial cell differentiation and androgen production. Endocrinology 138: 691-697.
Zamboni, L. 1974. Fine morphology of the follicle wall and follicle cell-oocyte association. Biol Reprod 10: 125-149.
Zarrow, M. X., and E. D. Wilson. 1961. The influence of age on superovulation in the immature rat and mouse. Endocrinology 69: 851-855.
Zeleznik, A. J., A. R. Midgley, Jr., and L. E. Reichert, Jr. 1974. Granulosa cell maturation in the rat: Increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. Endocrinology 95: 818-825.
Zhang, L. et al. 2008a. Genomic and epigenetic alterations deregulate microrna expression in human epithelial ovarian cancer. Proc Natl Acad Sci U S A 105: 7004-7009.
Zhang, P., and S. H. Mellon. 1997. Multiple orphan nuclear receptors converge to regulate rat p450c17 gene transcription: Novel mechanisms for orphan nuclear receptor action. Mol Endocrinol 11: 891-904.
Zhang, W. et al. 1999. Cr6: A third member in the myd118 and gadd45 gene family which functions in negative growth control. Oncogene 18: 4899-4907.
Zhang, X. et al. 2007. Inhibition of the egf receptor by binding of mig6 to an activating kinase domain interface. Nature 450: 741-744.
Zhang, Z. et al. 2008b. Mir-21 plays a pivotal role in gastric cancer pathogenesis and progression. Lab Invest 88: 1358-1366.
Zhang, Z. et al. 2009. Microrna-21 protects from mesangial cell proliferation induced by diabetic nephropathy in db/db mice. FEBS Lett.
Zhu, S., M. L. Si, H. Wu, and Y. Y. Mo. 2007. Microrna-21 targets the tumor suppressor gene tropomyosin 1 (tpm1). J Biol Chem 282: 14328-14336.