HORMONAL REGULATION OF MICRORNA EXPRESSION IN THE OVARY

BY

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ABSTRACT

The ovary is a dynamic organ that is charged with the responsibility of producing a viable gamete so that the circle of life can be reproduced for future generations. The ovary is also responsible for producing, secreting, and maintaining the proper hormone milieu of estrogens and progesterone for maintenance of pregnancy and the overall fitness of a woman's health. Understanding the mechanisms that regulate the interplay between hormone action and biological function is critical for furthering our knowledge of fertility and reproductive health. For decades, research has been conducted on understanding the transcriptional regulation of ovarian gene expression and how this relates to reproductive function. Recently, attention has turned to alternative forms of gene regulation, including post-transcriptional gene regulation. One mechanism of post-transcriptional gene regulation is the expression and function of microRNA (miRNA). These highly conserved, short, non-coding RNA molecules primarily silence gene expression by directly interfering with protein translation or causing the degradation of messenger RNA. The focus of these studies was to first determine if miRNA are necessary for female fertility. Conditional deletion of Dicer, a key processing enzyme in miRNA biogenesis, in ovarian granulosa cells, the oviduct, and uterus, led to a drastic decrease in ovulation rate and complete infertility in female mice. To further investigate the role of miRNA in ovulation, we next investigated miRNA-212 and -132. While these two co-transcribed miRNA were highly induced by the luteinizing hormone surge immediately prior to ovulation, they did not appear to have an effect on female fertility in the mouse. In a second series of studies, we analyzed the regulation of miRNA by hormones in two *in vivo* models. We found that miRNA expression was altered in theca cells from women suffering from polycystic ovarian syndrome (PCOS) and that

expression of miRNA was altered in the fetal ovaries of sheep exposed to an excess of prenatal androgens. Taken together, these studies provide evidence that miRNA are crucial for female fertility and ovarian function and that hormones influence the expression of ovarian miRNA in diseased states. These studies support the need for further study to understand the mechanisms through which these post-transcriptional regulators affect ovarian function, so that we can potentially use them as a therapeutic target to help overcome infertility and/or disease.

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The Ovary

1. **The structure and development of the ovary**

The ovary is a highly dynamic and functional organ that is responsible for the perpetuation of life. Beginning prior to birth, it houses, nurtures, and protects the female's eggs as they prepare to leave the ovary through ovulation, undergo fertilization, and provide the mother's genetic contribution that will ultimately define her offspring. From fetal development through the end of a woman's life, the ovary is the primary provider of estrogens and other hormones that are critical for not only reproduction, but numerous aspects of her health. The following is a review of literature that describes the development and multiple functions of the ovary as well as regulatory mechanisms of the ovarian cycle.

Structure of the Ovary

The adult mammalian ovary is divided into two main regions: the cortex and medulla. In humans, mice, rats, sheep, cows, and nearly all other mammals, the cortex contains the follicles, the functional unit of the ovary that encloses the oocyte. The exterior of the ovary is lined by a single, continuous layer of cuboidal or columnar epithelial cells known as the germinal or surface epithelium, derived from the coelomic epithelium during ovarian development (Harrison and Weir 1977). In the adult ovary, the tunica albuginea is a thin layer of connective tissue that lies directly adjacent to the ovarian surface epithelium. Throughout the cortex, follicles at different stages of development are embedded in the stroma, a layer of connective cells, secretory cells, and capillaries that support the follicle. The medullary region of the ovary is the most central portion of the ovary and is largely a loose core of connective tissue consisting of a

heterogeneous population of cells. The ovary is connected to the rest of the body vascularly, nervously, and physically through the hilum. This 'stalk', is the entry point of the vascular system, as well as containing the ligaments that connect the ovary to the mesovarium. Arteries from the hilum empty into the medulla, where a system of smaller blood vessels branch throughout the ovary and into the cortex to maintain and support follicles while serving as a means to transport ovarian hormones systemically.

Development of the Ovary

During fetal development, an undifferentiated, bi-potential gonad forms from the coelomic epithelium. Proliferation of this epithelial layer causes a thickening of the tissue to form the genital ridge. The primordial germ cells originate extra-gonadally in the endoderm of the yolk sac and migrate to the bilateral genital ridge (Zuckerman and Baker 1977). Once there, the primordial germ cells begin to undergo mitotic proliferation as the surrounding somatic epithelium cells continue to proliferate and form a thickening around the gametes. The sexually undifferentiated gonad then begins to re-organize into the outer cortex and inner medullary regions divided by a tunica albuginea. At the same time, a series of sex cords begin to surround the primordial germ cells. Differentiation of the biopotential gonad occurs when the cortex of the ovary continues to proliferate while the medulla region recesses (Zuckerman and Baker 1977). The primordial germ cells are primarily situated in the cortex region and the previous cord like structure regresses, leaving nests of germ cells that are surrounded by epithelial cells. In the ovary, the primordial germ cells will stop proliferating and go into meiotic arrest until they are activated later in life. The epithelial cells that surround the nests will

transition into pre-granulosa cells, associate with an individual primordial germ cell and become a primordial follicle (Zuckerman and Baker 1977).

In mammals, sexual determination is initiated by the sex-determining region of the Y chromosome (SRY) gene (Gubbay, et al. 1990, Koopman, et al. 1991, Lovell-Badge and Robertson 1990). Expression of this transcription factor in the developing male gonad sets off a cascade of genes that will in turn drive testis formation and inhibit pathways that lead to ovarian formation. Formation of the female gonad is considered be a passive genetic process that occurs when SRY is not expressed. Wnt4 is a primary factor in ovarian development and is believed to inhibit testis development while possibly feeding forward to promote ovarian development (Vainio, et al. 1999). Likewise, follistatin is regulated by Wnt4 and further antagonizes testis formation and is important for germ cell survival (Yao, et al. 2004). Intensive work has been dedicated to this field, for a more detailed review please see (Brennan and Capel 2004, Yao 2005).

The oviduct, uterus, and vagina, or secondary sex organs all develop from the Mullerian ducts, while the secondary sex organs of the male are formed from the Wolffian ducts. Both duct systems develop in the fetus; however one set will undergo regression while the other undergoes differentiation into a functional organ system. In the male, anti-Mullerian hormone (AMH, also referred to as Mullerian inhibiting substance, MIS) is produced by the developing testes, leading to apoptosis and degeneration of the Mullerian ducts. Testosterone, also produced by the testes, stimulates development of the Wolffian ducts and masculinizes the fetus. In the developing ovary, no AMH is produced, thus the Mullerian ducts undergo differentiation and form the

oviduct, uterus, and anterior region of the vagina. Without testosterone the Wolffian duct undergoes regression.

2. **The ovarian cycle**

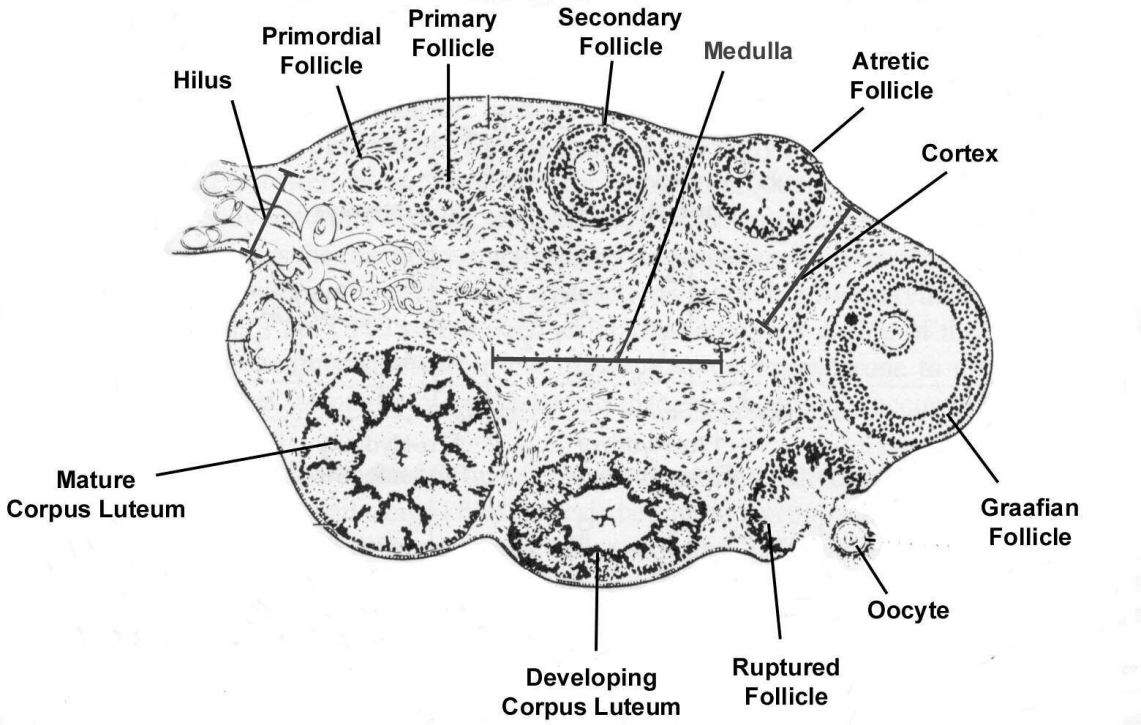
Each reproductive cycle, the mammalian ovary releases one or more mature oocytes (the egg) into the reproductive tract. The ovarian cycle is divided into two phases: the follicular phase and the luteal phase. The follicular phase involves the formation, growth, and maturation of follicles through a process termed folliculogenesis. Upon follicular maturation, ovulation occurs and the mature gamete is released from the ovary for potential fertilization. Following ovulation, the luteal phase begins as the ruptured follicular tissue differentiates into luteal tissue. These cellular changes and tissue remodeling lead to a shift in cellular function from aiding in the development of a mature gamete, to providing an environment conducive to the establishment and maintenance of pregnancy should the oocyte be fertilized. If fertilization does not occur, the luteal tissue regresses, allowing for a new follicle to mature, and the cycle starts over again. While in humans and other monovulatory species (i.e. cow, sheep, non-human primate) only one egg is released, and in litter bearing species such as the mouse, rat, or pig, multiple eggs are ovulated, the process of producing the mature gamete requires selection from thousands of eggs and can take a timeframe of up to several months. Great variance is observed in the processes of folliculogenesis and ovulation between mammalian species.

Folliculogenesis

The process of folliculogenesis, or the formation and maturation of the ovarian follicle that is responsible for protecting and nurturing the maturing oocyte, begins before birth in most mammalian species. Following the process of oocyte formation, and continuing through oogenesis, a thin layer of flattened pre-granulosa cells associates with and surrounds the oocyte to form a primordial follicle (Pedersen and Peters 1968). This process, termed primordial follicle assembly, begins during fetal development and is completed by birth in higher mammals, thus providing females with their full complement of gametes at the time of birth. In rodents, follicle assembly begins at embryonic day 17.5 and is completed by post-natal day 5 (Borum 1967). Prior to this point, oogonia are undergoing proliferation through mitotic division and are not associated with any somatic cells (Pepling and Spradling 2001). The primordial follicle, including the oocyte, is non-growing and remains quiescent in the resting follicular pool until later in life when it undergoes primordial follicle activation and enters the follicular pool as a primary follicle. At birth, women are estimated to have 400,000 follicles remaining out of the 6 to 7 million oogonia present at the peak of mitotic proliferation during gestation (Rajkovic, et al. 2006). The large number of oogonia and follicles lost is attributed to follicular atresia, a process that will continue to deplete follicular reserves throughout life.

Figure VI-1. Schematic diagram depicting the structure of the ovary. Figure adapted from Ojeda, 2004.

Figure V1-1


Hilus
Primordial Follicle
Primary Follicle
Secondary Follicle
Medulla
Atretic Follicle
Cortex
Mature Corpus Luteum
Graafian Follicle
Oocyte
Developing Corpus Luteum
Ruptured Follicle

Primordial follicle activation, or the transition from primordial to primary follicle, is an irreversible process that begins the growth and maturation process of follicle development. The granulosa cells of the primary follicle have transitioned to a cuboidal or columnar shape and form a distinct layer around the oocyte, which has begun to increase in size (Harrison and Weir 1977). A basement membrane, termed the basal lamina, forms adjacent to the outer most layer of granulosa cells and will act as a blood barrier to keep the avascular granulosa cell and oocyte distinct from the blood supply that will soon form around the follicle. Following proliferation of the granulosa cells to form a second, concentric circle around the oocyte and the formation of a thick, glycoprotein rich layer known as the zona pellucida around the oocyte, the follicle is classified as a secondary follicle. The oocyte of the secondary follicle continues to grow and the total volume of the follicle increases. During the latter stage of the secondary follicle, a layer of somatic/ mesenchymal cells originated from the ovarian interstitium and termed theca cells will associate tightly with the outside of the basement membrane (Magoffin 2005). Theca cells are embedded with capillaries, act as an important source of nutrients (i.e. glucose and amino acids), growth factors, and hormones for the growing follicle and oocyte, and will eventually become a key component in the production of steroids in the ovarian follicle. Continued proliferation of granulosa cells will result in multiple layers of cells around the oocyte and the eventual formation of a fluid filled cavity known as the antrum. The follicular fluid that comprises the antrum is thought to be a combination of liquid from the serum on the outside of the follicle combined with secreted factors from the granulosa cells. Numerous factors have been isolated from follicular fluid and include: gonadotropins, growth hormone, prolactin, steroid hormones, corticoids,

members of the TGFβ superfamily, insulin-like growth factors (IGFs), amino-acids, RNAs, sugars, as well as other compounds and is thought to be primary source of nutrition and signaling for the oocyte (Revelli, et al. 2009). As more fluid begins to fill the follicle it will coalescence into a large fluid filled cavity that will push the oocyte, surrounded by one or two layers of specialized granulosa cells referred to as cumulus cells to one side. The cumulus granulosa cell is specifically defined as the layer of cells that line the ring of the oocyte. They are crucial for communication between the rest of the follicle (and thus, the rest of the organism) and the oocyte (Diaz, et al. 2006). The cumulus cells appear to be biochemically and molecularly different in both enzyme and receptor expression when compared to the mural granulosa cells, or the cells that line the follicular wall and previously referred to as granulosa cells. Mural granulosa cells are largely steroidogenic and communicate with the theca, cumulus granulosa cells, and rest of the organism systemically. Granulosa cell differentiation appears to be due to a gradient of follicle stimulated hormone (FSH) exposure, as mural granulosa cells are closer to the vasculature outside of the follicle wall and have greater response to the hormone (Diaz, et al. 2007). At this point, the follicle has reached the antral stage of development and a 'stalk' of granulosa cells connects the cumulus-oocyte complex to the wall of the follicle, while allowing it to be bathed in follicular fluid. During the tertiary follicle transition, the layer of theca cells on the exterior of the basal lamina divide into the theca interna and theca externa. Theca interna cells are nearest the basement membrane, have large numbers of mitochondria, smooth endoplasmic reticulum, and lipid vesicles, and thus are primarily responsible for steroid synthesis and transport of signals into the follicle (Magoffin 2005). The external layer of theca cells is critical for

preparing the follicle for ovulation and is embedded with macrophages, fibroblasts, and smooth muscle cells (Hirshfield 1991). The antral follicle will continue to increase in total volume, both from continued granulosa cell proliferation and increased volume of follicular fluid. The pre-ovulatory follicle produces large amounts of estradiol and if selected for ovulation, will ovulate in response to a surge of luteinizing hormone (LH).

While the stages of follicular development are easy to classify and identify, the endocrine and molecular mechanisms that regulate this process and the ovarian cycle are very complex. The early stages of follicular development, including the formation of primordial follicles and subsequent transition to the primary follicular phase are generally thought to occur independent of the pituitary gonadotropins, FSH and LH (Eppig and O'Brien 1996, O'Shaughnessy, et al. 1997, Sokka and Huhtaniemi 1990). The factors responsible for signaling the primordial follicle to enter the growing pool and transition to the primary follicle are not completely understood, but it appears to be a balance between inhibitory and activating factors. Culture of fetal bovine ovaries in serum-free media led to the induction of follicular growth, thus suggesting the theory that an extra-ovarian factor must be responsible for keeping primordial follicles arrested (Wandji, et al. 1996). Recently, the transcription factor Foxo3 along with the PI3K signaling pathway was also implicated in inhibiting primordial follicle growth (John, et al. 2008, John, et al. 2007). A handful of genes and growth factors were identified to play a role in the transition from primordial follicle to primary follicle, including Kit ligand and its cognate receptor c-Kit, which were first hypothesized as a key regulator in primordial follicle activation (Parrott and Skinner 1999). Likewise, Nobox (newborn ovarian homeobox) and Sohlh1 and 2 are important transcription factors in the activation of primordial

follicles (Choi, et al. 2008, Choi, et al. 2007, Pangas, et al. 2006, Qin, et al. 2007). While these mechanisms appear to be involved in the processes linked to the resumption of oocyte growth, the actual upstream signal that regulates these mechanisms remains to be elucidated.

The transition from primary to pre-antral follicle also appears to be gonadotropin independent, as mice lacking the FSH receptor (FSHR) or the β-subunit of FSH exhibit normal follicular growth through the pre-antral stage (Abel, et al. 2000, Kumar, et al. 1997). Instead, the follicle appears to respond to paracrine cues from the oocyte as evidenced by the inability of ovaries lacking the oocyte derived growth factor GDF9 to progress past the primary stage of development (Dong, et al. 1996). The primary defect in these mice appears to be a decrease in granulosa cell proliferation (Elvin, et al. 1999) and lack of formation of the theca cell layer (Dong, et al. 1996). The formation of the follicular antrum appears to be the first point under control of the pituitary gonadotropin FSH (Kumar, et al. 1997). Following this stage of development, follicular growth or regression by atresia appears to be largely regulated by the gonadotropin hormones.

During each ovarian cycle, a cohort of antral follicles begins to grow in a process named recruitment (Ginther, et al. 1996). Follicles that have been recruited either undergo atresia or continue to grow into medium sized antral follicles. As the cohort of follicles grow, a single follicle (in monovular species) or multiple follicles (in litter bearing species) will emerge from the cohort, gaining a distinct growth advantage over the other follicles. The morphologically larger follicle is termed the dominant follicle and is capable of developing into an ovulatory follicle with proper hormonal stimulation, while the other smaller follicles (subordinate follicles) regress (Fortune, et al. 2001).

This larger follicle exerts 'dominance' over the smaller, subordinate follicles by producing hormones that change the endocrine environment to one that does not support further growth of the smaller follicles. Therefore, the subordinate follicles will undergo degeneration through a process termed atresia through apoptosis (Fortune 1994, Ginther, et al. 1996, Richards 1994).

Follicular Atresia

Throughout the lifespan of the female, the vast majority of follicles will undergo atresia. The process of atresia is an important mechanism to ensure that only the best and most healthy follicles and oocytes with the greatest chance at becoming successfully fertilized will be ovulated. The primary mechanism of atresia is through regulated and programmed cell death through apoptosis (Quirk, et al. 2004). During apoptosis, the nucleus of the cell containing the DNA condenses, fractures, and eventually will fragment. The cytoplasm begins to aggregate, the organelles disintegrate, and neighboring macrophages will phagocytize the apoptotic cell. Atresia can be initiated in either the oocyte or somatic cells of the follicle (Matsuda, et al. 2012). A large percentage of atresia occurs during follicular assembly, as oocytes that do not assemble with pre-granulosa cells will undergo apoptosis. Atresia of follicles that have assembled correctly will typically occur at the antral stage of development due to the lack of exposure/production of survival factors (i.e. the subordinate follicle). FSH is the most critical of survival factors and expression of its receptor (FSHR) is necessary to escape atresia. Thus, the signaling pathways set in motion by FSH, namely the production of cyclic AMP (cAMP) in the granulosa cell, turn on anti-apoptotic factors. Estrogens,

insulin-like growth factor I (IGF-1), interleukin-1 (IL-1), epidermal growth factor (EGF), and basic fibroblast like factor (bFGF) have all been identified as pro-survival factors in follicular growth and development (Matsuda, et al. 2012).

Ovulation

Ovulation is the culmination of growth, development, and selection of a healthy follicle that is needed for oocyte release into the Fallopian tube (human) or oviduct (other mammals). In most mammals, including the human, primate, sheep, and rodent, ovulation is induced by a surge of LH. The molecular basis of ovulation begins with events occurring in the peri-ovulatory follicle in the period leading up to the ovulatory event. The peri-ovulatory follicle produces large quantities of estradiol (the primary estrogen produced by the follicle). It is estimated that the human peri-ovulatory follicle produces approximate 400 to 800 ng of estradiol a day (Tagatz and Curpide 1973). Granulosa cell expression of steroidogenic enzymes such as Cyp19 (also named Aromatase) are high, to ensure synthesis and secretion of estradiol is great enough to enter circulation and stimulate the anterior pituitary in a feed forward mechanism to release a bolus of LH known as the LH surge. Upon binding to its cognate receptors on the peri-ovulatory follicle, this ovulatory dose of LH activates a number of signaling cascades (i.e. cAMP signaling) and the programming that drives estradiol synthesis, granulosa cell proliferation, and other factors necessary for follicular development is shut off. The LH surge also stimulates gene expression, turning on genes necessary for ovulation, cumulus expansion, and preparing the follicular tissue to undergo luteinization following ovulation

(Richards, et al. 2002). These changes that occur are both temporal and spatial, affecting the cumulus granulosa cells, mural granulosa cells, theca cells, and endothelial cells.

The event of ovulation occurs approximately 13-15 hours post-LH in the mouse (Runner and Gates 1954) and 32 to 36 hours post-LH in the human (Espey, et al. 2004). For ovulation to occur, the actual follicular wall and corresponding layers of the ovary must undergo rupture. The specific site of rupture is termed the stigma, and the tunica albuginea and surface epithelium of the ovary must be breached at this particular location. In addition to stimulating the expression of numerous genes, the LH surge causes an increase of blood flow at the site of the ovulatory follicle. This leads to increased dilation and permeability of the capillaries embedded in the layer of theca externa cells in the follicle, priming the follicular wall for rupture.

In the mural granulosa cells, LH rapidly induces expression of a number of genes necessary for ovulation, including progesterone receptor (PR), cyclooxygenase-2 (COX-2 or PTGS2), hyaluronan synthase (HA), CCAAT/enhancer binding protein β (C/EBPβ), early growth response protein-1 (Egr-1), as well as others (Richards 2005). Eight hours following the LH surge several other important genes including a disintegrin-like and metallopeptidase with thrombosopondin type 1 motif (ADAMTS-1) followed by matrix metallopeptidase 14 (MMP14), exhibit increased expression (Richards 2005). Cumulus granulosa cells do not express receptors for LH, so expression of factors such as the EGF ligands and associated receptors as well as Cox-2 are particularly important to mediate the LH signal from mural to cumulus granulosa cell.

Luteal Phase

Following ovulation, granulosa and theca cells differentiate into large and small luteal cells, respectively. In the days following ovulation, the corpus luteum (CL) increases in size by increasing the cytoplasmic:nuclear ratio of large lutein cells. This increase in cellular cytoplasm is accompanied by an increase in the cellular organelles associated with steroid production, such as mitochondria, smooth endoplasmic reticulum, and Golgi apparati (Smith, et al. 1994). Expression of mRNA for steroidogenic enzymes switches from that necessary for estradiol production to that needed for progesterone production. Progesterone is secreted by the CL and is required to establish and maintain pregnancy, if the ovulated oocyte becomes fertilized (Niswender, et al. 2000). Blood flow to the CL also increases shortly after ovulation (Schams and Berisha 2004). The CL goes through a period of intensive angiogenesis until nearly every luteal cell is in contact with one or more capillaries (Redmer and Reynolds 1996).

If fertilization does not occur, the CL undergoes a process called luteolysis in which the CL regresses. Luteolysis, or the demise of the corpus luteum, occurs in two stages (Niswender, et al. 2000). First, functional demise occurs resulting in decreased capacity for progesterone production and secretion. A decrease in progesterone coupled with other factors then leads to the second stage, or structural demise of luteal tissue (Niswender, et al. 1994). In most mammalian models of luteolysis, regression is dependent upon synthesis and release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus. If no embryo is present, $PGF_{2\alpha}$ is released from the uterus and induces luteolysis. In the presence of an embryo, the release of $PGF_{2\alpha}$ is inhibited to prevent luteal regression and maintain pregnancy. Structural demise is characterized by a decrease in luteal cell

number and size. Apoptosis is dramatically increased in the endothelial cells of capillaries in the corpus luteum, resulting in the degradation of vascular tissue and the accompanied decrease in blood flow. As less blood reaches the luteal tissue, a reduction in oxygen and nutrient delivery to luteal cells facilitates corpus luteum regression (Niswender, et al. 2000, Smith, et al. 1994).

Gonadotropin signaling and the regulation of ovarian function

Regulation of folliculogenesis is largely controlled by communication between the ovary, pituitary and hypothalamus - the hypothalamic-pituitary-gonadal axis. Two critical hormones, released from the anterior pituitary in response to gonadotropin releasing hormone (GnRH) from the hypothalamus, are LH and FSH. These gonadotropic polypeptide hormones bind to the appropriate G protein-coupled receptor and affect follicular development and steroid production by modulating the cAMP and/or the protein kinase A (PKA) second messenger systems. The mechanism of cAMP activity begins with FSH/LH binding to its receptor, leading to a conformation change and activation of the G_s subunit. The G_s subunit is released from the complex and activates adenylate cyclase which catalyzes conversion of ATP to 3'-5'-cyclic AMP (cAMP). The process of cAMP formation is often mimicked experimentally by use of the pharmacological agent forskolin, a compound that activates adenylate cyclase and increases cAMP formation. Alternatively, the non-degradable and easily permeable compound, 8-Bromo-cAMP can be used to mimic the LH surge. The primary effector of cAMP is the PKA pathway, a tetrameric complex consisting of two regulatory subunits and two catalytic subunits. Upon formation, cAMP molecules bind to the regulatory

subunits, allowing for release of the catalytic subunits. The primary PKA substrate appears to be cAMP response-element binding protein (CREB), a transcription factor that when phosphorylated by PKA regulates gene expression. CREB binds to cAMP response elements (CRE) sites in the promoter regions of genes. Numerous genes involved in ovarian function are regulated by cAMP/CREB, including Cyp19 and progesterone receptor. In addition to transcriptional regulation, PKA can also directly affect the phosphorylation of proteins, as evidenced by the phosphorylation of StAR and associated increase in steroidogenic activity (Arakane, 1997). A primary regulatory factor in cAMP signaling is the enzyme family of phosphodiesterases (PDEs) that degrade cAMP molecules. LH and FSH can also signal independently of cAMP via G_i subunit activation of phospholipase C (PLC) and subsequent activation of IP3 and diacylglycerol (DAG) (Hsieh, et al. 2011). Granulosa cells become responsive to FSH when the FSH receptor begins to be expressed in the secondary follicle. LH receptors are expressed in theca cells, as well as in granulosa cells of selected antral follicles (Fortune, et al. 2001). Estradiol production is the result of cooperation between theca and granulosa cells (Magoffin 2005). Throughout the follicular stage of the ovarian cycle, the increasing levels of estradiol secreted by the growing follicle stimulates the hypothalamus in a feed-forward mechanism to increase the release of GnRH and subsequent secretion of FSH and LH from the pituitary (Fortune 2003). Activated estrogen receptors in the anterior pituitary stimulate an increase in the release of gonadotropins. FSH and LH in turn, stimulate granulosa and theca cell steroid production. This positive feed-forward mechanism causes greater estrogen production. As a result, more estrogen is produced and released as the follicles mature. When the

dominant follicle reaches ovulatory size, the increased production of estradiol triggers the hypothalamus to increase pulse frequency and amplitude of GnRH pulses so that ultimately a large pulse of LH and FSH to be released from the pituitary. This gonadotropin surge, often called the LH surge because it is released in a greater concentration relative to FSH, causes the follicle to rupture and release the oocyte. The remaining follicular tissue involutes and begins to undergo luteinization. Progesterone produced from the CL exerts negative feedback on hypothalamic activity, keeping the GnRH pulse amplitude and frequency low and slow, thus inhibiting the surge release of gonadotropins from the pituitary, preventing ovulation from occurring. As the CL regresses, progesterone production decreases and allows hypothalamic and gonadotropin secretion to increase. Follicular growth and maturation marks the beginning of the next ovarian cycle.

3. **Steroidogenesis**

The primary hormone products of the ovary are 17β-estradiol from dominant follicles and progesterone from the corpus luteum, however several intermediates including androstenedione and testosterone are secreted in lesser quantities from the ovary. In addition to the many previously described functions of estradiol in the ovary and female reproductive system, it also has important roles throughout the female body (i.e. bone strength). Progesterone is similarly important, as it is critical in regulating the uterus for a successful pregnancy.

Cholesterol is the precursor of all ovarian steroids hormones. Steroid producing cells in the ovary can synthesize cholesterol *de novo* or uptake cholesterol from

circulating serum lipoproteins or cholesterol esters (Strauss and Miller 1991). If cholesterol levels are low in steroidogenic cells, *de* novo synthesis of cholesterol begins with the formation of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA by the HMG-CoA synthase enzyme (Ho, et al. 2004). HMG-CoA reductase then uses the HMG-CoA substrate to produce mevalonic acid and a series of enzymatic reactions will create the 30 carbon intermediate squalene molecule. Following cyclization, the 26 carbon cholesterol molecule is formed (Ho, et al. 2004). However, as expression of HMG-CoA, the rate-limiting step of cholesterol synthesis is low in ovarian steroidogenic cells, most cholesterol reaches the ovary via serum lipoproteins that have been digested from dietary sources (Christenson and Devoto 2003). Ovarian steroidogenic cells can uptake cholesterol bound to low-density lipoproteins (LDL) or high-density lipoproteins (HDL). LDL molecules contain cholesterol in the form of cholesterol esters, which cannot be used directly for steroidogenesis due to their esterification (Ho, et al. 2004). Cholesterol esters are stored in lipid droplets, and must be hydrolyzed to free cholesterol by neutral cholesterol hydrolase. In humans, LDL is the primary transporter of cellular cholesterol, while in cows, sheep, rats, and mice, HDL is the primary form of cholesterol. LDL enters the cell through multiple methods, including binding to the plasma membrane of the cell and undergoing endocytosis to form a clathrin coated pit in the interior of the cell (Christenson and Devoto 2003). A lysosome will then bind the vesicle, release a lipase that will dissociate the cholesterol ester from the lipoprotein and both will be released into the cytosol. The primary mechanism of HDL entry into the cell is through scavenger receptor B1 (SRB1) (Ho, et al. 2004). This receptor can also transport LDL into the cell and causes the freeing and movement of the

cholesterol ester into the cell, while the lipoprotein stays on the exterior of the cell. Following conversion of cholesterol ester to free cholesterol, the molecule can be transferred to the mitochondria for steroid synthesis.

Two types of enzymes are primarily responsible for a series of cleavages and oxidations that ultimately turn cholesterol into a number of steroid hormone derivatives. Cytochrome P450 enzymes (abbreviated CYP) are a large class of proteins involved in steroid biosynthesis in endocrine glands including the ovary, testis, and adrenals. P450 enzymes are bound to the membrane of the mitochondria or endoplasmic reticulum and catalyze the hydroxylation and cleavage of a steroid substrate (Payne and Hales 2004). They are named for the shift in absorbance peak from 420nm to 450nm upon catalytic reduction (Hall 1986) and have a heme iron group that requires oxygen and reducing agents derived from NADPH for catalytic activity (Strauss and Miller 1991). The second major class of enzymes involved in ovarian steroidogenesis is the hydroxysteroid dehydrogenases (abbreviated HSD). These proteins carry out a series of reactions that catalyze the interconversion of steroidal alcohols and carbonyls (Strauss and Miller 1991).

The critical rate limiting step in production of steroid hormones in the ovary is the transport of cholesterol from the outer to the inner membranes of the mitochondria by steroidogenic acute regulating protein (StAR) (Strauss, et al. 1999). Delivery of cholesterol to the inner membrane of the mitochondrial allows for the first committed step of steroidogenesis to occur by the cytochrome P450-side change cleavage (Cyp11a1) enzyme. This series of irreversible cleavage reactions removes the side chain of the 26 carbon cholesterol to form the 21 carbon steroid pregnenolone. From this point forward,

all steroid metabolism is conducted in the endoplasmic reticulum. Following the synthesis of pregnenolone and ending with the formation of androstenedione, steroidogenesis can proceed through the Δ4 or Δ5 pathways, so named for the location of the double bond in the A ring of the steroid (Conley and Bird 1997). Conversion of pregnenolone to progesterone with the 3βHSD enzyme will cause future reactions to go through the Δ4 pathway, due to the Δ4-5 isomerase activity of the enzyme. Alternatively, the enzyme Cyp17, will cause steroidogenesis to progress through the Δ5 pathway, with dual hydroxylase and desmolase activity that will convert pregnenolone into 17α-hydroxy-pregnenolone and subsequently the 19 carbon androgen, dehydroepiandrosterone (DHEA) (Rice and Savard 1966). 3βHSD can then catalyze DHEA into androstenedione, at which point all steroids are considered Δ4. Cyp17 can also catalyze the Δ4 hydroxylation of progesterone to 17α-hydroxy-progesterone and subsequent desmolation to androstenedione. The decision of which pathway to follow for steroidogenesis is largely species dependent, as the affinity of Cyp17 for 17α-hydroxy-progesterone in humans, primates, sheep, and cattle is low, thus leading primarily to the Δ5 pathway (Conley and Bird 1997). However, in other species such as the pig, rat, and mouse, such inefficiency is not observed, and either the Δ4 or Δ5 pathways can be used (Conley and Bird 1997). Metabolism of androstenedione can lead to synthesis of testosterone via 17βHSD (Labrie, et al. 1997). Estradiol, the primary estrogen (18 carbons) produced by the ovary, is synthesized by aromatization of testosterone by the enzyme Cyp19, also referred to as aromatase (Brodie, et al. 1976).

Ovarian steroid hormone synthesis of the follicle is an intricate interplay and compartmentalization between the granulosa and theca cells. In an elegant experiment

where theca, granulosa, or luteal cells alone or combined where transplanted into eye of a rat, it was found that theca or granulosa cells alone were not sufficient to produce estrogen (Falck 1959). The combination of theca and granulosa cells or the presence of luteal cells did produce an estrogenic effect (Falck 1959). These early observations as to the necessity of both major cellular components of the follicle were the initial basis of the 'two-cell, two-gonadotropin' hypothesis of estrogen synthesis in the ovary. Following several decades of research, we now better understand the compartmentalization of steroidogenesis in the ovarian follicle. Theca cells, on the exterior of the follicle, express receptors for LH (Magoffin 2005). Upon LH stimulation, expression of Cyp17 is turned on in the theca cell and steroidogenesis through androstenedione will proceed. In the early antral follicle, granulosa cells only express receptors for FSH (Erickson, et al. 1979), thus preventing transcriptional up regulation of enzymes regulated by LH and necessary for steroidogenesis through androgen synthesis. Instead, FSH signaling increases expression of Cyp19 (aromatase) in granulosa cells of small antral follicles, so that androgens that diffuse across the basement membrane from the theca cell can act as substrate for estradiol synthesis (Liu and Hsueh 1986). However, upon acquiring dominance, and reaching the pre-ovulatory stage, granulosa cells will begin to express LH receptors and thus turn on expression of Cyp17 and allow synthesis of estradiol from cholesterol (Fortune, et al. 2001). This compartmentalization of steroid production allows for suppression of estradiol production during the luteal phase of the ovarian cycle, thus keeping feedback on the hypothalamus/pituitary low. The ability of granulosa cells of the dominant follicle to express LH receptors and produce estradiol from cholesterol allows for the increased synthesis and release of estradiol during the follicular

phase. This furthers allows for the feed forward activity of estradiol on the hypothalamus/pituitary and ultimately initiate the subsequent LH surge. Alternatively, luteal cells express high levels of Cyp11, allowing for large levels of progesterone to be secreted from the corpus luteum (Doody, et al. 1990).

4. **Regulation of gene expression in the ovary**

Transcriptional regulation of gene expression is a primary and well-defined method of controlling cellular and biological function. Numerous examples exist of the regulation of genes in the ovary, including the regulation of Cyp19 or progesterone receptor in response to LH signaling as described above. Transcriptional regulation allows for a stimulus, such as LH, to rapidly turn off and on gene expression of mRNA that is necessary for a cell to respond to and carryout a specified function. However, changes in mRNA expression levels are not always reliable predictors of protein expression levels (Waters, et al. 2006). Comparisons of transcriptomes and associated proteomes have suggested that only 20 to 40% of changes in the proteome are due to transcriptional regulation (Brockmann, et al. 2007, Seliger, et al. 2009). The lack of direct correlation between transcriptome and proteome suggests that another mechanism of gene regulation is at play. One potential mechanism is post-transcriptional gene regulation, which encompasses any event that affects the mRNA between the time of transcription and the end of translation. Post-transcriptional gene regulation involves a broad number of mechanisms, including the 5'capping and 3'poly-adenylation of the mRNA, alternative splicing, regulation of mRNA half-life, and inhibition of translation. In the ovary, several examples of these mechanisms have been identified. For instance,

in granulosa cells the LH receptor has been reported to undergo accelerated degradation due to the binding of a RNA binding protein named LRBP (LHR RNA binding protein and later identified as mevalonate kinase) (Kash and Menon 1999, Lu, et al. 1993, Nair, et al. 2002, Nair and Menon 2004). LRBP binds to the LHR mRNA, thus preventing translation by interacting with the ribosome complex and also increasing the rate of LHR mRNA decay (Menon, et al. 2009).

Another example of post-transcriptional gene regulation in the ovary involves theca cells collected from ovaries of women suffering from polycystic ovarian syndrome (PCOS). This condition is largely defined by the presence of excess androgen production and synthesis, and numerous laboratories have attempted to elucidate the cause. One laboratory has reported that the transcriptome of PCOS theca cells exhibits altered gene expression compared to theca cells from non-polycystic ovaries (Wood, et al. 2004). Furthermore, the mRNA half-life of Cyp17 is increased nearly two fold in theca cells isolated from PCOS women vs. normal women (Wickenheisser, et al. 2005). Several post-transcriptional mechanisms including altered mRNA stability or 5'capping could be involved, although the authors state that the reason for this change in Cyp17 half-life is unknown. One potential mechanism for altering mRNA half-life is post-transcriptional gene regulation by microRNA, a class of short, non-coding RNA molecules further discussed in the next chapter.

VII: CHAPTER 2

microRNA: Post-transcriptional gene regulation

1. Discovery and nomenclature of miRNA

MicroRNA (miRNA) are a class of small, non-coding RNA molecules, approximately 22 nucleotides (nt) long, that are involved in the post-transcriptional regulation of gene expression. The initial discovery of miRNA occurred in 1993 by the Victor Ambros laboratory, when it was hypothesized that the *C. elegans* gene *lin*-4 was not coding a protein, but rather two short RNA molecules (Lee, et al. 1993). Upon further examination, it was determined that one RNA was a 22nt long molecule and the other was a longer RNA that was predicted to form a stem loop structure encompassing the same 22nt sequence. Analysis of the 22nt sequence revealed that it was complementary to a region in the 3'untranslated region (3'UTR) of the *lin-14* gene, which had previously been hypothesized to be negatively regulated by *lin-4* (Lee, et al. 1993, Wightman, et al. 1991, Wightman, et al. 1993). At the time, it was thought that this mechanism was specific to *C. elegans*, and so even though multiple groups continued to study these 'tiny' RNAs, they were not reported again in the literature for several years.

The first miRNA found to be conserved across species was *let-7* in 2000 by the laboratory of Gary Ruvkun when he established that the sequence was evolutionarily conserved in Drosophila, zebrafish, annelids, and mollusks, and humans (Pasquinelli, et al. 2000). Since then, research on the identification, mechanism, and function of miRNA in all biological processes has increased exponentially, with over 21,000 miRNA identified in dozens of species (Kozomara and Griffiths-Jones 2011). The number of publications on miRNA has grown from the single digits in the early 2000s to over 5,000 publications in the year 2011 alone.

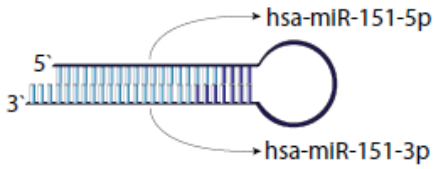
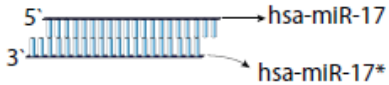
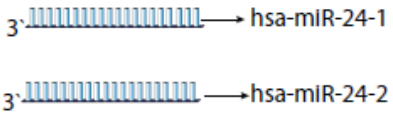
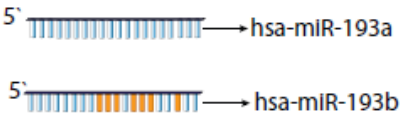
MicroRNA nomenclature

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. 2008). The current nomenclature for animal miRNA is described below and is diagramed in the Figure VII-1. 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 (e.g. 'hsa' indicates human and 'mmu' indicates mouse). To distinguish between the precursor and mature forms of miRNA, a lowercase 'r' ('mir') represents the precursor form of the miRNA, and 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 might or might not be separated by a dash depending on the suffix) denotes either the processing 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. In a few cases, miRNA genes have been identified at the same chromosomal location and are found on opposing

DNA strands (sense versus antisense) and, thus, have unique mature miRNA sequences. The miRNA on the antisense chromosome used to be identified with an ‘as’ suffix and that on the sense chromosome with an ‘s’ suffix. However, as of April of 2011, miRNA on the antisense strand are given a new number if they are not deemed similar. 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 is paralogous (shares 70% of mature sequence) to hsa-miR-19a, which originates from chromosome 13.

Figure VII-1. MicroRNA nomenclature.

Figure VII-1

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 antisense).	

2. MicroRNA structure and biogenesis

MicroRNA are transcribed from diverse regions of the genome. Based on computational as well as experimental evidence, miRNA have been identified in introns and exons of non-coding RNA, introns and exons of coding RNA, and from discrete intergenic regions (Sayed and Abdellatif 2011). There appears to be no definitive set of characteristics to mark miRNA genes, however most intergenic miRNA have promoter regions similar to protein-coding RNAs and are thus transcriptionally regulated in a similar matter (i.e. nuclear hormone receptors, etc) (Jegga, et al. 2007). Some miRNA are clustered together in regions of the genome. For example, the miR-17-92 cluster is a group of six miRNA (miR-17, -18a, -19a, -19b, -20a, and -92a) that are co-transcribed as one poly-cistronic transcript.

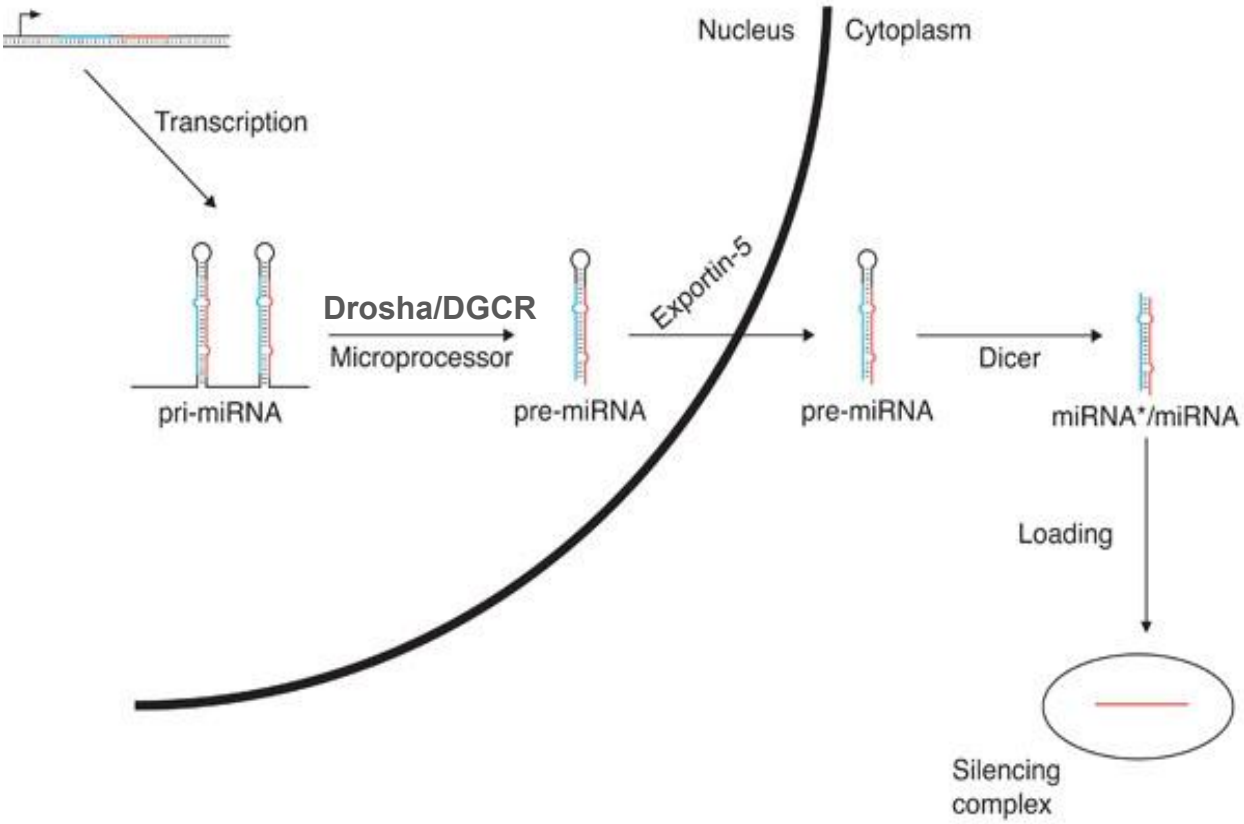
The majority of miRNA are transcribed by RNA polymerase II in a manner similar to transcription of protein-coding genes (Lee, et al. 2004). However, a handful of miRNA genes have been found to have a high number of Alu repeats and are thus transcribed by RNA polymerase III (Borchert, et al. 2006). As RNA pol II is the primary mechanism of miRNA transcription, a long, primary RNA transcript (pri-miRNA) that undergoes 5'methyl capping and 3'poly adenylation is formed (Cai, et al. 2004). The pri-miRNA will form a secondary structure that includes at least one, but possibly several, hairpins loops, which will be cleaved by the RNA endonuclease (RNAse) III enzyme Drosha in the nucleus to form the pre-miRNA (Figure VII-2) (Lee, et al. 2003). RNase III endonucleases are a family of multi-domain enzymes that bind and cleave double stranded RNA with tandem RNase III domains adjacent to a double stranded RNA binding domain (Blaszczyk, et al. 2001). The cofactor DiGeroge syndrome critical

region 8 (DGCR8) is necessary for Drosha cleavage of pri-miRNA due to two double stranded RNA binding domains that recognize the single stranded RNA-double stranded RNA junction that exists where the hairpin begins (Han, et al. 2004). Together, the Drosha-DGCR8 complex forms what is termed the Microprocessor complex that is responsible for cleaving the pre-miRNA stem loop(s) from the pri-miRNA transcript (Denli, et al. 2004, Gregory, et al. 2004). Pre-miRNA are approximately 70 nucleotides long with an approximately 33bp stem loop composing the majority of the structure. Following processing of pre-miRNA, Exportin 5 (EXP5) transports pre-miRNA from the nucleus to cytoplasm (Bohnsack, et al. 2004, Lund, et al. 2004, Yi, et al. 2005). The cofactor RanGTPase is necessary for transport and release of the pre-miRNA into the cytoplasm, following the hydrolysis of GTP (Bohnsack, et al. 2004). Once in the cytoplasm, the pre-miRNA is cleaved by the RNAse III enzyme Dicer to produce the mature, 17-23 nucleotide long miRNA (Bernstein, et al. 2001, Hutvagner, et al. 2001). Dicer is similar in structure and function to Drosha in that it has RNase III domains and a double stranded DNA binding domain, however, it also includes DEAD-box (Asp-Glu-Ala-Asp) and RNA helicase domains and a PAZ (Piwi Argonaute Zwille) domain (Fortin, et al. 2002). The PAZ domain acts as a sort of measuring stick to cleave the mature miRNA to the correct length, as it binds the 3'end of the dsRNA hairpin, thus allowing the RNAase III domains to cleave approximately 22 nucleotides away (Macrae, et al. 2006). Following production of the mature miRNA by Dicer cleavage, the double stranded complex is loaded onto an Argonaute (Ago) protein for formation of the RNA induced silencing complex (RISC), which ultimately regulates gene expression (Pillai, et al. 2004). In mammals, the Argonautes are a family of four proteins (Ago1-4) which are

the key effectors of miRNA mediated post-transcriptional gene regulation (Ender and Meister 2010). All Ago proteins are ubiquitously expressed and contain a PIWI domain and a PAZ domain, similar to that found in Dicer, however, only Ago2 has RNA endonuclelytic or 'slicer' activity that causes target mRNA degradation (Miyoshi, et al. 2005). Other members of the Ago family, as well as Dicer, GW182, and TAR RNA-binding protein (TRBP) compose the RISC and are involved in the mediation of translational repression (Kawamata and Tomari 2010).

Figure VII-2. MicroRNA biogenesis.

Figure VII-2


Nucleus
Cytoplasm
Transcription
pri-miRNA
Drosha/DGCR
Microprocessor
pre-miRNA
Exportin-5
pre-miRNA
Dicer
miRNA*/miRNA
Loading
Silencing
complex

While it was thought for well over a decade that all processing of miRNA went through the canonical biogenesis pathway described above, it was recently described that miRNA maturation can be Drosha and/or Dicer independent (Yang and Lai 2011). Examples of Drosha/DGCR8 independent biogenesis include the formation of pre-miRNA from introns. These molecules, termed miRtrons, are short hairpins formed from intronic regions that have been spliced from primary mRNA transcripts (Berezikov, et al. 2007). As they exist as a short, double stranded, RNA hairpin molecule, they are able to undergo Dicer cleavage as normal, and be loaded onto the RISC and function as a normal, canonical miRNA. Many of these miRNA, for example miR-62, have been identified by the cloning of small RNA from Drosha or DGCR8 knockout mice (Barbiarz 2008, Chong 2010, Yi 2009). Deletion of Drosha or DGCR8 led to embryonic lethality in the mouse due to the loss of canonical miRNA processing (Fukuda, et al. 2007, Gregory, et al. 2004, Wang, et al. 2007), however a number of these miRtrons were observed even in the absence of Drosha or DGCR8. To date, one example of a Dicer independent miRNA has been identified in vertebrates. Processing of miR-451 is believed to progress directly from Drosha cleavage to loading onto the Argonaute 2 (Ago2) protein. Ago2 is unique because it is has 'slicing', or RNA cleavage ability, and it is believed to cleave the loop from pre-miR-451 (Cheloufi, et al. 2010, Cifuentes, et al. 2010, Yang, et al. 2010).

3. Mechanisms of action

Sequence complementary between the mature miRNA and the target mRNA is the key component of miRNA mediated gene regulation. The first miRNA to be

identified, *lin-4,* targeted the mRNA *lin-14* in the 3'UTR (Lee, et al. 1993, Wightman, et al. 1993). The majority of miRNA:mRNA target interactions are believed to occur in the 3'UTR region, however recent evidence suggest that targeting can sometimes occur in the 5'UTR or actual coding region of the mRNA (Lee, et al. 2009, Younger, et al. 2009). It is believed that the amount of homology between the mature miRNA and target mRNA is the key factor in determining the actual mechanism employed by the RISC to prohibit translation. When there is exact homology between miRNA and target it is believed that RISC causes degradation of the target message via Ago2 endonucleolytic slicing capabilities (Miyoshi, et al. 2005).

In most cases, however, it is thought that there is only partial complementarity between miRNA and target and mechanisms that directly interfere or inhibit translation occur. The critical region of the miRNA that is needed for targeting mRNA is the seed sequence, or the 2 through 8 nucleotides from the 5' edge of the miRNA (Lewis, et al. 2005). For miRNA mediated regulation to occur, these sequences must be homologous with the mRNA target. Multiple miRNA that share the same seed sequence are referred to as miRNA families and together target the same group of mRNA targets. Multiple miRNA families have been identified (i.e. the highly expressed *let-7s)* and often are found to play important roles in mediating disease states (Jerome, et al. 2007). The presence of the seed sequence has allowed for the development of multiple target prediction algorithms that provide researchers insight and help into figuring out the function of specific miRNA. The most common and reputable algorithms are available online and include Targetscan (www.targetscan.com (Lewis, et al. 2005)), PicTar ((Krek,

et al. 2005), http://pictar.mdc-berlin.de/), and miRanda (http://www.microrna.org/microrna/home.do (Betel, et al. 2008).

Anti-sense homology of the miRNA seed sequence aligns the RISC with the target mRNA and allows for this effector protein complex to inhibit translation through three proposed mechanisms. The hypothesized mechanisms include mRNA deadenylation, inhibition of translation initiation and inhibition of translation elongation (Sayed and Abdellatif 2011). In all proposed models, the RISC binds to the mRNA target and interferes with or prevents translational machinery from binding to the 5' end of the transcript. However, depending on the mode of repression, additional proteins may associate with the RISC to act as effector molecules. In the case of mRNA deadenylation, several other proteins bind to the RISC, including chromatin assembly factor 1 (CAF1) which interacts with Ago2 to recruit the deadenylase protein complex CCR4-NOT to the 5' end of the mRNA (Fabian, et al. 2009). Deadenylation of the mRNA transcript leads to its de-stability, which will in turn decrease its half-life and thus the amount of protein that is translated. Inhibition of the initiation of translation occurs when eIF6 is part of the RISC and prevents the 80S ribosomal subunit from joining the translation machinery (Chendrimada, et al. 2007). Ago2 is also believed to contain a cap-binding domain that prevents eIF4E, a member of the translation complex, from binding to the initiation complex, thus preventing translation from occurring (Kiriakidou, et al. 2007). The final mechanism of miRNA action is hypothesized to be the premature termination of protein translation. It is thought that translation of the target mRNA initiates normally, however the RISC interferes with elongation of protein synthesis by

slowed ribosome movement or ribosome 'drop-off' from the target mRNA (Filipowicz, et al. 2008).

Regardless of the multiple potential mechanisms proposed for miRNA post-transcriptional gene regulation, it appears that miRNA are not a molecular switch that can simply turn protein synthesis of a particular mRNA on or off. A study looking at global protein synthesis in response to loss of a miRNA found that the repressive effect on proteins was relatively small and rarely exceeded more than a four-fold change in expression (Selbach, et al. 2008). However, the same study also found that loss of an individual miRNA can down regulate expression levels of hundreds of proteins. Other studies have demonstrated that over 60% of human genes are under the control of miRNA (Friedman, et al. 2009). Taken together, this suggests that even minor tweaking or 'fine-tuning' of protein expression by miRNA will collectively make a large scale difference.

4. Functional significance of miRNA mediated gene expression

MicroRNA are critical for nearly every biological function examined. Genetic deletion studies of key miRNA biogenesis processing enzymes (Drosha and Dicer) render mice embryonic lethal due to developmental defects (Bernstein, et al. 2003, Chong, et al. 2010, Fukuda, et al. 2007, Gregory, et al. 2004). Conditional deletion studies of Drosha and Dicer have found that global loss of miRNA influences nearly every physiological system examined, including: vascular development (Bernstein, et al. 2003, da Costa Martins, et al. 2008, Pan, et al. 2011), lung (Harris, et al. 2006), embryonic development (Zhang, et al. 2011), kidneys (Sequeira-Lopez, et al. 2010), inner ear development

(Soukup, et al. 2009), neural development (Cuellar, et al. 2008, Davis, et al. 2008), spermatogenesis (Papaioannou, et al. 2009), female reproductive function (Luense, et al. 2009) and many others (for a more inclusive review, (Sayed and Abdellatif 2011).

Not surprisingly, aberrant miRNA expression and/or function can lead to innumerable disease states, including lack of proper development, immune function, and nearly every cancer (Hermeking 2012, Osman 2012, Sayed and Abdellatif 2011). MicroRNA have been identified as serum or local biomarkers in several of these conditions and have tremendous potential use as clinical markers for disease (Ajit 2012). Furthermore, targeting miRNA or using miRNA mimics have great potential as therapeutic means to treat disease states (Elmen, et al. 2008, Iorio and Croce 2012).

SECTION II

MICRORNA ARE ESSENTIAL FOR FEMALE FERTILITY

VIII: CHAPTER 3

The role of Dicer in female fertility

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1. Abstract

Dicer is an RNAse III endonuclease that is essential for the biogenesis of microRNAs and small interfering RNAs. These small RNAs post-transcriptionally regulate mRNA gene expression through several mechanisms to affect key cellular events including proliferation, differentiation and apoptosis. Recently, the role of Dicer function in female reproductive tissues has begun to be elucidated through the use of knockout mouse models. Loss of Dicer within ovarian granulosa cells, luteal tissue, oocyte, oviduct and, potentially, the uterus renders females infertile. This review discusses these early studies and other data describing the current understanding of microRNAs and small interfering RNAs in female reproduction.

2. Dicer-mediated post-transcriptional gene regulation

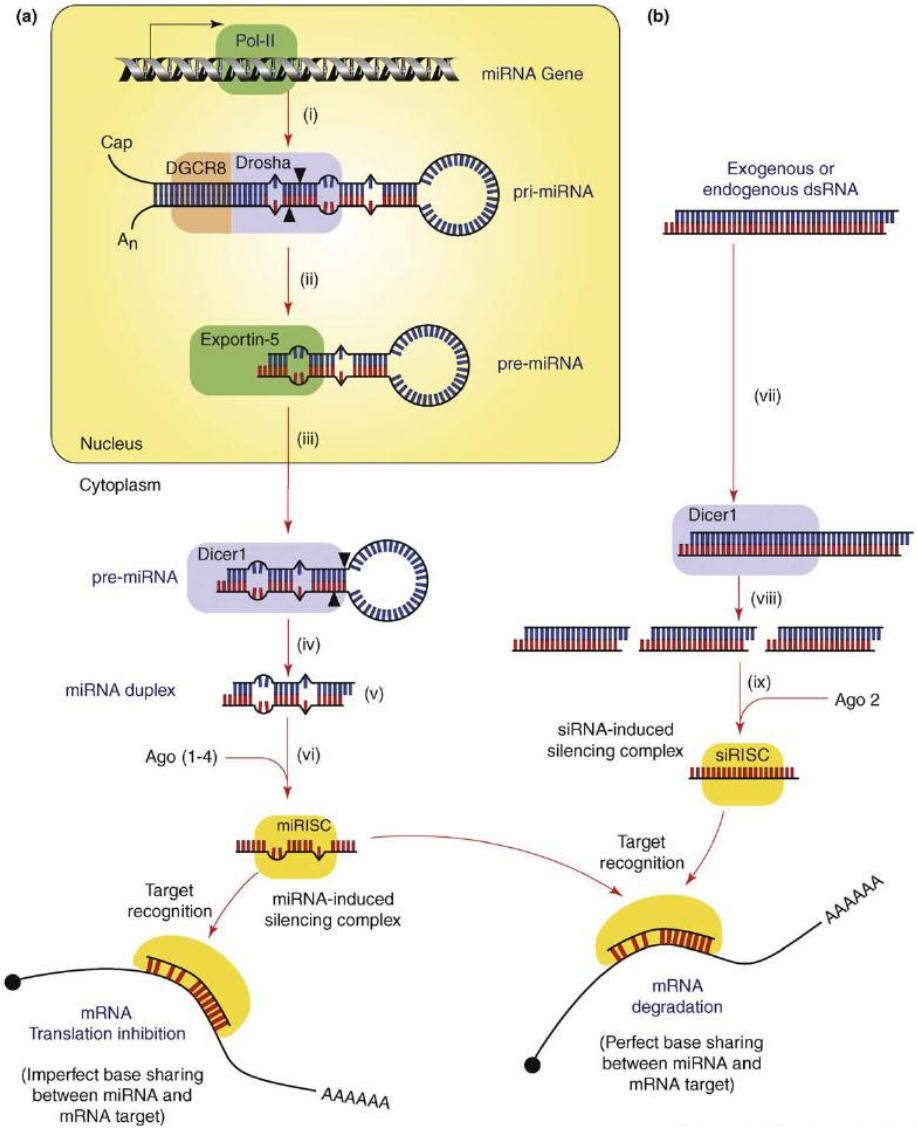
Regulation of fertility in the female is a dynamic and highly regulated process that requires the coordinated actions of multiple tissues and organ systems (e.g. hypothalamus, pituitary, ovary and reproductive tract) to develop a fertilizable gamete, as well as provide a suitable environment for fertilization and subsequent fetal development. To attain this optimal environment, the female reproductive system must be highly responsive to subtle changes in hormones and other external cues. A large body of evidence supports a role for transcriptional regulation in mediating these changes, and recent evidence suggests a hereto underappreciated role for post-transcriptional gene regulation in reproductive tissue and organ function (Carletti and Christenson 2009). Post-transcriptional gene regulation encompasses all aspects of messenger RNA (mRNA) turnover, processing, storage and translation, and provides cells with additional mechanisms to regulate protein content after transcription events. Recently, study of post-transcriptional gene regulation has surged because of the discovery of small non-coding RNAs, including microRNAs (miRNA) and small interfering RNAs (siRNAs) (Ruvkun 2008). Incorporation of miRNA and siRNA into RNA-induced silencing complexes (RISCs) enables the targeting of specific mRNA transcripts, ultimately providing cells with a post-transcriptional regulatory mechanism to either induce or inhibit protein production in response to stimuli, independent of commencement or cessation of mRNA transcription (for reviews, see Refs (Filipowicz, et al. 2008, van den Berg, et al. 2008)). This review focuses on the RNA endonuclease III (RNAse III) Dicer

and its enzymatic products, miRNA and siRNA, which elicit post-transcriptional regulatory responses.

Dicer is a cytosolic multidomain protein comprising an RNA helicase, a domain of unknown function (DUF283), a Piwi Argonaute Zwille (PAZ) domain, two RNAse III domains and a double-stranded-RNA-binding domain (dsRBD) (Macrae, et al. 2006, Provost, et al. 2002). The RNA helicase unwinds long double stranded RNA (dsRNA) precursors, whereas the dsRBD and PAZ domains are essential for Dicer binding to dsRNA and for determining the length of the siRNA or miRNA products, respectively. Cleavage of miRNA or siRNA precursors is dependent on the two RNAse III domains within the Dicer protein. Dicer is essential in miRNA and siRNA biogenesis (Figure VIII 1), and its function is crucial to the cell; general knockout of Dicer1 (hereafter referred to as 'Dicer') in the mouse causes morphologic abnormalities and stunted growth in embryonic day (E)7.5 embryos and lethality by E11.5 (Bernstein, et al. 2003). To further explore Dicer function, multiple groups developed Dicer alleles with the second RNAse III domain flanked by loxP sites ($Dicer^{fl/fl}$) to facilitate conditional knockdown (cKO) of $Dicer^{fl/fl}$ via tissue-specific recombination by Cre recombinase (Andl, et al. 2006, Harfe, et al. 2005, Mattiske, et al. 2009, Mudhasani, et al. 2008, Yi, et al. 2006). Although miRNA are abundant in mammalian somatic tissues and, thus, most affected by the loss of Dicer, several reports using deep sequencing methods have suggested that siRNAs might also have an important role or roles in both somatic tissues and germ cells (Tam, et al. 2008, Watanabe, et al. 2008). Therefore, whereas loss of Dicer within somatic tissues has typically been linked to altered miRNA biogenesis, changes observed in conditional $Dicer^{fl/fl}$ knockout mice might be due to a combined loss of miRNA and siRNA.

Figure VIII-1 Biogenesis of miRNA and siRNA. (a) (i) MicroRNAs (miRNA) are transcribed by RNA polymerase II from intragenic or intergenic regions of the genome. (ii) This transcript, the primary miRNA (pri-miRNA), can range from 100 to 1000 nucleotides in length and shares many properties of mRNA transcripts, including a 7-methylguanasine (m7G) 50-cap and poly-A tail. Within pri-miRNA, ~70 bp stem loops are recognized and cleaved by the complex of DiGeorge syndrome critical region gene 8 (DGCR8) and the RNAse III endonuclease enzyme Drosha to produce the precursor miRNA (pre-miRNA). (iii) Exportin 5 then transports the pre-miRNA from the nucleus to the cytoplasm, where (iv) the RNAse III endonuclease Dicer cleaves the stem away from the loop of the pre-miRNA to produce the (v) mature miRNA duplex. (vi). One strand of this ~21 nt long duplex is incorporated into the RNA-induced silencing complex (RISC) to regulate the translation and/or degradation of target mRNAs. (b) Whereas exogenous addition of double-stranded RNA, or dsRNA (siRNA), to the cell is a common experimental manipulation, recent evidence suggests that endogenous forms of siRNA also exist in animals. (vii) Endogenous siRNA forms when long complementary strands of RNA bind to form dsRNA. The individual strands of these dsRNA are products of pseudogenes, transposable elements or protein-coding genes and are the result of complementary transcriptional products from cis or trans loci (the same or different chromosomes, respectively) or the result of inverted repeat sequences that have folded to form a hairpin structure. (viii) Within the cytoplasm, dsRNA is cleaved into multiple ~21 nt fragments by Dicer. (ix) One strand from these duplexes is incorporated into a siRNA-induced silencing complex (siRISC) to bind target mRNA and facilitate siRNA-mediated transcript degradation.

Figure VIII-1



MicroRNAs are derived from highly conserved genes that bind partially complementary sequences in the 3`untranslated region and/or coding regions of target mRNA transcripts to regulate gene expression (for a review, see Refs (Filipowicz, et al. 2008, van den Berg, et al. 2008)). To date, 706 and 547 miRNA have been identified in the human and mouse, respectively (http://microrna.sanger.ac.uk/) (Griffiths-Jones, et al. 2008). Because each miRNA is derived from a specific pre-miRNA hairpin loop and represents a specific gene product, a standardized nomenclature for mammalian miRNA has been established (see Figure XX-1). The functions of miRNA are diverse and play a part in numerous processes, including cellular proliferation and differentiation, embryonic development, and apoptosis (for a review, see Refs (Bushati and Cohen 2007, Stefani and Slack 2008, Williams 2008)). Exogenous siRNAs injected into cells have been widely used to knockdown gene expression (for a review, see Ref. (Svoboda 2008)). Endogenous siRNAs are derived from dsRNA (i.e. pseudogenes, transposable elements or protein-coding genes). Because of the nature of their biogenesis, siRNAs are typically thought to be fully complementary to their target transcripts, and a standardized nomenclature or estimate of the number of mammalian siRNAs has yet to be established (Figure VIII-1). It remains possible that siRNAs associated with RISCs could also act like miRNA and not require full complementation to affect post-transcription gene regulation. Endogenous siRNAs derived from pseudogenes have been shown to target specific mRNA transcripts and regulate transposable elements in the mammalian oocyte (Tam, et al. 2008, Watanabe, et al. 2006, Watanabe, et al. 2008). The biological significance of siRNA retrotransposon silencing is, thus far, unique to the oocyte, and its exact role has yet to be defined (Watanabe, et al. 2006).

Limited examples of post-transcriptional gene regulation exist within the somatic cells of the reproductive system (Dell'Aquila, et al. 2004, Menon, et al. 2006). However, as the function of Dicer and its products (miRNA and siRNAs) are studied in the female reproductive tract, vital roles for such post-transcriptional gene regulation in female fertility are becoming evident (Carletti and Christenson 2009). In addition, because of the involvement of miRNA in these crucial biological pathways, it is not surprising that they have been implicated in a number of reproductive diseases and cancers of the reproductive system (for a review, see Refs (Luo and Chegini 2008, Pan and Chegini 2008, Wei and Soteropoulos 2008)). Gaining a better understanding of Dicer generated miRNA and siRNAs will provide important insight into regulation of the female reproductive system and perhaps the mechanisms that regulate fertility and etiology of reproductive diseases.

3. Dicer in the oocyte and early embryo

Female germ cells (oocytes) enclosed in layers of somatic cells (granulosa and theca) form ovarian follicles. The somatic cells support the growth and development of the oocyte and ultimately release (ovulate) a mature, transcriptionally quiescent oocyte that can be fertilized by sperm in the oviduct to form the developing zygote. The zygote undergoes a series of reductive cell divisions and eventually becomes an embryonic blastocyst. In the mouse, the embryo is dependent upon maternal transcripts produced during oocyte development until the two-cell stage when transcription of the embryonic genome begins. Oocytes and fertilized eggs contain 10–15-fold higher levels of Dicer

transcripts than any other cells and/or tissues (BioGPS; http://biogps.gnf.org/) (Su, et al. 2002) and are one of few known mammalian cells and/or tissues in which Dicer expression is regulated (Nicholson and Nicholson 2002). Expression of the Dicer transcript remains steady in the growing mouse oocyte during folliculogenesis (Watanabe, et al. 2008) and through the germinal vesicle and metaphase II stages (Cui, et al. 2007, Murchison, et al. 2007). After fertilization, the amount of Dicer mRNA decreases by approximately half and remains low in the two-cell embryo through the blastocyst stage (Cui, et al. 2007, Murchison, et al. 2007). Expression of total miRNA during this same period is highest in the mature oocyte and one-cell zygote before decreasing by half in the two-cell embryo (Tang, et al. 2007). Increased miRNA expression observed in the four-cell embryo (Tang, et al. 2007) could result from the resumption of transcription of the embryonic genome, post-transcriptional regulation of Dicer or increases in other miRNA-processing factors, such as Drosha or Exportin 5 (Figure VIII-1)

To examine Dicer function within the oocyte and early embryo, $Dicer^{fl/fl}$ mice were crossed with mice expressing Cre recombinase driven by the oocyte-specific zona pellucida 3 (ZP3) promoter (Dicer ZP3-cKO) or alkaline phosphatase, liver/bone/kidney (Alpl) promoter (Dicer Alpl-cKO) (Mattiske, et al. 2009, Murchison, et al. 2007, Tang, et al. 2007). In the Dicer ZP3-cKO model, Cre expression is turned on shortly after initiation of oocyte growth (Mattiske, et al. 2009, Murchison, et al. 2007, Tang, et al. 2007), whereas in the Dicer Alpl-cKO model, Cre expression is turned on as early as the primordial germ cell (Mattiske, et al. 2009). Early folliculogenesis and oocyte development in Dicer ZP3-cKO mice seemed normal, evidenced by normal ovulation

rates and oocytes indistinguishable from wild type littermates (Murchison, et al. 2007). The importance of communication between the oocyte and cumulus granulosa cells in follicular and oocyte development is well documented (Dong, et al. 1996). Therefore, the lack of a somatic cell phenotype in follicles with oocytes lacking Dicer suggests that oocyte miRNA- or siRNA-mediated post-transcriptional gene regulation does not play a crucial part in regulating somatic cells during folliculogenesis.

Although early development and growth of oocytes from Dicer ZP3-cKO females was unaffected, these oocytes were less likely to extrude a polar body after mating, and immunostaining indicated multiple spindles and chromatin condensation defects (Mattiske, et al. 2009, Murchison, et al. 2007, Tang, et al. 2007). Similar spindle defects were observed in oocytes derived from Dicer Alp1-cKO mice (Mattiske, et al. 2009). Further analysis of cultured oocytes collected from Dicer ZP3-cKO mice found that meiosis proceeds normally until metaphase II, when abnormal spindle formation was observed (Mattiske, et al. 2009). Transplantation of wild-type germinal vesicles (nucleus of oocyte) into enucleated oocytes from Dicer ZP3-cKO mice resulted in defective spindle formation, whereas reciprocal transplantation of mutant germinal vesicles into enucleated wild-type oocytes resulted in normal spindle formation in 74% of oocytes (Mattiske, et al. 2009). These data suggest that meiotic defects arise from the ooplasm of the oocyte and not the germinal vesicle (Mattiske, et al. 2009). Furthermore, expression analysis of oocytes indicated an overabundance of mRNA transcripts in Dicer ZP3-cKO oocytes, consistent with a loss in miRNA inhibition of translation or mRNA degradation (Tang, et al. 2007).

The roles of individual miRNA and siRNAs in the oocyte are largely unknown, with a few recent exceptions (Murchison, et al. 2007, Tang, et al. 2007). Loss of Dicer within oocytes was shown to decrease siRNA levels, whereas corresponding siRNA target transcripts, protein phosphatase 4 regulatory subunit 1 and retrotransposon long terminal repeat 10, increased 1.5-fold and 5-fold, respectively (Tam, et al. 2008, Watanabe, et al. 2008). The biological role of siRNA retrotransposon silencing is unique to the oocyte, thus far, and its exact role has yet to be defined (see Ref. (Svoboda 2008) for more information on RNA silencing in oocytes and early embryos).

4. Dicer within somatic cells of the ovary

The granulosa and thecal cells of the ovarian follicle support oocyte growth and produce key endocrine hormones (steroids and proteins) that regulate the reproductive system. In response to an ovulatory surge of luteinizing hormone (LH), the mature oocyte is released (ovulation), and with the exception of a few cells that exit the ovary with the oocyte, the remaining somatic tissue that comprised the follicle undergoes luteinization (hypertrophy and vascularization) to form the corpus luteum. These LH-mediated ovarian events are essential for female fertility. Consistent with other observations showing that Dicer is not dynamically regulated (Nicholson and Nicholson 2002), levels of Dicer in granulosa cells before and after the LH surge did not change (Fiedler, et al. 2008). Dicer expression in other somatic tissues of the ovary (thecal cells, corpus luteum and interstitium) has not been examined directly but is not anticipated to change (BioGPS) (Su, et al. 2002). In disease states, however, recent studies examining

ovarian cancer cells found that Dicer and Drosha levels decrease in tumor cells (Merritt, et al. 2008). Functional deletion studies of Dicer point to a clear and important role for miRNA and/or siRNA in ovarian function and female fertility (Gonzalez and Behringer 2009, Hong, et al. 2008, Nagaraja, et al. 2008, Otsuka, et al. 2008, Pastorelli, et al. 2009).

Otsuka et al. created a general hypomorphic mutation ($Dicer^{hypo}$; ~75% reduction in Dicer protein) using a gene-trap method and observed that $Dicer^{hypo}$ females were infertile because of luteal deficiency. Transplantation of wild-type ovaries into $Dicer^{hypo}$ females restored fertility, indicating that loss of fertility was due to an ovarian defect (Otsuka, et al. 2008). Serum progesterone levels in $Dicer^{hypo}$ mice remained low after mating, and histological analyses of ovaries revealed a lack of luteal tissue vascularization. In these mice, the global reduction of Dicer would be predicted to decrease all miRNA; however, selective ovarian bursal replacement of two known angiogenic miRNA, miR-17-5p and let-7b, was sufficient to partially restore luteal vascular development and progesterone production through day 5.5 post-coitus, although pregnancy was not maintained (Otsuka, et al. 2008). The inability to restore fertility could be due to an insufficient amount of exogenous miRNA, clearance of exogenous miRNA or the need for additional miRNA in other processes necessary for fertility (e.g. oviductal and uterine function and implantation) (Otsuka, et al. 2008). Alternatively, because Dicer was reduced in all cells in this model, the fertility defect could be due to a loss of function of non-reproductive cells (i.e. endothelial cells) within the ovary.

Mice with targeted deletion of Dicer within ovarian granulosa cells of developing follicles were generated by crossing $Dicer^{fl/fl}$ mice with mice expressing Cre recombinase driven by the anti-Mullerian hormone receptor 2 promoter (Dicer Amhr2-cKO)

(Gonzalez and Behringer 2009, Hong, et al. 2008, Nagaraja, et al. 2008, Pastorelli, et al. 2009). Within the ovary, Amhr2-Cre recombinase is expressed in granulosa cells of pre-antral and antral follicles (Jamin, et al. 2002b, Jorgez, et al. 2004). Studies by our group (Hong, et al. 2008) and others (Gonzalez and Behringer 2009, Pastorelli, et al. 2009) crossed the Amhr2-Cre mice to the same Dicer-floxed mouse line (Harfe, et al. 2005), whereas Nagaraja et al. (Nagaraja, et al. 2008) used a different Dicer-floxed line (Yi, et al. 2006). Both lines of Dicer mice exhibited defects in ovarian function (Hong, et al. 2008, Nagaraja, et al. 2008). The loss of Dicer expression in ovarian granulosa cells reduced natural (Hong, et al. 2008) and equine chorionic gonadotropin (eCG)- and human chorionic gonadotropin (hCG)-stimulated ovulation rates when compared with wild-type mice (Hong, et al. 2008, Nagaraja, et al. 2008). Increased numbers of atretic follicles (Nagaraja, et al. 2008) and trapped oocytes in luteinizing follicles were observed in Dicer Amhr2-cKO mice (Hong, et al. 2008, Nagaraja, et al. 2008). Both studies found that loss of Dicer did not impact serum estrogen levels or mating behavior (Hong, et al. 2008, Nagaraja, et al. 2008). Collectively, these studies indicate that Dicer affects ovulation rate, probably by influencing the total number of pre-ovulatory follicles that achieve proper development and/or by affecting the ability of follicles to ovulate. Interestingly, evidence for trapped oocytes in luteinized follicles was not reported in the general Dicer^{hypo} mouse model (Otsuka, et al. 2008). Discrepancies between the Dicer^{hypo} and Dicer Amhr2-cKO models could be due to the steroidogenic cell-specific deletion of Dicer in the Amhr2-cKO model and total cell deletion in the Dicer^{hypo} model. Detailed studies of the different stages of follicular development (especially during the peri-ovulatory period), paired with Dicer-expression analyses, are needed to elucidate how

and when loss of Dicer impacts follicular development, ovulation and/or luteinization. It is possible that Dicer is necessary for proper recruitment of follicular waves and loss of Dicer leads to fewer follicles available for growth, maturation and ovulation.

In contrast to the clear evidence regarding ovulation rate, the two lines of Dicer Amhr2-cKO mice present conflicting evidence regarding whether loss of Dicer in ovarian granulosa cells impacts oocyte function (Hong, et al. 2008, Nagaraja, et al. 2008). Nagaraja et al. reported that cultured, fertilized oocytes collected from the oviduct of stimulated Dicer Amhr2-cKO females failed to progress to the two-cell embryonic stage at the same rate (29.0%) as fertilized oocytes collected from wild type females (82.8%). Conversely, Hong et al. reported that 97% and 100% of fertilized oocytes collected from Dicer Amhr2-cKO and wild-type females, respectively, one day after mating (no pharmacologic stimulation), progressed to the two-cell stage. The differences between these studies might have resulted from pharmacologic stimulation versus natural follicular cycle or because of different Dicer-floxed strains (Harfe, et al. 2005, Yi, et al. 2006).

Functional analyses of Dicer in the ovary implicate miRNA in ovarian function, and the individual miRNA involved in specific functions are now beginning to be determined. Laboratories have identified as many as 177 miRNA in whole ovaries of newborn, two-week-old and adult mice (Choi, et al. 2007, Mishima, et al. 2008, Ro, et al. 2007). Several of these miRNA (let-7a, miR-143, miR-21, miR-125, let-7b and let-7c) have also been described in granulosa cells of primary, secondary and antral follicles (Yao, et al. 2009). Human granulosa cell-culture studies have implicated a number of miRNA in the inhibition of steroid production, with 36, 51 and 57 specific miRNA

decreasing progesterone, estradiol and testosterone synthesis, respectively (Sirotkin, et al. 2009). Conversely, 10, 0 and 1 miRNA were found to stimulate human granulosa cell production of progesterone, estradiol and testosterone, respectively (Sirotkin, et al. 2009). Whether these miRNA are expressed and exhibit similar function within ovarian follicular or luteal tissues remains to be tested. In a cell-specific study, Fiedler et al. (Fiedler, et al. 2008) identified 212 known miRNA within granulosa cells isolated from peri-ovulatory follicles, 13 of which were regulated by the LH surge (Fiedler, et al. 2008). Two of these LH-regulated miRNA (miR-132 and miR-212) were found to post-transcriptionally regulate C-terminal-binding protein 1 (Fiedler, et al. 2008), a protein that was shown recently to repress the expression of steroidogenic factor-1 (Dammer and Sewer 2008), a nuclear receptor involved in ovarian, adrenal and testis function development and function (e.g. steroidogenesis (Lala, et al. 1992)). In addition to the Otsuka et al. study, these are the only studies linking specific miRNA to biological endpoints in the ovary, illustrating the need for additional research.

5. Dicer in the oviduct and uterus

Oocyte fertilization and early pre-implantation embryonic development occurs within the oviduct. Subsequently, the developing embryo passes through the utero-tubal junction and enters the uterus, where implantation, placentation and embryonic and fetal development occur. In addition to granulosa cells, Amhr2-Cre is expressed in the oviduct, uterus, cervix and anterior portion of the vagina (Jamin, et al. 2002b). Loss of Dicer in mouse oviducts results in a dramatic phenotype consisting of shortened tubule

length, loss of oviductal coils and large fluid-filled sacs (Gonzalez and Behringer 2009, Hong, et al. 2008, Nagaraja, et al. 2008, Pastorelli, et al. 2009). Histological analysis of oviducts from Dicer Amhr2-cKO females revealed loss of the smooth muscle layer and disorganization of the epithelium in the oviduct, particularly in the isthmus (region near the uterus) of the oviduct (Gonzalez and Behringer 2009, Hong, et al. 2008, Nagaraja, et al. 2008). Collection of embryos on day 4 post-mating found all embryos retained in the oviduct of Dicer Amhr2-cKO females, whereas embryos in wild-type females had all traversed the utero-tubal junction and resided within the uterus (Hong, et al. 2008). Similarly, affinity chromatography beads similar in size to pre-implantation embryos that were injected into oviducts were unable to enter the uterus in Dicer Amhr2-cKO mice (Gonzalez and Behringer 2009). Because ovulated Dicer Amhr2-cKO oocytes were fertilized after mating (Hong, et al. 2008), this would argue that the block is unidirectional and/or caused by an effect on smooth muscle contractility, cilia or oocyte size that prevents transport. Nagaraja et al., identified 28 down regulated miRNA in oviducts from the Dicer Amhr2-cKO mouse model, 23 of which were predicted to target at least one member of the Wnt or Hox family of genes (Nagaraja, et al. 2008). In fact, the defect in oviductal transport of the Dicer Amhr2-cKO mouse phenocopies that seen in mice deficient in Wnt/β-catenin signaling (Arango, et al. 2005, Deutscher and Hung-Chang Yao 2007). Indeed, b-catenin levels were reduced in oviducts and uteri of Dicer Amhr2-cKO females (Hong, et al. 2008). In addition to developmental effects, it seems that loss of miRNA in the oviduct might also influence factors secreted into the oviductal lumen; embryos collected three days after mating from the oviducts of Dicer Amhr2-cKO

females were developmentally delayed compared to those collected from wild-type animals (Hong, et al. 2008).

Like the oviductal phenotype, the uteri of Dicer Amhr2- cKO animals were also developmentally compromised (with three of four reports noting severe defects (Gonzalez and Behringer 2009, Hong, et al. 2008, Nagaraja, et al. 2008). The lengths, diameters and weights of uteri collected from eCG-stimulated juvenile Dicer Amhr2-cKO females were smaller than those of uteri from wild-type littermates (Hong, et al. 2008, Nagaraja, et al. 2008). Histological analysis revealed the presence of all tissue layers within the uterus (Gonzalez and Behringer 2009, Hong, et al. 2008, Nagaraja, et al. 2008), although reduced numbers of uterine glands and a thinner myometrial layer were observed in one Dicer Amhr2-cKO mouse model (Gonzalez and Behringer 2009, Hong, et al. 2008). Gonzalez et al. further noted that the uterine glands reside in close proximity to the myometrial layer, mimicking a human condition referred to as adenomyosis (Parrott, et al. 2001). The uteri of these mice (n = 3) were not able to sustain pregnancy after embryo transfer. Interestingly, histological analysis failed to observe any defect in the other Dicer floxed mouse line (Nagaraja, et al. 2008), and adenogenesis (i.e. gland formation and location), estrogen responsiveness and stimulus-induced decidualization reaction all seemed normal (Nagaraja, et al. 2008).

Expression of Dicer mRNA and protein, as well as the Argonaute proteins, is abundant in the mouse uterus on days 4 through 8 of pregnancy (day 1 = presence of vaginal plug), suggesting that miRNA synthesis is ongoing (Chakrabarty, et al. 2007). Furthermore, microarray analysis of miRNA present in the uterus on day 4 of pregnancy (receptive phase) and at implantation sites suggests that miRNA are important for

establishing pregnancy (Chakrabarty, et al. 2007, Hu, et al. 2008). Comparison of miRNA expression within uterine tissues collected on days 1 and 4 of pregnancy identified 32 miRNA up regulated on day 4 (Chakrabarty, et al. 2007). Expression of miR-101 and miR-199* were stimulated by estradiol and found to post-transcriptionally regulate prostaglandin synthase-2, an enzyme necessary for implantation in mice. Further analysis of the pregnant uterus found 13 miRNA up regulated in implantation sites compared to inter-implantation sites (Chakrabarty, et al. 2007). Analysis of miR-21, one of the up regulated miRNA, found that expression was dependent upon the presence of an activated embryo (Chakrabarty, et al. 2007). Uterine miRNA also seem to be regulated by estrogen; 49 miRNA were found to be differentially regulated in response to estradiol (Nothnick 2008). Taken together, these studies suggest a role for uterine miRNA in implantation and pregnancy.

6. Future directions

To date, the study of Dicer function in female reproductive tissues is limited to a few reports (Figure VIII-2). However, these studies all clearly demonstrate that Dicer and its enzymatic products are crucial for female fertility. Conditional deletion of Dicer in the oocyte, ovary, oviduct and uterus provide convincing evidence that miRNA and/or siRNAs are necessary for the overall development and function of the female reproductive system. Recent studies have demonstrated that expression of Amhr2-Cre recombinase is leaky and that recombination occurs in the brain, pituitary, heart and tail, in addition to the known tissues expressing Amhr2 (Hernandez Gifford, et al. 2009,

Pastorelli, et al. 2009). Additional studies using more refined temporal and cell-specific Cre-recombinases are needed to further our understanding of post-transcriptional gene regulation in the female reproductive system (Figure VIII-2). Identification of individual miRNA or siRNAs in each female reproductive organ will enable researchers to better understand the mechanisms of gene regulation that allow successful reproduction. Thus far, little is known about the role of siRNAs in somatic cells of the reproductive system, although they seem to be highly abundant (Tam, et al. 2008, Watanabe, et al. 2008). Additional research is needed to determine the functional role siRNA molecules exert on tissue and organ function. Recently, antagomirs and locked nucleic acid oligonucleotides have been used to down regulate specific miRNA expression in a variety of cells in vitro and tissues in vivo (Elmen, et al. 2008, Krutzfeldt, et al. 2005). It is anticipated that establishing the spatiotemporal expression patterns of miRNA in the female reproductive tract will provide targets for drug and therapeutic treatments for reproductive diseases such as endometriosis, uterine leiomyoma and ovarian, uterine and cervical cancers. Moreover, understanding the role that post-transcriptional gene regulation has in reproduction will facilitate the elucidation of the etiologies leading to reproductive failure and, we hope, provide methods and targets for treatment of infertility and provide new means of contraception.

7. Acknowledgements

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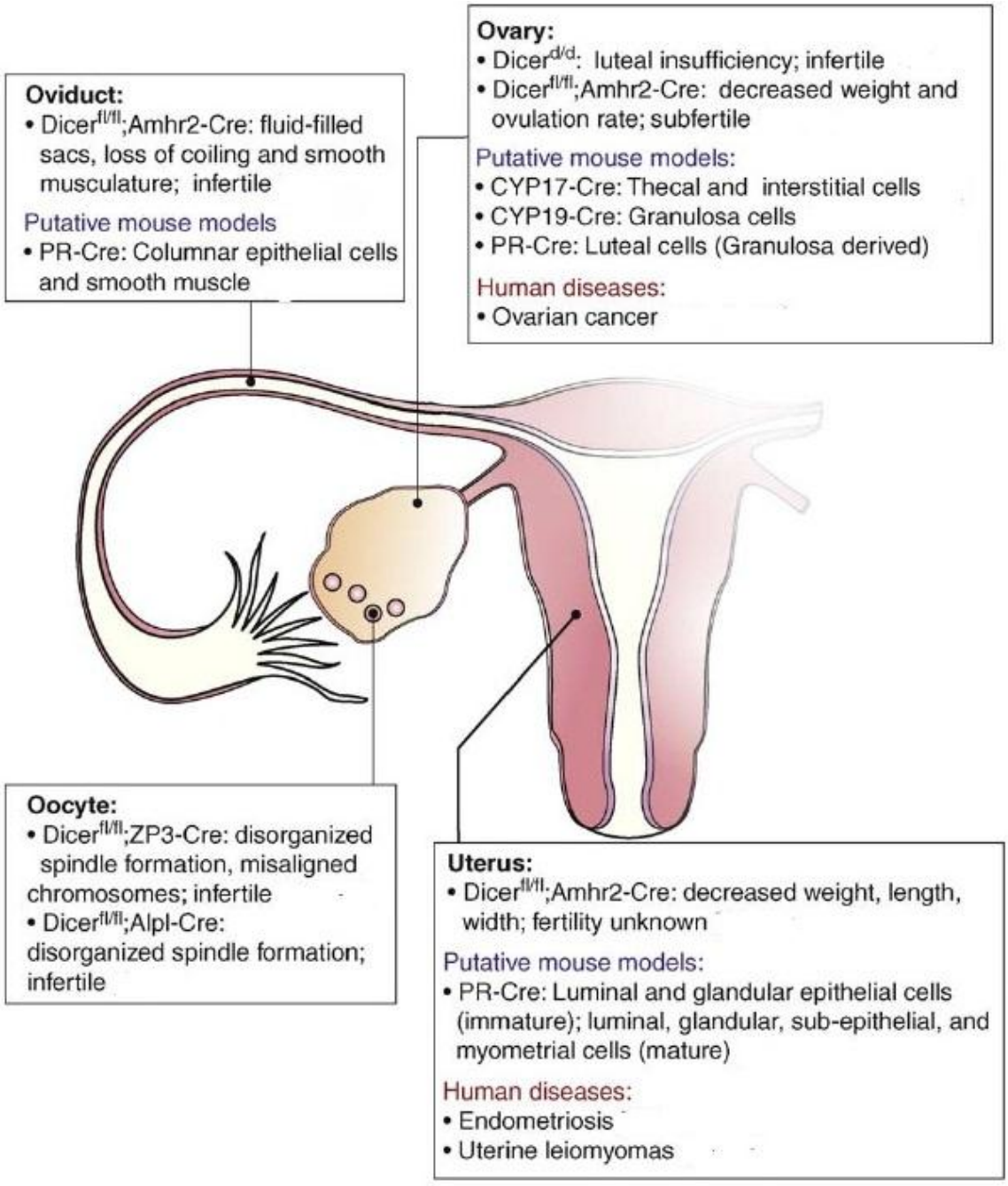
Figure VIII-2. Effects of Dicer deletion on female fertility. The function of Dicer and its products (siRNA and miRNA) in female fertility have been investigated in Dicer1-floxed mouse lines expressing Cre-recombinase in female reproductive tissues. The descriptions of phenotypes caused by Dicer deletion are listed for each tissue. Additional female reproductive tissue Cre-recombinases are available. It is anticipated their use will further refine the temporal and cellular importance that Dicer (i.e. miRNA/siRNA) has on female fertility. Lastly, a number of expression analysis studies have linked human reproductive diseases to altered miRNA or siRNA profiles they are listed. Presently, most of these studies are biomarker studies because specific miRNA/siRNAs have not yet been demonstrated to be causative. $Dicer^{fl/fl}$;Amhr2-Cre is referred to as Dicer Amhr2-cKO, $Dicer^{fl/fl}$;ZP3-Cre is referred to as Dicer ZP3-cKO and $Dicer^{fl/fl}$;Alpl-Cre is referred to as Dicer Alpl-cKO in the text of this review. Gene abbreviations: Alpl, alkaline phosphatase (liver/bone/kidney); Amhr2, anti-Mullerian hormone receptor 2; Cre, cre recombinase; CYP17, cytochrome P450 subfamily 17; CYP19, cytochrome P450 subfamily 19; hypo, hypomorph; ZP3, zona pellucida 3; PR, progesterone receptor.

Figure VIII-2



IX: CHAPTER 4

Dicer1 is essential for female fertility and the development of the female reproductive tract

A paper published in the journal
***Endocrinology*, December 2008**

1. **Abstract**

The ribonuclease III endonuclease, Dicer1 (also known as Dicer), is essential for the synthesis of the 19–25 nucleotide noncoding RNAs known as micro-RNAs (miRNA). These miRNA associate with the RNA-induced silencing complex to regulate gene expression post-transcriptionally by base pairing with 3'untranslated regions of complementary mRNA targets. Although it is established that miRNA are expressed in the reproductive tract, their functional role and effect on reproductive disease remain unknown. The studies herein establish for the first time the reproductive phenotype of mice with loxP insertions in the Dicer1 gene ($Dicer1^{fl/fl}$) when crossed with mice expressing Cre-recombinase driven by the anti-Mullerian hormone receptor 2 promoter ($Amhr2^{Cre/+).}$ Adult female $Dicer1^{fl/fl}$;$Amhr2^{Cre/+}$ mice displayed normal mating behavior but failed to produce offspring when exposed to fertile males during a 5-month breeding trial. Morphological and histological assessments of the reproductive tracts of immature and adult mice indicated that the uterus and oviduct were hypotrophic, and the oviduct was highly disorganized. Natural mating of $Dicer1^{fl/fl}$;$Amhr2^{Cre/+}$ females resulted in successful fertilization as evidenced by the recovery of fertilized oocytes on d1 of pregnancy, which developed normally to blastocysts in culture. Developmentally delayed embryos were collected from $Dicer1^{fl/fl}$; $Amhr2^{Cre/+}$ mice on d3 of pregnancy when compared with controls. Oviductal transport was disrupted in the $Dicer1^{fl/fl}$;$Amhr2^{Cre/+}$ mouse as evidenced by the failure of embryos to enter the uterus on d4 of pregnancy. These studies implicate Dicer1/miRNA mediated post-transcriptional gene regulation in reproductive somatic tissues as critical for the normal development and function of these tissues and for female fertility. (Endocrinology 149: 6207–6212, 2008).

2. Introduction

Sustained reproductive function requires that the female reproductive tract, including the uterus, oviduct, cervix, and ovary, correctly develops and functions together. To obtain the precise temporospatial control over cellular protein expression that is necessary for organogenesis, a myriad of gene transcription, translation, and posttranslational regulatory mechanisms are invoked. Despite the general importance of the female reproductive system, our understanding of organogenesis and the molecular mechanisms that regulate the development and differentiation of these tissues lags behind other organ systems. Moreover, recent observations suggest that in addition to transcription, translation, and posttranslational modifications, posttranscriptional gene regulation may play a more pronounced role in cell, tissue, and organ function. Recently, micro-RNAs (miRNA) have been demonstrated to play a novel, yet not thoroughly defined role in post-transcriptional regulation of gene expression.

MicroRNAs are a recently described class of small non-coding regulatory RNAs that regulate gene expression post-transcriptionally (Bartel 2004). MicroRNAs are proposed to be involved in diverse developmental and pathological processes (Wienholds and Plasterk 2005). Biogenesis of miRNA is a multistep process that culminates with the ribonuclease (RNase) III endonuclease, Dicer1, cleaving the 70- to 110-bp hairpin precursor miRNA and forming 19–25 nucleotides long double-stranded miRNA (Murchison and Hannon 2004). Subsequently, one strand of this pair associates with the Argonaute proteins to form the RNA-induced silencing complex, which can then affect posttranscriptional gene regulation. In mammals the miRNA-RNA-induced silencing complex primarily binds the 3`untranslated regions of target mRNAs, with partial

complementarity to either repress or enhance translation (Bushati and Cohen 2007, Filipowicz, et al. 2008, Vasudevan, et al. 2007).

Dicer1 (through the generation of miRNA and subsequent post-transcriptional regulation of specific gene products) has been proposed to play a role in the normal development of the lung (Harris, et al. 2006), limbs (Harfe, et al. 2005), and skeletal muscle (O'Rourke, et al. 2007), as well as the female germ line (Murchison, et al. 2007). Recently, post-transcriptional regulation and miRNA have been proposed to play a role in embryo implantation (Chakrabarty, et al. 2007), as well as in the human endometrium and the pathophysiology of endometriosis (Pan, et al. 2007). Collectively, these studies suggest that Dicer1 and its miRNA products play a pivotal role in the molecular regulation of multiple organ systems, which may include reproductive functions such as oocyte maturation, embryo implantation, and uterine pathophysiological conditions. To date, there is no information on the role of Dicer1 and miRNA in the development and subsequent function of the female reproductive organs. As such, the objective of the current study was to examine the phenotypical consequences of conditional deletion of the Dicer1 gene product from the developing female reproductive system and its impact on female fertility.

3. Materials and Methods

Generation of conditional Dicer1 knockdown

The University of Kansas Medical Center's Institutional Animal Care and Use Committee approved all procedures involving mice before use. Mice homozygous for loxP insertions flanking the second RNase III domain in the Dicer1 gene ($Dicer1^{fl/fl}$; generously provided by Dr. Clifford Tabin, Harvard Medical School) were crossed with mice heterozygous for Cre recombinase knocked into the anti-Mullerian hormone receptor 2 locus ($Amhr2^{Cre/+}$; generously donated by Dr. Richard Behringer, Baylor College of Medicine) to produce mice that exhibit knocked-down expression of Dicer1 in the ovarian granulosa cells and the derivatives of the Mullerian duct (i.e. oviduct, uterus, and cervix). The resulting progeny were genotyped as previously described (Harfe, et al. 2005, Jamin, et al. 2002b).

Breeding studies

To assess fertility of these mice, female $Dicer1^{fl/fl}$;$Amhr2^{Cre/+}$ mice (42 d of age, n=8) were mated with adult wild-type males of known fertility. $Dicer1^{fl/fl}$;$Amhr2^{Cre/+}$ females were continually exposed to males for a minimum of 5 months. Female mice were checked daily for the presence of a seminal plug to confirm mating. To characterize the general morphology of the $Dicer1^{fl/fl}$;$Amhr2^{Cre/+}$ female reproductive tract, mice at several developmental ages, after natural mating or after a follicular stimulation protocol, were killed, and ovarian, oviductal and uterine function, and morphology were evaluated.

To assess ovarian and uterine function and examine fertility, adult littermate females (42 d of age) of $Dicer1^{fl/fl}$;$Amhr2^{Cre/+}$ and wild-type control (i.e.

Dicer1$^{fl/+}$;Amhr2$^{+/+}$ or Dicer1$^{fl/fl}$;Amhr2$^{+/+}$) genotypes were naturally mated, and killed on d 1, 3, 4, 6–7 post-coitus (d1=day seminal plug observed; n= 9, 6, 8, and 7 for wild-types, and n=8, 6, 8, and 6 for Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ on each respective day of pregnancy). Ovulation and fertilization rates were determined by counting the oocytes recovered from cumulus-oocyte complexes expressed from the oviducts of mice killed on d1 of pregnancy. After collection the fertilized embryos were cultured for 5 d as previously described to assess embryonic development (Biggers, et al. 2000, Summers, et al. 2005). Total body, ovarian, and uterine weights were recorded for d1 pregnant mice.

In vivo embryonic development, oviductal transport, and implantation rates were assessed on d3, 4, 6–7 pregnancy, respectively. Embryos collected on d3 and 4 pregnancy were classified for developmental stage as previously described (Biggers, et al. 2000, Summers, et al. 2005). Oviductal transport was assessed by determining the location of the embryos within the oviducts on d4. The oviducts and uterine horns were carefully bisected immediately below the utero-tubal junction, and the oviduct and uteri flushed independently into separate collection dishes. It was found that oviducts of Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ females could not be flushed, therefore, the oviducts were dissected along their entire length to release embryos. The embryos were counted, and the stage of development was recorded. On d6–7 pregnancy, the number of implantation sites was determined by injection of Chicago Sky Blue dye into the tail vein 1 min before euthanization (Paria, et al. 1993).

Ovarian, oviductal, and uterine tissues from all mice were fixed and embedded in paraffin or flash frozen for Western blot analysis. To examine uterine and ovarian function under controlled conditions, immature littermate females (22 d of age) of

Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ (n=6) and control wild-type (i.e. Dicer1$^{fl/+}$ or Dicer1$^{fl/fl}$ lacking the Amhr2$^{Cre/+}$; n=12) genotypes were administered 2 IU equine chorionic gonadotropin (eCG) for 46 h, followed by 2 IU human chorionic gonadotropin (hCG) for 16–17 h. Ovulation rates were determined by counting the oocytes recovered. Uterine and oviductal tissues were collected from the eCG plus hCG-treated mice, as well as from untreated immature d 10 and 26 female Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ and littermate control mice for Western blot analysis and histological analyses. Serum blood samples were obtained from all adult and treated immature (d 25) mice for subsequent determination of progesterone and estrogen concentrations by RIA (Terranova and Garza 1983).

Western blot analysis

Combined uterine and oviductal tissues (d 10) or pooled oviductal tissues alone (d 25 immature eCG plus hCG treated mice) were homogenized in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA). The resulting protein lysates were centrifuged at 16,000 g for 5 min to pellet the cellular membrane debris. Supernatants were transferred to new tubes and stored at 80 C until use. Protein samples (10µg), as determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA), were loaded onto 12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes using standard methods. Immunoblots were blocked with 5% milk solution and incubated overnight at 4 C with antibodies to β-catenin (BD Biosciences, San Jose, CA) and α-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, protein-antibody complexes were visualized using West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) following the manufacturer's protocol. ODs for the immunoblots were determined on a Gel-Pro Analyzer (Media Cybernetics, Inc., Bethesda, MD).

Histological analysis

Ovaries, oviducts, and uteri collected for histology were immediately fixed in either 4% paraformaldehyde or Bouin's solution, before embedding in paraffin. Sections from the midregion of the uterus, and serial tissue sections (8 μm) from ovarian and oviductal tissues were stained with hematoxylin and eosin.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 4;GraphPad Software Inc., San Diego, CA). Uterine and ovarian weights, ovulation rates, percentage of embryonic development, and progesterone levels were analyzed by the Student's t test.

4. Results

Female Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice (n=8) are infertile, as evidenced by the failure to produce offspring over a 5-month breeding study. The mating behavior of female Dicer1$^{fl/fl}$; Amhr2$^{Cre/+}$ mice appeared normal, as shown by the presence of seminal plugs after exposure to male mice. Moreover, several mice (not exposed to males) exhibited normal 4- to 5-d estrous cycles as detected by vaginal smears. Female wild-type mice used as controls (e.g. Dicer1$^{fl/fl}$ or $^{fl/+}$ lacking Amhr2$^{Cre/+)}$ showed evidence of a pending pregnancy within 14 d of male exposure, whereas Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ females failed to exhibit visible signs of pregnancy. Male Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice (n=5) were fertile, as evidenced by their ability to sire multiple litters with wild-type females.

To establish whether the cause of female infertility is related to a gross developmental defect, the reproductive tracts of untreated immature (d25) Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ and littermate control mice were examined (Fig. IX-1A). The length and diameter of the Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ uterine horn was remarkably shorter compared with control uteri. Similar to the uterus, the oviducts of Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice were truncated in length (less than one half the length), and both tissues appeared more transparent when observed through a dissecting microscope than corresponding tissues from control mice (compare Fig IX-1, B and C). In addition, sac-like structures were observed within the oviduct of Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice (Fig IX-1C). Similar oviductal and uterine morphological findings for the eCG plus hCG-treated immature (d 25) and for the adult d1 pregnant Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice were observed (data not shown). The uterine weights of the d1 pregnant Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice (1.61±0.20 mg/g; uterine weight/total body weight) were ($P<0.05$) reduced compared with age-

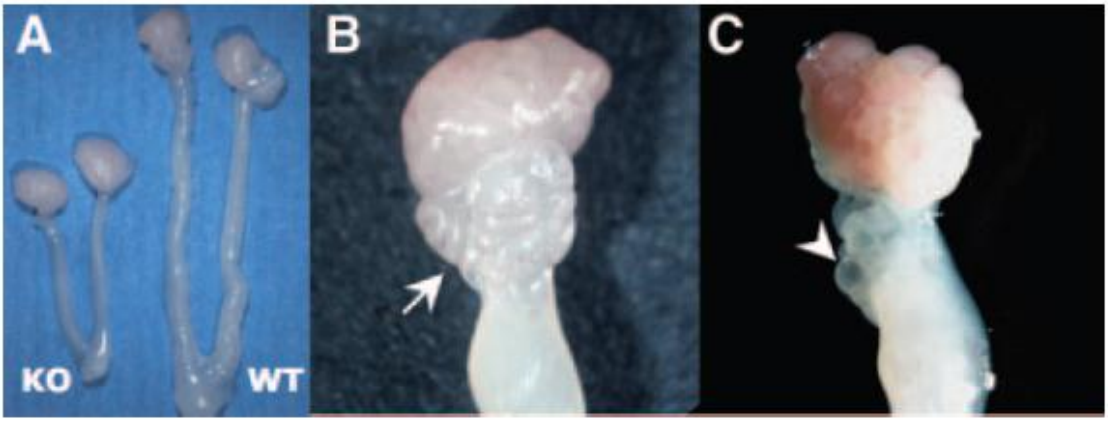
matched mice (5.00±0.28 mg/g). Again, distended sac-like structures filled with clear fluid (Fig. 1C) were concentrated at the end of the oviduct nearest the utero-tubal junction, in contrast to the typical mucosal folding characteristic of oviducts from control mice.

The oviducts of the immature and pregnant Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice were extremely fragile and did not appear patent, as evidenced by unsuccessful attempts to flush the oviduct. Oviducts ruptured easily; thus, to collect embryos, oviducts were manually dissected rather than flushed. However, naked oocytes/embryos could be visualized through the transparent wall of the oviduct, implicating that the oviduct was capable of early gamete collection and transport. In contrast, wild-type control mice were readily flushed and contained a large number (10 –15) of oocytes or embryos in immature and adult mice, respectively.

Figure IX-1. Morphological changes in the female reproductive tract of mice with anti-Mullerian hormone receptor-2-Cre recombinase targeted deletion of *Dicer1*.

A, Immature female reproductive tracts (magnification, x1.5) from wild-type (WT) and *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ [knockout (KO)] mice. The uteri of the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ mice are roughly two thirds the length of the wild-type mice. **B and C**, Magnified view (x6) of the oviduct and ovary from control (B) and *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ mice (C). Note the extensive coiling of the wild-type oviduct (*white arrow*) and the lack of such coiling in the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ knockout oviduct, and the presence of fluid-filled sacs near the utero-tubal junction (*white arrowhead*).

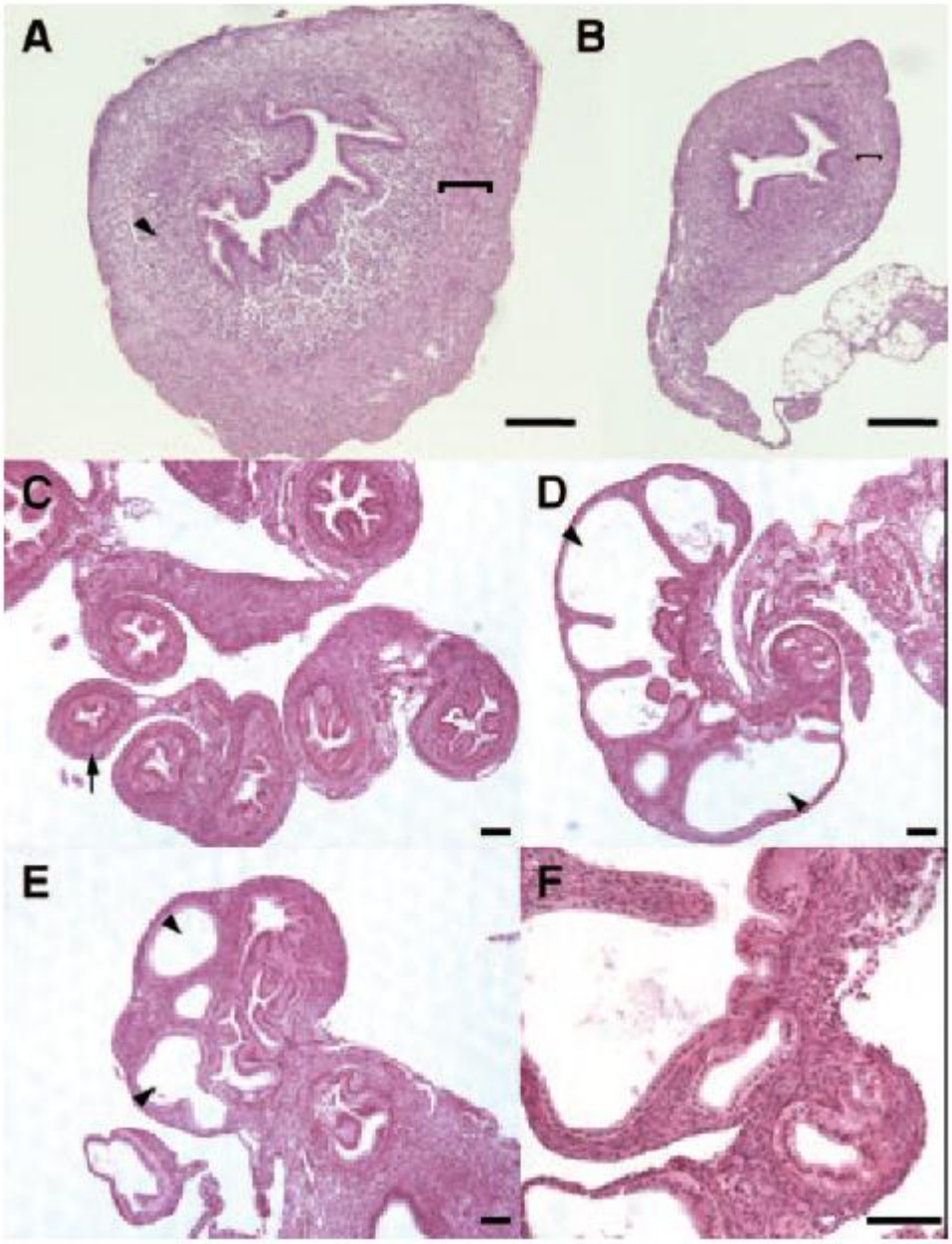
Figure IX-1



The uteri of Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice did not appear to lack primary cell types because uterine glands, and stromal and myometrial tissues all appeared to be present (Fig IX-2, A and B). However, the uteri of immature eCG plus hCG treated Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice did appear to have a thinner myometrial layer and reduced numbers of uterine glands than that observed in control littermates. Similar findings were observed for pregnant mice. However, the differences in uterine morphology and weights between the Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ and wild-type mice were not attributable to changes in progesterone (d1 pregnant mice; See Fig IX-5) or estrogen levels (eCG plus hCG treated mice; data not shown). Similarly, the isthmus of the oviduct, the portion with the greatest musculature, exhibited an almost complete loss of smooth muscle tissue, and the characteristic mucosal folds were replaced by distended sac-like structures (Fig. IX-2, C–E). Histological evaluation of the oviduct also showed a generally disorganized epithelial cell layer (Fig.IX-2F).

Figure IX-2. Histological analysis of the uterus and oviduct of immature Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ and littermate wild-type control mice. A and C, Uteri and oviduct of wild-type mouse. B and D–F, *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ uteri and oviductal tissue. Immature 25-d-old mice were sequentially administered eCG (46 h), followed by hCG (16 h) before tissue harvesting. Tissues were fixed in Bouin's solution, paraffin embedded, sectioned (8 _M), and hematoxylin and eosin stained. The uteri (B) of the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ were reduced in size and appeared to contain much less smooth muscle [lateral and transverse smooth muscle are marked with *brackets* when compared with the wild-type (A)]. Uterine glands were prevalent in the wild-type uteri (A) and sparse in the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ uteri (*arrowhead*). The oviducts of wild-type mice were highly coiled, and the smooth muscle around the isthmus region was well developed (C, *arrow*). Similar regions in the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ were not observed because this region of the oviduct was populated with sac-like structures. D shows a region of the oviduct that is transitioning from a tubule-like structure to the sac-like structures seen more anterior in E, which resided near the utero-tubal junction; *arrowheads* denote several of the saclike structures. F shows a magnified view of epithelium from *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ mice. *Bars*, 500 μm for A and B. *Bars*, 200 μm for C–F.

Figure IX.2



In addition to the gross morphological changes in the uterus and oviduct, ovarian weight was also reduced ($P < 0.05$) in the d1 pregnant Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ (0.22±0.009 mg/g; ovarian weight/total body weight) mouse compared with control mice (0.27±0.014 mg/g). Moreover, numbers of naturally ovulated cumulus-oocyte complexes were less ($P<0.05$) in the Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice (7.0±1.1; mean±sem) than control mice (10.7±0.9). Similar to the naturally mated animals, the ovulation rate for the immature eCG plus hCG-treated wild-type females (n=12) was significantly greater ($P<0.05$) than that of Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ females [(n=6) 16.2±1.4 vs. 3.67±1.50, respectively]. However, gross morphological and histological examination of the pregnant ovaries from both genotypes showed no marked differences (data not shown). However, examination of immature Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice given eCG alone (46 h) indicated that these ovaries contained fewer large antral follicles (data not shown). Overall, the reduced ovulation rates and ovarian weights, as well as histological observations, suggest that fewer pre-ovulatory follicles developed in the Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$. However, in depth analyses of follicular dynamics and characterization of Dicer1 will be necessary to further address ovarian Dicer1 function.

Culture of the d1 (pronuclear stage) embryos indicated that embryos from Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice were capable of normal in vitro development (Table 1X-1). Embryonic development through blastocyst formation and the percentage of hatching was not different across genotypes. In contrast, *in vivo* embryonic development assessed on d 3 pregnancy indicated that embryos from Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ females were markedly delayed when compared with wild-type mice (Fig.1X-3 and Table1X-2). Moreover, the incidence of fragmentation and degeneration of these embryos increased in

Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ derived embryos after 1d culture compared with wild-type derived embryos (Fig. 3). Embryos collected from Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice on d4 pregnancy also displayed increased fragmentation and degeneration (data not shown).

To further evaluate oviductal function, the location of embryos within the reproductive tract was examined on d4 of pregnancy. All embryos in wild-type females (n=9) were located in the uterus, whereas no embryos were found in the uterus of Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice (n=8; Table1X-3). The majority of the embryos within the oviduct of Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice were found in the upper one third of the oviduct. The few embryos that had progressed through the oviduct to the isthmus were mostly fragmented, and some zonae pellucidae had been lost (data not shown). Implantation was assessed in another group of mice at d6 and 7 of pregnancy. All wild-type mice exhibited implantation sites (9.8±0.4 implantation sites per dam), whereas no evidence of implantation was observed in Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice.

Table IX-1. In vitro embryonic development of embryos collected from d1 pregnant Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ and wild-type females.

	Day of embryo culture				
	2	3	4	5	
Stage of embryo development	≥2 cells	≥4 cells	≥ Compacting morula	≥ Blastocyst	≥ Hatching blastocyst
Genotype of donor female (n = embryos; dams)					
Wild-type (n = 75; 9)	100%	96%	90.7%	86.7%	54.7%
Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ (n = 37; 7)	97%	86%	91.7%	72.2%	38.9%

Percentage of two or more cell embryos was calculated from total numbers of fertilized oocytes (n = 75 and 37) for each genotype. Percentages of four or more cell embryos and all subsequent groups were calculated from the number of two or more cell embryos present on d 2 culture.

Figure IX.3. Embryos from d3 pregnant *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ and wild type female mice. A and C, Embryos from wild-type mice immediately after collection (A) and after 24 h culture (C). B and D, Embryos from *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ mice immediately after collection (B) and after 24 h culture (D). Increased fragmentation and degeneration were observed in embryos collected from *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ females compared with wild-type controls after 24 h culture. *Bars*, 100 µm.

Figure IX.3

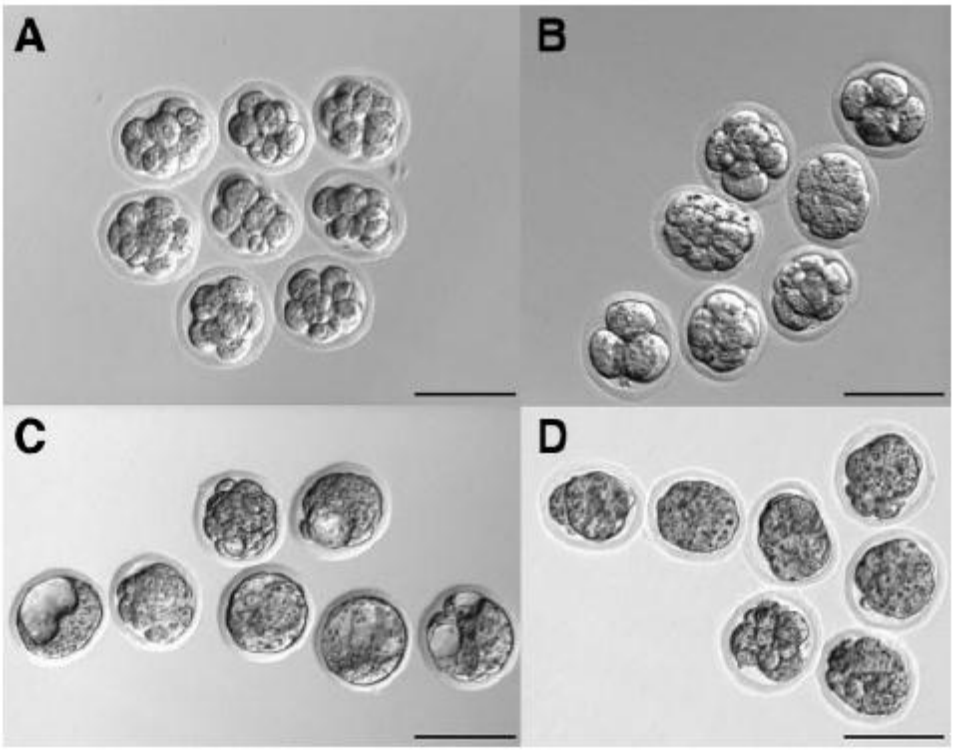


Table IX.2. In vivo embryonic development in d3 pregnant *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ and wild type female mice

Genotype	Embryonic stage			
	1 cell	4–6 cells	8 cells	>8 cells
Wild-type (n = 7)	0	0	54	7
Dicer1$^{fl/fl}$;*Amhr2*$^{Cre/+}$ (n = 8)	14	8	25	0

Numbers of embryos found in each region of oviduct and uterus from a wild-type (n = 7) and *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ (n 8) mice.

Table IX.3. Location of embryos on d4 of pregnancy in *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ and wild type female mice.

Genotype	Oviduct				Uterus
	Upper	Mid	Lower	Total	
Wild-type (n = 9)	0	0	0	0	91
Dicer1$^{fl/fl}$;*Amhr2*$^{Cre/+}$ (n = 9)	43	13	7	63	0

Numbers of embryos found in each region of oviduct and uterus from a total of nine mice of each genotype.

Serum progesterone concentrations indicated that Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ and control mice had similar levels through d4 of pregnancy (Fig 1X-4). On d6 of pregnancy, Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice exhibited a ($P<0.05$) minor (24%) decline in progesterone levels compared with the controls. In a single embryo transfer experiment, in vitro fertilized oocytes derived from Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice were able to establish pregnancy in recipient females with normal fetal development to at least embryonic d15 (E15) (data not shown).

To determine whether the loss of Dicer1 affects Wnt signaling, we examined β-catenin expression. We observed reduced levels of β-catenin in combined uterine/oviductal tissues of immature d10 Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice compared with wild-type mice (Fig.1X-5). In addition, oviductal tissues from d26 eCG plus hCG-treated Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ mice also showed a loss in β-catenin protein expression. β-catenin levels were 53 and 31% lower in the Dicer1 mice on d10 and 26, respectively, after normalization with the loading control, α-actin.

Figure IX-4. Serum progesterone concentrations in pregnant *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ (KO) and wild-type (WT) mice. Serum collected on d1, 3, 4, and 6 of pregnancy was analyzed for the concentration of progesterone by RIA. Data are presented4 as mean concentration of progesterone±SEM.

Figure IX.4

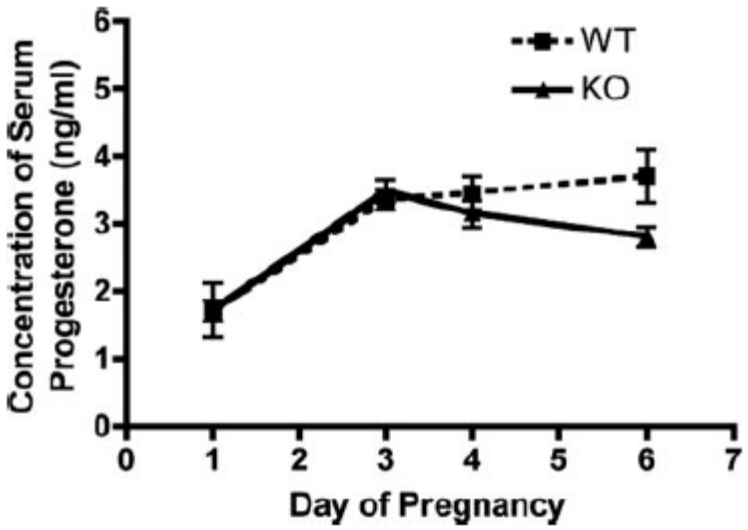
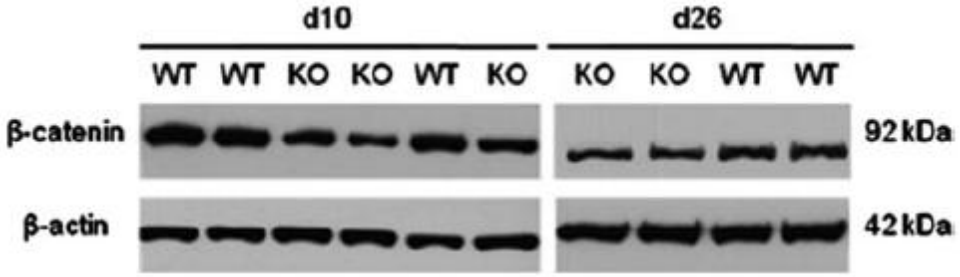

Concentration of Serum Progesterone (ng/ml)
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Day of Pregnancy
WT
KO

Figure IX-5. Oviductal and uterine β-catenin levels in *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ (KO) and wild-type (WT) female mice. Oviductal and uterine tissue pooled together from d10 or oviductal tissue alone from d26 mice was analyzed by Western blot for β-catenin protein levels .α-Actin was used as a loading control.

Figure IX-5


d10
d26
WT WT KO KO WT KO
KO KO WT WT
β-catenin
92kDa
β-actin
42kDa

5. Discussion

Conditional knockdown of the miRNA-processing enzyme, Dicer1, using the Amhr2-driven Cre-recombinase yielded female mice sterile, whereas having no effect on male fertility. A previous study using a similar approach to delete Dicer1 specifically in the oocyte via ZP3-driven Cre-recombinase, also rendered female mice infertile (Murchison, et al. 2007). In these later mice, oocyte development and folliculogenesis appeared normal until the resumption of meiosis, at which point defects in spindle formation and chromatin separation in the oocytes were observed. Female mice were also found to be infertile in a recent study in which Dicer1 expression was globally knocked down using a gene-trap method (Otsuka, et al. 2008). In this study a defect in vascularization of luteal tissue was observed, leading to insufficient progesterone secretion and a failure to maintain pregnancy. Global knockdown of Dicer1 had no effect on ovulation rate, suggesting that granulosa cell function was not compromised in their model (Otsuka, et al. 2008). Conversely, granulosa cell gene expression is modulated in the Amhr2-Cre recombinase model (Boerboom, et al. 2005, Boerboom, et al. 2006, Deutscher and Hung-Chang Yao 2007, Jamin, et al. 2002a, Jeyasuria, et al. 2004, Jorgez, et al. 2004), and we observed reduced ovulation rates and pre-ovulatory follicular development in our mice. Nevertheless, the infertile phenotype we observed in $Dicer1^{fl/fl}$;$Amhr2^{Cre/+}$ could not be attributed to reduced luteal function because progesterone levels were similar through the early stages of embryonic development (d4) and only showed a minor decline on d6 pregnancy. Our data instead point to a loss in oviductal development and function as the primary cause of Dicer1 mediated infertility in our model.

The marked gross morphological changes in the uterus and oviduct observed in our model of Dicer1 deletion suggest that either disruption of tissue development and/or function leads to female infertility. The Amhr2 promoter driving Cre recombinase expression has been used extensively to knock down gene expression in tissues derived from the Mullerian duct (i.e. oviductal, uterine, and cervical tissues) (Deutscher and Hung-Chang Yao 2007, Jamin, et al. 2002a). The Amhr2-driven Cre-recombinase activity has been detected in the Mullerian duct mesenchyme as early as E12.5 in ROSA reporter mice (Jamin, et al. 2002a) and as late as E15.5 in mice using a fluorescent reporter (Deutscher and Hung-Chang Yao 2007). Moreover, the mesenchymal expression of the fluorescent reporter was also not uniform within the embryonic Mullerian duct (Arango, et al. 2005, Deutscher and Hung-Chang Yao 2007). Furthermore, postnatal Amhr2-lacZ and Amhr2-Cre expression was also greater in the circular smooth muscle cells of the myometrium (Arango, et al. 2008, Arango, et al. 2005). However, histological observations of the uteri and oviduct from $Dicer1^{fl/fl};Amhr2^{Cre/+}$ mice suggest that the cellular layers normally comprising these tissues are present at decreased levels compared with wild-type mice.

This was particularly true for the smooth muscle that is present in the isthmus of the oviduct and myometrium. Indeed, the isthmus region of the oviduct was not readily identifiable in the $Dicer1^{fl/fl};Amhr2^{Cre/+}$ female mice. Therefore, the loss of smooth musculature in these tissues is consistent with elevated Amhr2 expression. Consistent with the loss and disruption of oviductal cell layers, the oviduct of the $Dicer1^{fl/fl};Amhr2^{Cre/+}$ mouse was not able to support normal embryonic development, nor was it able to facilitate transport of embryos to the uterus. The ability of in vitro cultured

pronuclear (d1) embryos collected from Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ donors to develop at a similar rate as those derived from wild-type females offers further support that oviductal function is disrupted. Finally, the ability of in vitro fertilized oocytes derived from Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ donor mice to establish a pregnancy when transferred to wild-type recipients provides conclusive proof that the ovary is not the primary cause of infertility in this mouse.

Collectively, these observations suggest that Dicer1 and its product (miRNA) play key roles in uterine and oviductal development. Disruptions of oviductal and uterine morphology have previously been seen in mice with deletions of the homeobox genes, Hoxa9, 10, 11, and 13, as well as genes in the Wnt pathway, including Wnt-7a and β-catenin (Branford, et al. 2000, Miller and Sassoon 1998, Post and Innis 1999). The loss of uterine musculature, lack of uterine glands, and failure of the oviduct to undergo coiling phenocopies some of the observations seen when Wnt-7a was knocked out (Carta and Sassoon 2004, Miller, et al. 1998, Miller and Sassoon 1998) or when -catenin was knocked out using Amhr2-Cre (Arango, et al. 2005, Deutscher and Hung-Chang Yao 2007). However, both Wnt-7a and β-catenin had additional reproductive tract phenotypes that were not mimicked by the deletion of Dicer1. In these models, combined uterine and oviductal disruptions have been shown to occur mostly in early development. To determine whether the loss of Dicer1 might lead to disrupted Wnt signaling, immunoblot analysis of oviductal tissues from age-matched d25 immature mice, and combined uterine/oviductal tissues from 10d old mice were analyzed. β-catenin levels were lower in the Dicer1$^{fl/fl}$;Amhr2$^{Cre/+}$ uteri and oviducts. Ongoing studies are examining whether the loss of miRNA is affecting the posttranscriptional gene regulation

of this specific transcription factor within developing reproductive tracts. In conclusion, these studies indicate that posttranscriptional gene regulation in somatic tissues of the female reproductive system as regulated by Dicer1, and its product, miRNA, plays an essential role in female fertility. The loss of Dicer1 resulted in developmental and functional consequences at both the reproductive tract and gonadal level. Ongoing studies are underway to identify the specific miRNA and their target genes that affect both the development and function of these tissues.

X: CHAPTER 5

Conditional deletion of the LH regulated miR-132/212 in granulosa cells does not affect female fertility

1. Abstract

Our laboratory has recently identified two miRNA, miR-212 and miR-132, which are highly induced by the luteinizing hormone (LH) surge in the ovary. These miRNA are co-transcribed and share an identical seed sequence that targets the 3'UTR of complementary mRNAs. Furthermore, these miRNA are regulated by cAMP/CREB, the primary mechanism of LH signaling in the ovary. Interestingly, miR-132 and miR-212 have been shown to amplify CREB signaling (a downstream component of cAMP activation) in a feed-forward mechanism in neuronal cells. Thus, we have hypothesized that miR-132 and miR-212 might be involved in mediation of cAMP/CREB signaling in ovarian somatic cells. The aim of this study was to determine if miR-212/132 are involved in cAMP/CREB signaling and ovarian function. Bioinformatic analysis of potential targets of miR-132/212 (Targetscan) revealed a number of members involved in the regulation of Raf signaling, a key component in CREB signaling, including: SMAD4, RASA1 (RAS p21 protein activator 1), SPRED1 (sprout-related, EVH1 domain-containing protein 1), DACH1 (dachshund homolog 1), ERBIN (ERBB2 interacting protein), APC (adenomatous polyposis coli), and SLK (STE20-like kinase). Conditional deletion of miR-212/132 in ovarian granulosa cells using the Amhr2-Cre and Aromatase-Cre promoters did not appear to have an effect on ovarian function or female fertility. Based on these findings, we believe that even though miR-212/132 are highly induced by LH, they do not appear to play a critical or non-redundant role in ovarian function, specifically ovulation.

2. Introduction

Each reproductive cycle, an ovulatory dose of luteinizing hormone (LH) initiates several ovarian events, including: ovulation, luteinization, cumulus cell expansion, and oocyte maturation. These events are critical and necessary for the proper release of the mature egg cell from the ovary, fertilization, and maintenance of pregnancy. Understanding the cellular events and associated regulatory mechanisms involved in mediation of the LH surge provides potential opportunities for regulation of the reproductive cycle and new methods of contraception.

LH is released from the anterior pituitary gland and binds to the transmembrane, G-protein coupled LH receptor on thecal and granulosa cells of ovarian follicles. Binding of LH causes a rapid increase in intracellular levels of the second messenger cyclic-AMP (cAMP), which in turn activates a number of cellular signaling cascades, including the PKA system (Richards 2001). These signaling cascades in turn regulate transcription of genes essential for ovulation, luteinization, and other processes, as well as regulation of post-transcriptional regulatory events, such as microRNA (miRNA) expression. MicroRNAs are a recently identified class of small, non-coding RNAs that regulate post-transcriptional gene expression through inhibition of translation or mRNA degradation by complementary pairing with the 3'-untranslated region (3'UTR) of the target mRNA (Filipowicz, et al. 2008, Lee and Ambros 2001). Our laboratory, and others, have recently reported that decreased global expression of miRNA specifically in ovarian granulosa cells results in a dramatic decrease in ovulation rate in mice (Hong, et al. 2008, Nagaraja, et al. 2008, Pastorelli, et al. 2009). Furthermore, our laboratory has recently identified 3 miRNA (miR-212, miR-132, miR-21) that are rapidly increased in

response to the LH surge (Fiedler, et al. 2008). Inhibition of miR-21 function in granulosa cells blocked ovulation and increased cellular apoptosis (Carletti, et al. 2010). Further studies of miRs-132 and 212 found a rapid and robust increase in expression of these transcripts increasing 17- and 21-fold, respectively, within 2 hours of hCG administration (Fiedler, et al. 2008). Our laboratory has also found that bovine granulosa cells collected from peri-ovulatory follicles 16hrs post-hCG administration exhibited ~300 and ~90-fold induction of miR-132 and miR-212 expression, respectively, compared to 0hr hCG (unpublished data) indicating that this increase in miR-212/132 expression occurs in a possibly conserved manner in species other than the mouse. Additionally, we have previously reported the rapid increase of miR-132 and miR-212 in response to cAMP in granulosa cells *in vivo* (Fiedler, et al. 2008). Taken together, these studies suggest that LH regulates miRNA expression and that this may play a critical role in mediation of LH signaling in the ovary. Recently, studies in neuronal cells have described that miR-212 and miR-132 increase cAMP signaling via a feed-forward mechanism (Hollander, et al. 2010, Im, et al. 2010, Magill, et al. 2010, Remenyi, et al. 2010). Interestingly, miR-212 and miR-132 are co-transcribed from a single promoter to form a single primary transcript. This transcript is then further processed to form individual, mature miR-132 and miR-212. Furthermore, mature miR-132 and miR-212 share an identical seed sequence, or the region of the mature miRNA that binds to the complementary sequence of the 3'UTR of the target mRNA. Taken together, this suggests similar functional roles for miR-132 and miR-212 in regulation of post-transcriptional gene expression. Our laboratory has previously shown a rapid increase in miR-212 and 132 following LH, however the mechanism or downstream function of

these miRNA have yet to be explored. Thus, we hypothesized that miRs-212 and 132 are involved in a feed forward loop of LH/cAMP signaling. The aim of this study was to determine if miR212 and 132 are involved in cAMP/CREB signaling in the ovary and to determine if miR-212 and 132 have a functional role in ovulation/luteinization and overall ovarian function.

3. Methods

In vitro miR212/132 inhibition

Granulosa cells were collected and subsequently cultured from unstimulated, immature CF-1 mice (d25) as previously described (Carletti, et al. 2010). Briefly, 25 day old mice were sacrificed by cervical dislocation and ovaries removed and placed in M199 collection media (Sigma, St. Louis). Ovaries were subsequently treated with a 0.5M sucrose solution for 15 mins, rinsed with collection media, and granulosa cells collected by puncturing large, antral follicles with a 26 gauge insulin needle. The resulting cellular material was centrifuged at 1,000xg for 5 min, counted for number of live and dead cells, and plated at 2x10^6 cells per 10cm dish in granulosa cell culture media (DMEM:F12 (Sigma) with 10% fetal bovine serum (Sigma) and 50ng/ml gentamycin (Invitrogen, Carlsbad, CA)). Twenty four hours after plating, media was changed to fresh culture media and 24hrs later cells exposed to serum free DMEM:F12 media. After 24hrs of serum starvation, cells were transfected with Lipofectamine 2000 (Invitrogen) and locked nucleic acids (LNAs, Exiqon, Norway) specific to miR-212 (LNA-212) and miR-132 (LNA-132) or a non-specific control (LNA-NS). Cells were exposed to transfection complexes for 4 hrs and then treated with 1mM 8-Br-cAMP (Sigma) for 1, 2, 12, or 24hrs. Cells were collected in cell lysis buffer for protein isolation.

Bioinformatic analysis for miR212/132 targets

To identify putative targets of miR212/132 several bioinformatic approaches were used. The miRGator functional analysis online tool (http://genome.ewha.ac.kr/miRGator/, (Nam, et al. 2008)) was used to predict gene

ontology classes enriched for putative miR212/132 targets. Specific putative targets were obtained by using the Targetscan algorithm (www.targetscan.com (Lewis, et al. 2005)) with focus on targets important to ovarian function (Luense, et al. 2011).

Generation of conditional miR212/132 knockdown

The University of Kansas Medical Center's Institutional Animal Care and Use Committee approved all procedures involving mice before use. Mice homozygous for loxP insertions flanking the miR212/132 gene ($miR212/132^{fl/fl}$; generously provided by Dr. Richard Goodman, Oregon Health and Science Center) were crossed with mice heterozygous for Cre recombinase knocked into the anti-mullerian hormone receptor 2 locus ($Amhr2^{Cre/+}$; generously donated by Dr. Richard Behringer, Baylor College of Medicine) or mice expressing Cre recombinase driven by the Cyp19 (aromatase) promoter ($Cyp19^{Cre/+}$; generously donated by Dr. Jan Gossen, Schering Plough Corporation). The Amhr2-Cre promoter is expressed in ovarian granulosa cells of the pre-antral follicle and other derivatives of the Mullerian duct (i.e. oviduct, uterus, and cervix) (Hong, et al. 2008, Jorgez, et al. 2004), while Cyp19-Cre expression is restricted to granulosa cells of antral follicles and the corpus luteum as evidenced by breeding of Cyp19-Cre mice to the Rosa26 reporter line (Fan, et al. 2008a, Fan, et al. 2008b). The resulting progeny were genotyped as previously described (Fan, et al. 2008b, Jorgez, et al. 2004, Magill, et al. 2010).

Validation of miR212/132 knock-down

To validate that miR-212 and 132 were deleted from granulosa cells of conditional knock-down mice, immature mice (d22) were stimulated with 2 IU of eCG

(Calbiochem, Billerica, MA) for 48hrs. Mice were sacrificed by cervical dislocation and ovaries removed and placed in Dulbecco's PBS (Sigma). Granulosa cells were expressed from the ovaries by inserting a needle into antral follicles and releasing the cells as previously described (Carletti, et al. 2010). To remove any oocytes, the resulting cellular material was passed over a filter and subsequently centrifuged at 1,000xg to pellet any cellular material. Total RNA was isolated from the resulting cellular pellet by TRIZOL extraction (Sigma) per manufacturer's protocol. To synthesis cDNA, 50ng of total RNA was reverse transcribed using the miRCURY Universal RT Kit (Exiqon, Denmark) as described by the manufacture. Quantitative RT-PCR was performed using the miRCURY LNA primer assays for miR-212 and miR-132 in combination with the SybrGreen amplification kit (Exiqon). The small RNA U6 was used as a normalizer (Exiqon).

Breeding studies

To assess fertility of these mice, female miR212/132$^{fl/fl}$;Amhr2$^{Cre/+}$ mice and miR212/132$^{fl/fl}$;Cyp19$^{Cre/+}$ (42 d of age, n=2 and n=5, respectively) were mated with adult wild-type males of known fertility. miR212/132$^{fl/fl}$;Amhr2$^{Cre/+}$ and miR212/132$^{fl/fl}$;Cyp19$^{Cre/+}$ females were continually exposed to males for a minimum of 2 months. Female mice were checked daily for the presence of a seminal plug to confirm mating.

Ovulation studies

To determine if mice lacking the miR212/132 gene were capable of ovulation, immature littermate females (22 d of age) of miR212/132$^{fl/fl}$;Amhr2$^{Cre/+}$ (n=6) and control

wild-type (i.e. miR212/132$^{fl/+}$ or miR212/132$^{fl/fl}$ lacking the Amhr2$^{Cre/+}$; n=5) genotypes or miR212/132$^{fl/fl}$;Cyp19$^{Cre/+}$ (n=6) and control wild-types (n=6) were administered 2 IU equine chorionic gonadotropin (eCG) for 46 h, followed by 2 IU human chorionic gonadotropin (hCG, Sigma) for 16–17 h. Animals were euthanized by cervical dislocation followed by collection of cumulus-ooycte-complexes from the oviduct. Ovulation rates were determined by counting the oocytes recovered.

Western blot analysis

Granulosa cells from LNA treated cell culture were collected in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) and the resulting protein lysates were centrifuged at 16,000x g for 5 min to pellet the cellular membrane debris. Supernatants were transferred to new tubes and stored at 80 C until use. Protein samples (10µg), as determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA), were loaded onto 12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes using standard methods. Immunoblots were blocked with 5% milk solution and incubated overnight at 4 C with antibodies to phospho-CREB (Millipore) and α-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, protein-antibody complexes were visualized using West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) following the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 4;GraphPad Software Inc., San Diego, CA). Litter size and ovulation rates were analyzed by the Student's t-test. P values less than 0.05 were considered significant.

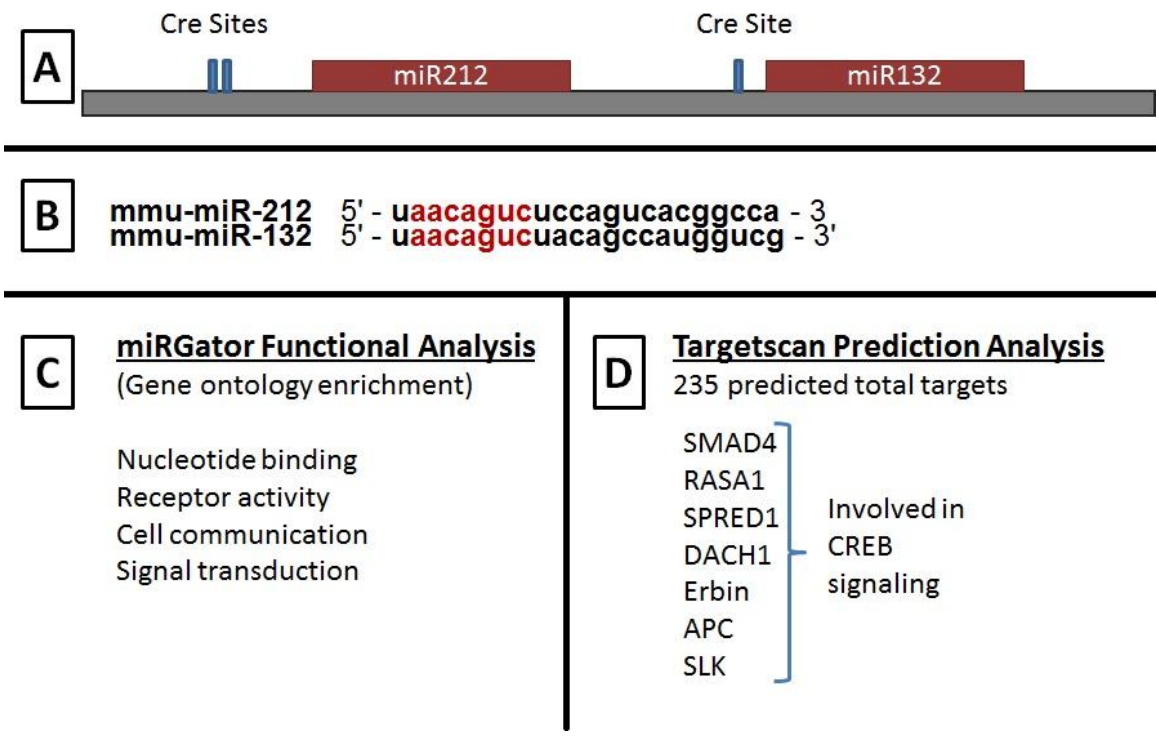
4. Results

Bioinformatic analysis of miR-212/132 targets

To identify putative mRNA targets of miR-212/132, two different bioinformatics approaches were utilized. First, miRGator, an online bioinformatic tool to categorize and determine enrichment of gene ontologies represented in putative mRNA targets, was utilized. Gene ontologies that were most represented in putative miR212/132 targets included nucleotide binding, receptor activity, cell communication, and signal transduction (Figure X-1C). Next, individual targets of miR-212/132 were obtained from the miRNA target prediction algorithm Targetscan and analyzed for targets of interest. A total of 235 mRNAs were predicted to be targeted by miR-212/132 (Figure X-1D). Of these targets, several appeared to be involved in cAMP/CREB signaling (SMAD4, RASA1 (RAS p21 protein activator 1), SPRED1 (sprout-related, EVH1 domain-containing protein 1), DACH1 (dachshund homolog 1), ERBIN (ERBB2 interacting protein), APC (adenomatous polyposis coli), and SLK (STE20-like kinase), the key signaling pathway involved in transcription of miR-212/132.

FigureX-1. Structure and putative function of miR212/132. **A**) Schematic representation of miR212/132 genomic locus. Cre sites represent location of cAMP response binding elements. **B)** Seed sequences of miR212 and miR132. Bases colored in red represent the seed sequence and are essential for miRNA:mRNA targeting. **C)** Gene ontology functional classes enriched in miR212/132 targets using the miRGator functional analysis tool. **D)** Putative miR212/132 targets in the CREB signaling pathway using Targetscan algorithm.

Figure X-1

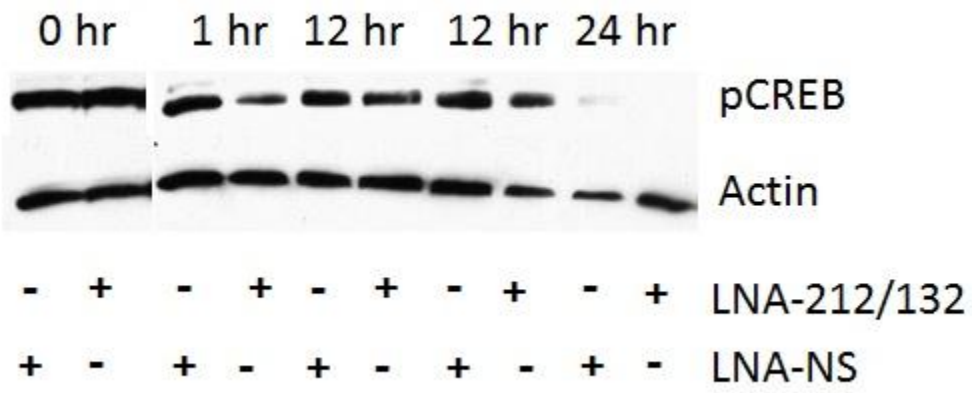

A
Cre Sites
Cre Site
miR212
miR132
B
mmu-miR-212 5' - uaacagucuccagucacggcca - 3
mmu-miR-132 5' - uaacagucuacagccauggucg - 3'
C
miRGator Functional Analysis
(Gene ontology enrichment)
Nucleotide binding
Receptor activity
Cell communication
Signal transduction
D
Targetscan Prediction Analysis
235 predicted total targets
SMAD4
RASA1
SPRED1
DACH1
Erbin
APC
SLK
Involved in
CREB
signaling

Inhibition of miR212/132 in vitro *appears to decrease CREB phosphorylation*

As phosphorylation of CREB is the key component in initiation transcriptional regulation in response to LH/cAMP/CREB signaling, levels of phosphor-CREB were measured in granulosa cells with miR-212/132 inhibited by locked-nucleic acid molecules (LNAs). No differences were observed in CREB phosphorylation at 0 hours, however by 1hr phospho-CREB levels appeared to decrease compared to non-specific treated control cells (Figure X-2). CREB phosphorylation appeared to remain low at 12hr compared to non-specific treated controls.

Figure X-2. Phospho-CREB protein levels in ovarian granulosa cells. Western blot of granulosa cell protein extracts transfected with LNA-212 and 132 or LNA-NS probed for phospho-CREB (pCREB) or actin. The time above each set of lanes represents the length of 8-Br-cAMP treatment after 4hr transfection of LNA.

Figure X-2

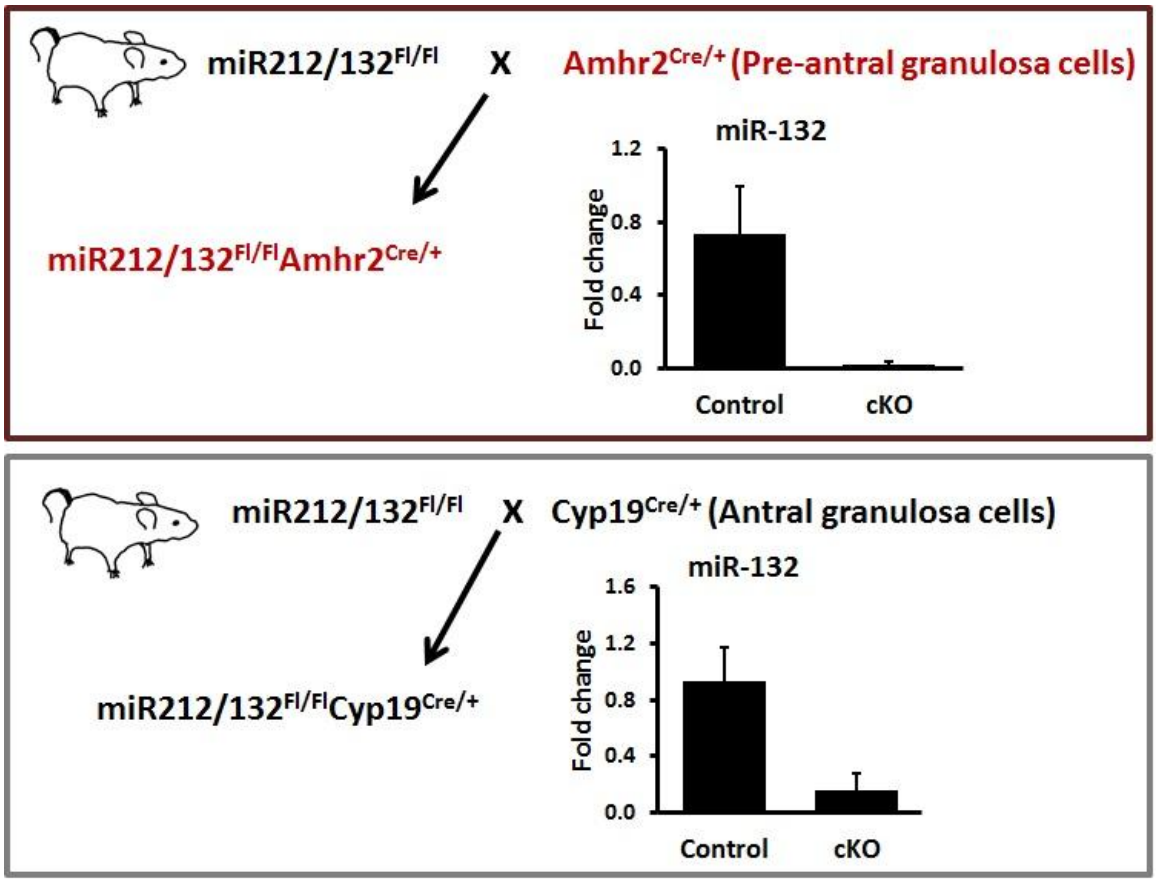


Generation of miR-212/132 conditional knock-out mice

To determine if miR-212/132 is necessary for fertility, two conditional knock out mouse lines were generated to knock-out miR-212/132 at two different points of ovarian function. Deletion of miR-212/132 with the Amhr2-Cre promoter decreased expression of miR-132 in conditional knock-out (cKO) mice compared to wild-type mice (Figure X 3A). However, expression of miR-212 was non-detectable in both cKO and control mice. Similar deletion of miR-212/132 with the Cyp19-Cre promoter resulted in nearly non-detectable levels of miR-132 in cKO mice (Figure X.3B). Expression of miR-212 was also non-detectable in both cKO and control mice.

Figure X-3. Experimental design and validation of generation of conditional miR212/132 knock-out mice. Mice with loxP insertions flanking the miR212/132 gene were crossed with Cre expression promoters for Amhr2 (n=2 per genotype) (**A)** and Cyp19 (n=5 per genotype) **(B)** to generate cell specific conditional knock-out (cKO) of miR212/132. Bar graphs represent expression levels of miR-132 in granulosa cells collected from mice 48hr after eCG stimulation. Levels of miR-212 were not detectable in control or cKO mice.

Figure X-3

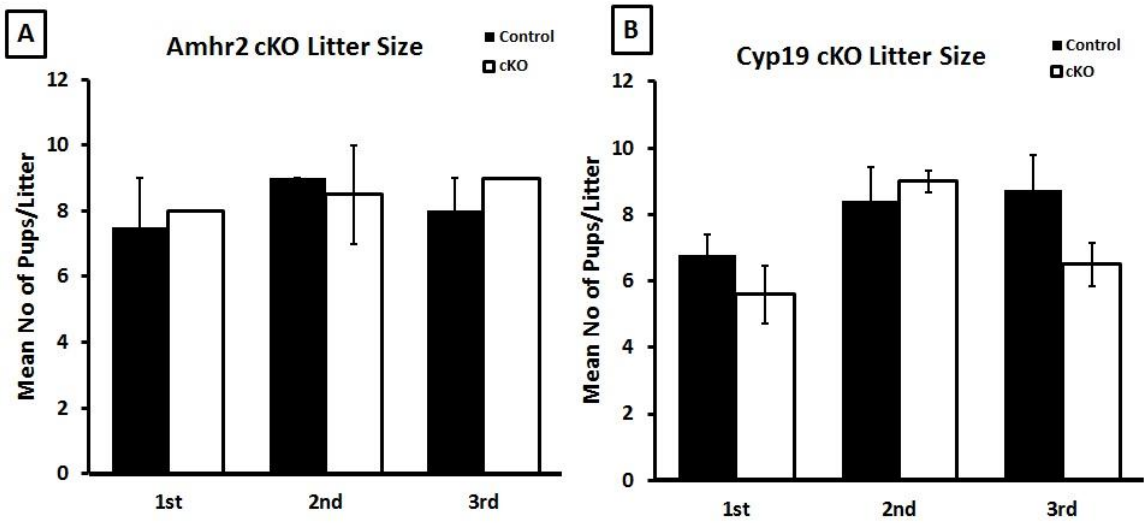

miR212/132Fl/Fl X Amhr2Cre/+ (Pre-antral granulosa cells)
miR212/132Fl/FlAmhr2Cre/+
miR-132
Fold change
1.2
0.8
0.4
0.0
Control
cKO
miR212/132Fl/Fl X Cyp19Cre/+ (Antral granulosa cells)
miR212/132Fl/FlCyp19Cre/+
miR-132
Fold change
1.6
1.2
0.8
0.4
0.0
Control
cKO

miR-212/132 conditional knock-out mice are fertile

Amhr2-cKO and Cyp19-cKO mice bred to males of known fertility appeared to normal mating behavior, as evidenced by the presence of a copulatory vaginal plug. All Amhr2-cKO (Figure X-4A) and Cyp19-cKO (Figure X-4B) mice delivered live pups that were of same size and approximate weight of control mice. There were no obvious physical defects or problems with pups from cKO dams and both male and female pups were present.

Figure X-4. Conditional knock-out of miR212/132 does not affect fertility. Mean size of litters from female conditional knock out mice (cKO) using the Amhr2-Cre promoter (n=2 per genotype) **(A)** or Cyp19-Cre promoter (n=5 per genotype) **(B)** compared to wild type (Control) mice. The number under the X axis represents the 1st and subsequent litter number.

Figure X-4

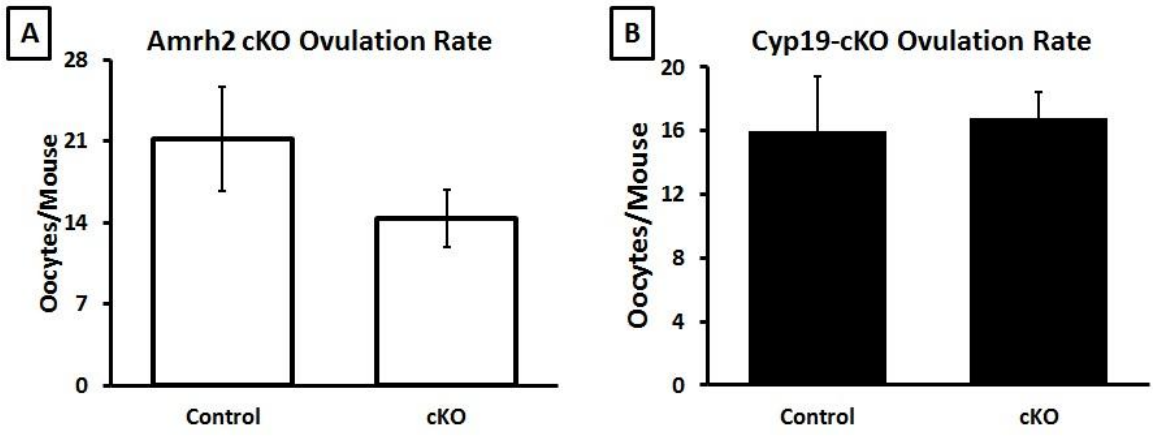

A
Amhr2 cKO Litter Size
Control
cKO
Mean No of Pups/Litter
12
10
8
6
4
2
0
1st
2nd
3rd
B
Cyp19 cKO Litter Size
Control
cKO
Mean No of Pups/Litter
12
10
8
6
4
2
0
1st
2nd
3rd

miR-212/132 conditional knock-out mice undergo normal ovulation

Amhr2-cKO and Cyp19-cKO mice stimulated with a low dose of eCG and hCG underwent ovulation on a normal timeframe, as evidenced by the presence of cumulus-oocyte-complexes in the oviduct 16hrs post-hCG. The mean number of oocytes collected from Amhr2-cKO (Figure X-5A) and Arom-cKO (Figure X-5B) mice were the same as collected from wild-type littermate controls.

Figure X-5. Conditional knock-out of miR212/132 does not affect ovulation rate. Mean number of oocytes retrieved from oviduct of female conditional knock out mice (cKO) using the Amhr2-Cre promoter (n=6 per genotype) **(A)** or Cyp19-Cre promoter (n=6 per genotype) **(B)** compared to wild type (Control) mice.

Figure X-5


A
Amrh2 cKO Ovulation Rate
Oocytes/Mouse
28
21
14
7
0
Control
cKO
B
Cyp19-cKO Ovulation Rate
Oocytes/Mouse
20
16
12
8
4
0
Control
cKO

5. Discussion

Bioinformatic analysis of putative targets of the LH regulated miR-212/132 reveals several proteins associated with CREB signaling. As CREB signaling is a major downstream effector of LH/cAMP signaling in ovarian granulosa cells, and due to the reports of several other groups that miR-212/132 appeared to form a feed forward loop to amplify CREB signaling, (Hollander, et al. 2010, Im, et al. 2010, Magill, et al. 2010, Nudelman, et al. 2010) we hypothesized that miR-212/132 was involved in cAMP/CREB signaling mediation and that it was involved in ovarian function (i.e. ovulation/luteinization). However, conditional deletion of miR212/132 in ovarian granulosa cells appears to have no effect on female fertility or ovulation rate.

Due to the rapid increase in miR-212/132 expression in response to LH in granulosa cells, (Fiedler, et al. 2008), their co-transcription as one primary-miRNA, and the sharing of a seed sequence, we anticipated they played a functional role in ovarian biology. Functional analysis of miR-21, another miRNA found to be highly induced by LH in granulosa cells was shown to impact ovulation, as inhibition of ovarian miR-21 with locked nucleic acids (LNA) blocked ovulation and caused the formation of luteinized follicles (Carletti, et al. 2010). This phenotype was strikingly similar to what our laboratory previously observed in conditional deletion of the miRNA processing enzyme Dicer (Hong, et al. 2008). Based on these findings, we expected to uncover a similar phenotype of altered ovarian function when miR-212/132 was deleted in ovarian granulosa cells.

We expected to observe a defect in the Cyp19-cKO mice, as Cre expression, and thus miR-212/132 deletion would occur in the antral follicle. The Cyp19-Cre mouse has been previously used with much success to delete Kras, Pten, (Fan, et al. 2008a, Fan, et al. 2008b) , β-catenin (Fan, et al. 2010), and EGFR (Hsieh, et al. 2011). Crossing of the Cyp19-Cre mouse with the Rosa26 reporter line indicated genetic recombination in granulosa cells of antral follicles and the corpus luteum, but no leaky expression in other tissues (Fan, et al. 2008a). Taken together, these studies suggest that the Cyp19-Cre is a suitable mouse line to conditionally delete target genes in the peri-ovulatory period. We additionally crossed the miR212/132$^{fl/fl}$ mouse line with the Amhr2-Cre delete mouse line to delete the miR-212/132 gene much earlier in follicle development. The Amhr2-Cre is a widely used mouse line that has been previously used by our laboratory to delete Dicer (Hong, et al. 2008) and dozens of other laboratories to delete a number of genes involved in ovarian function (Jamin, et al. 2002b). While the Amhr2-Cre mouse has been widely used in laboratories and numerous publications have denoted its activity, there has been much discussion for several years about the inefficient expression of the Cre recombinase in Amhr2 expressing cells. The promoter has also been described to be 'leaky', or ectopically expressed in cells that do not usually express Amhr2. Recently, a study investigating the conditional deletion of β-catenin published many of these long-known anecdotal observations (Hernandez Gifford, et al. 2009). Still, we did observe a decrease in expression of miR-132 in both Cyp19-Cre and Amhr2-Cre, thus suggesting that recombination and deletion is occurring. It is unclear why the expression levels of miR-212 were nearly non-detectable in wild-type transgenic animals. One possible reason is that miR-132 is preferentially regulated during miRNA biogenesis, i.e. Drosha, thus

releasing pre-mir-132, while leaving mir-212 in the primary transcript. Furthermore, our transgenic Amhr2-cKO and Cyp19-cKO mice were both maintained on C57B6 backgrounds, while our previous studies investigating the *in vivo* and *in vitro* upregulation of miR-212/132 in response to LH was conducted using CF-1 mice (Fiedler, et al. 2008). It is conceivable that these differences in expression patterns could be due to strain variation.

One potential explanation for a lack of phenotype could be that loss of these miRNA simply does not make enough of a difference in protein expression to alter ovarian function. It has been reported that the effect of miRNA regulation on protein expression is relatively small, never more than a 4-fold change, and often much less (Selbach, et al. 2008). Furthermore, over 60% of mRNA are anticipated to be targeted by miRNA (Friedman, et al. 2009) and each individual miRNA can potentially target thousands of mRNA (Lewis, et al. 2005). It is also entirely possible that another unknown or undiscovered miRNA is highly regulated by LH and is compensating for loss of miR-212/132. The original study when our laboratory identified LH regulated miRNA was conducted in 2007 and included only 357 miRNA (Fiedler, et al. 2008). Today, over a 1000 individual miRNA have been identified and the number continues to increase. Taken together, it is highly possible that miRNA function is more of fine-tuning or gene network wide regulatory mechanism and deletion of a single miRNA may not be enough to observe a phenotype.

SECTION III

Hormonal regulation of miRNA in the ovary

XI: CHAPTER 6

Hormonal regulation of ovarian disease

1. Androgen excess

Androgen excess is an endocrine disorder affecting approximately 6-8% of women of reproductive age (Azziz 2004, Azziz, et al. 2004). This disorder is most commonly caused by non-classical adrenal hyperplasia (NCAH), androgen secreting neoplasms, severe insulin resistance, and ovarian thecal cell androgen hypersecretion. Additionally, some women are exposed to excess androgens through anabolic steroids and other environmental sources. Women suffering from excess androgen production/exposure exhibit hirsutism, androgenic alopecia, acne, ovulatory dysfunction, and fertility problems. Many of these presenting symptoms can be treated successfully and assisted reproductive technologies (ART) can be utilized to help overcome fertility problems, however, the *in utero* environment of the womb is still susceptible to exposure to excess androgens. For example, in the sheep, fetal exposure to excess maternal androgens leads to reproductive dysfunction upon sexual maturity (Clarke, et al. 1977, Padmanabhan, et al. 2006, Wood, et al. 1991). Similar evidence in the monkey and rodent has led to the hypothesis that fetal tissue exposed to excess testosterone during gestation may undergo altered developmental programming leading to adult onset of disease (Abbott, et al. 2005, Demissie, et al. 2008). Based on these observations, we have chosen to use the prenatally androgenized ewe as a model to investigate the effects of excess maternal testosterone on the health of the offspring, thus allowing us to link fetal developmental programming with adult phenotypes. This model is an excellent choice for the study of adult onset of reproductive disease because of similar ovarian development and physiology with humans, ease of manipulating *in utero* conditions, a well characterized phenotype, and it is an economically viable research animal.

2. Developmental Programming and Ovarian Development

The *in utero* environment of the womb is critical for normal fetal development. Insults to this environment can cause abnormal fetal programming leading to adult onset of disease (Barker 1990). Studies conducted by David Barker in the 1980s and 1990s describe the correlation between maternal health and nutrition and cardiovascular disease in adult offspring (Barker 1990). This work led to the formation of the developmental origin of adult disease hypothesis, also known as the Barker hypothesis, which states that 'adverse influences early in development, and particularly during intrauterine life, can result in permanent changes in physiology and metabolism, which result in increased disease risk in adulthood' (de Boo and Harding 2006). The Barker hypothesis not only describes the importance of the *in utero* environment to the health of the adult offspring, but also highlights the malleability of the developing fetus to stimuli. The fetal gonad is highly susceptible to hormonal stimuli during development, as proper development is dependent upon specific and precise exposure to steroids (Kezele and Skinner 2003, Nilsson and Skinner 2009). Hormones regulate gene expression by binding to steroid hormone receptors, members of the nuclear hormone receptor superfamily of transcription factors, which in turn bind to DNA response elements to transcriptionally activate/inactivate genes (Whitfield, et al. 1999). Additionally, steroid hormones can also exert non-genomic effects on target cells (Simoncini and Genazzani 2003).

In the sheep, gonadal differentiation begins around gestational day 30 (Quirke, et al. 2001), with primordial germ cells undergoing meiosis from d55 to d100 (term = 147 days) (Juengel, et al. 2006). By d75 the maximum number of primordial germ cells is established due to sustained loss via apoptosis (Juengel, et al. 2006). Primary follicles

are first observed around d90, secondary and pre-antral follicles by d120, and antral follicles are observed by d135 (Juengel, et al. 2006). The developing ovine ovary gains capacity for steroid production by d35 when StAR, Cyp11a1, Cyp17, and Cyp19 expression is observed in the innermost regions of the ovarian cortex (Quirke, et al. 2001) and estrogen is synthesized (Lun, et al. 1998). Ovarian development in the human and primate mirrors that observed in the sheep, particularly in regards to timing of gonadal differentiation during the 1st trimester of gestation and to the establishment of the follicular pool prior to birth. In the mouse, primordial germ cells are first observed as early as d7.25 (Ginsburg, et al. 1990), gonad differentiation begins prenatally (10.5d) (Gubbay, et al. 1990), however follicle assembly begins shortly after birth (Pepling 2012, Skinner 2005). Since ovarian formation and development largely occurs during fetal life, especially in the human and sheep, insults to the *in utero* environment during gestation can affect ovarian function after birth as observed in the rodent (Sloboda, et al. 2009), sheep (Padmanabhan, et al. 2006), and monkey (Dumesic, et al. 2007).

Prenatal androgenized sheep model

Administration of testosterone to pregnant ewes effects fetal development. Studies conducted by Clarke et al describe developmental, behavioral, and reproductive consequences to prenatal testosterone exposure in sheep (Clarke, et al. 1976, 1977). Pregnant ewes implanted with 1 mg capsules of testosterone from days 30 to 80 of pregnancy produced female offspring with complete masculinization of the external genitalia and exhibition of aggressive behavior (Clarke, et al. 1976). Half of the prenatally testosterone treated animals failed to ovulate and none underwent a normal

estrus cycle. The timing of testosterone administration appears to be critical as ewes treated with testosterone from d50 to d100 of pregnancy produced offspring with a less severe masculinization of external genitalia, and only 25% of animals failed to ovulate (Clarke, et al. 1976, 1977). Animals that received prenatal testosterone administration from days 70 to 120 and 90 to 140 of pregnancy showed little to no effects on development and reproduction (Clarke, et al. 1976, 1977). Similar phenotypic effects are observed in the rhesus monkey, as exogenous testosterone administration beginning before day 60 of pregnancy (term = 167 days) causes masculinization of the external genitalia (Dumesic, et al. 2007).

More recent studies conducted by the V. Padmanabhan laboratory (University of Michigan), have further investigated the effects of prenatal androgen exposure on the fetus while comprehensively studying the developmental, metabolic, and reproductive consequences (Padmanabhan 2007, Padmanabhan, et al. 2010a). In this model, pregnant ewes are administered 100 mg of testosterone propionate twice weekly from days 30 to 90 of pregnancy (West, et al. 2001). Because testosterone can act through the androgen receptor or undergo aromatization to estrogen and act through estrogen receptors, an additional group of ewes were injected with dihydrotestosterone (DHT), a non-aromatizeable androgen, from d30 to 90 of pregnancy to determine if observed effects were due to androgen or estrogen actions. Offspring born from ewes that were exposed to excess testosterone during gestation suffered from low birth weight (Manikkam, et al. 2004) and intrauterine growth retardation (Steckler, et al. 2005). Prenatal testosterone exposure did not alter the age of attaining puberty in female sheep (Sharma, et al. 2002). Sexually mature female sheep prenatally treated with testosterone exhibit neuroendocrine

feedback defects, including reduced hypothalamic sensitivity to progesterone and estrogen positive and negative feedback which in turn causes hypersecretion of LH (Sharma, et al. 2002, Veiga-Lopez, et al. 2009, Veiga-Lopez, et al. 2008).

Ovarian function is also severely disrupted in prenatally testosterone treated adult sheep. The ovaries of testosterone treated females are multi-follicular due to increased follicular recruitment and follicular persistence, exhibiting a polycystic ovarian phenotype similar in appearance to the ovaries of women who suffer from PCOS (Manikkam, et al. 2006, Steckler, et al. 2005, West, et al. 2001). Similar polyfollicular phenotypes are observed in rhesus monkeys prenatally exposed to testosterone (Dumesic, et al. 2007). Both sheep and monkeys exposed to excess gestational testosterone exhibit increased anovulation compared to control treated animals (Dumesic, et al. 2007, Manikkam, et al. 2006). Additionally, protein expression of PPARγ and adiponectin, components of the insulin signaling pathway which plays an important role in mediation of folliculogenesis and steroidogenesis in the ovary, are altered in testosterone treated fetal d90 and 10 month old (postnatal) ovaries, respectively (Ortega, et al. 2010). Ewes exposed to excess prenatal testosterone exhibit insulin resistance (Padmanabhan, et al. 2010b) and altered expression of the insulin like growth factor (IGF) system (Recabarren, et al. 2005). Similar association of hyperandrogenemia and hyperinsulinemia has also observed in prenatally androgenized primate models (Dumesic, et al. 2005) and women suffering from PCOS (Burghen, et al. 1980).

3. Ovarian Steroid Receptor Expression

In the prenatally androgenized ewe model, exogenous testosterone treatment can act directly through the androgen receptor (AR) or undergo aromatization into estradiol and act through estrogen receptor alpha (ERα) or beta (ERβ). AR, ERα, ERβ, progesterone receptor (PR), and other steroid hormone receptors are members of the nuclear hormone receptor superfamily of transcription factors (Couse and Korach 1999). Upon steroid hormone binding, the ligand activated receptor complex transcriptionally regulates gene expression by binding an associated response element in the promoter region of the target gene (Whitfield, et al. 1999). Steroid hormone receptors regulate numerous genes and provide a mechanism for rapid mediation of hormonal stimuli. Expression of steroid hormone receptors is species and spatial-temporal specific. In the fetal ovine ovary, ERα expression is limited to the surface epithelium while ERβ is ubiquitously expressed in all cell types in all follicles regardless of size (Juengel, et al. 2006). AR is first expressed in the connective tissue of the fetal ovine ovary on d55 and throughout the ovary on d75 (Juengel, et al. 2006). Expression of PR was not observed in ovine ovaries prior to d75 of gestation (Juengel, et al. 2006). In the adult sheep, ERα is expressed only in the granulosa cells of early antral and antral follicles, ERβ is expressed in the oocyte, granulosa, and theca cell of all follicles, AR is expressed in the granulosa and theca cells in follicles starting at the secondary stage, and PR is expressed in the granulosa and theca of early antral and antral follicles (Juengel, et al. 2006). In the mouse, steroid hormone receptors appear to play little role in ovarian development as ER- and PR-null mice appear to undergo normal ovarian development (Couse and Korach 1999, Lydon, et al. 1996). However, in the adult, expression of ERβ is primarily in

granulosa cells of growing follicles while ERα is predominantly expressed in thecal and interstitial cells (Britt and Findlay 2003). Steroid hormones play an important role in regulating intraovarian function. Androgens are important for initiation of follicular development and promote granulosa cell survival and follicular growth through the early antral stage (Walters, et al. 2008). In antral follicles androgens function as inhibitors of follicular development and promote granulosa cell apoptosis (Billig, et al. 1993). Estrogens are necessary for proper folliculogenesis (Goldenberg, et al. 1972), steroidogenesis (Fortune and Hansel 1979), and protecting granulosa cells from undergoing atresia (Billig, et al. 1993). Taken together, steroid hormones and their associated receptors play important roles in controlling ovarian function.

4. Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is a condition of excess androgen synthesis and secretion that affects roughly 5-7% of women of reproductive age and is a leading cause of infertility (Azziz, et al. 2004). Women with PCOS also exhibit a number of different conditions including: hypersecretion of LH, hirsutism, alopecia, acne, the presence of polycystic ovaries, an- or oligo-ovulation, obesity, insulin resistance, type 2 diabetes, and metabolic disease. Due to the large and varied number of characteristics observed in women with PCOS, diagnosis is often difficult. Currently, two guidelines exist to diagnose PCOS, the Rotterdam criteria and the NIH/NICHD guidelines. The Rotterdam criteria requires women to exhibit 2 of the 3 following symptoms: oligo- or anovulation, excess androgen activity, and the presence of polycystic ovaries (Rotterdam 2004) while the NIH criteria requires women to exhibit oligo-ovulation and signs of excess androgen

that are not due to other factors that PCOS (Zawadski and Dunaif 1992). Thus far, successful treatment of the disease relies on management of the symptoms (i.e. infertility treatment, hormonal contraceptives, management of obesity and/or type 2 diabetes) (Carlsen and Vanky 2010). Although intensive study has been done on this disease that was first described by Stein and Leventhal in 1935 (Leventhal and Cohen 1951), the actual cause remains to be elucidated. Numerous studies have attempted to link genetic loci to this condition, however no candidate gene or single nucleotide polymorphism has been able to explain the condition (Strauss, et al. 2012).

The hallmark feature of PCOS is the synthesis of excess androgen production. The primary cause of this abnormal hormone profile is believed to be the excess synthesis of androgens by theca cells (Gilling-Smith, et al. 1997). The increased production of androgens is believed to be due to ovarian thecal cell hyperplasia and sensitivity to gonadotropins, thus increasing cellular steroidogenic potential (Magoffin 2006). Ovaries of women suffering from PCOS often have the presence of multiple, persistent follicular cysts on the ovary that are unable to undergo ovulation, yet do not undergo atresia to allow the beginning of another ovarian cycle (Chang 2007). Women with PCOS often have increased serum levels of the gonadotropin luteinizing hormone (LH; (Rebar, et al. 1976)), which binds directly to the thecal cell membrane and promotes increased androgen production.

Few mechanisms aside from genetic linkage or transcriptional regulation have been investigated with regards to PCOS. Interestingly, a previous study using theca cells has identified that Cyp17 mRNA half-life is increased in PCOS cells compared to normal cells (Wickenheisser, et al. 2005). One potential mechanism regulating mRNA half is

through miRNA mediated post-transcriptional gene regulation, thus suggesting that further study in this regulatory mechanism is warranted.

XII: CHAPTER 7

Developmental Programming: Gestational Testosterone Treatment Alters Fetal Ovarian Gene Expression

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1. **Abstract**

Prenatal testosterone (T) treatment leads to polycystic ovarian morphology, enhanced follicular recruitment/depletion, and increased estradiol secretion. This study addresses whether expression of key ovarian genes and microRNA are altered by prenatal T excess and whether changes are mediated by androgenic or estrogenic actions of T. Pregnant Suffolk ewes were treated with T or T plus the androgen receptor antagonist, flutamide (T+F) from d30 to 90 of gestation. Expression of steroidogenic enzymes, steroid/gonadotropin receptors, and key ovarian regulators were measured by RT-PCR using RNA obtained from fetal ovaries collected on d65 [n=4, 5, and 5 for T, T+F, and control groups, respectively] and d90 (n = 5, 7, 4) of gestation. Additionally, fetal d90 RNA were hybridized to multispecies microRNA microarrays. Prenatal T decreased ($P < 0.05$) *Cyp11a1* expression (3.7-fold) in d90 ovaries and increased *Cyp19* (3.9-fold) and 5α-reductase (1.8-fold) expression in d65 ovaries. Flutamide prevented the T-induced decrease in *Cyp11a1* mRNA at d90 but not the *Cyp19* and 5α-reductase increase in d65 ovaries. Cotreatment with T+F increased *Cyp11a1* (3.0-fold) expression in d65 ovaries, relative to control and T-treated ovaries. Prenatal T altered fetal ovarian microRNA expression, including miR-497 and miR-15b, members of the same family that have been implicated in insulin signaling. These studies demonstrate that maternal T treatment alters fetal ovarian steroidogenic gene and microRNA expression and implicate direct actions of estrogens in addition to androgens in the reprogramming of ovarian developmental trajectory leading up to adult reproductive pathologies.

2. Introduction

Adverse *in utero* environment, including exposure to abnormal nutritional, environmental, metabolic, and hormonal insults, can alter the developmental trajectory of the fetus and lead to the adult onset of disease (Barker 1990). Developmental insults have been documented to disrupt cardiovascular, metabolic, and reproductive systems in numerous species (*i.e.* human, monkey, sheep, rodent) (Barker 1990, Nijland, et al. 2008, Simmons 2007). For example, female sheep fetuses exposed to excess testosterone (T) manifest low birth weight (Manikkam, et al. 2004), intrauterine growth restriction (Steckler, et al. 2005), postnatal catch-up growth (Manikkam, et al. 2004), reproductive neuroendocrine defects (Robinson, et al. 1999, Sarma, et al. 2005, Sharma, et al. 2002, Veiga-Lopez, et al. 2009, Veiga-Lopez, et al. 2008, Wood and Foster 1998), ovarian defects (Manikkam, et al. 2006, Ortega, et al. 2009, Smith, et al. 2009, Steckler, et al. 2007, Steckler, et al. 2005, West, et al. 2001), oligo/anovulation (Manikkam, et al. 2006, Veiga-Lopez, et al. 2008), luteal compromise (Manikkam, et al. 2006, Steckler, et al. 2007), insulin resistance (Padmanabhan, et al. 2010a, Padmanabhan, et al. 2010b), and hypertension (King, et al. 2007), with many of these characteristics being similar to those observed in women with polycystic ovarian syndrome (Chang 2007).

The metabolic and reproductive dysfunctions observed in adult female sheep in response to excess fetal T exposure demonstrate the important role steroid hormones play in reprogramming of fetal organ differentiation. At the ovarian level, prenatal T treatment leads to polycystic ovaries (West, et al. 2001) and increases follicular recruitment (Smith, et al. 2009, Steckler, et al. 2005) and persistence (Manikkam, et al.

2006, Steckler, et al. 2007). Sheep prenatally treated with the nonaromatizable androgen, dihydrotestosterone (DHT) fail to produce a multifollicular phenotype (West, et al. 2001) or follicular persistence (Steckler, et al. 2007), thus suggesting these outcomes are programmed via aromatization of T to estrogen. A comprehensive understanding of the early perturbations underlying reprogramming of the adult phenotype in response to excess gestational T is essential to develop interventions aimed toward prevention. Immunohistochemical studies focusing on few select proteins have found that phenotypic changes are preceded by increased expression of proteins for androgen receptor [AR (Ortega, et al. 2009)] and peroxisome proliferator-activated receptor γ [PPARγ (Ortega, et al. 2010)] as early as fetal d90 in prenatal T-treated sheep, thus pointing to involvement of steroidogenic and metabolic signaling pathways. These predictions are also supported by findings of elevated estradiol and insulin levels in prenatal T-treated females (Padmanabhan, et al. 2010b, Veiga-Lopez, et al. 2008).

Recent studies implicate microRNA (miRNA) as key regulators in changing gene expression in developing tissues and organs (for review, see (Stefani and Slack 2008)), with an abundance of miRNA in newborn and adult mice ovaries (Ahn, et al. 2010, Choi, et al. 2007, Fiedler, et al. 2008, Hossain, et al. 2009, Ro, et al. 2007, Tripurani, et al. 2011). Our recent studies have found that miRNA play key roles in gonadotropin-mediated regulation of ovarian function (Carletti, et al. 2010, Hong, et al. 2008). The aim of this study therefore was to gain an understanding of early perturbations in the ovarian transcriptome, specifically the expression of steroidogenic enzymes, steroid hormone receptors, gonadotropin receptors, and key ovarian regulators and identify changes in fetal ovarian miRNA expression in response to prenatal T treatment.

3. Materials and Methods

Animals, prenatal treatments, and tissue collection

All procedures were approved by the University Animal Care and Use Committee at the University of Michigan. Two-to 3-yr-old Suffolk ewes were purchased locally and group fed 0.5 kg of shelled corn and 1.0–1.5 kg of alfalfa hay/ewe per day (2.31 Mcal/kg of digestible energy). The day of mating was determined by visual confirmation of paint markings left on the rumps of ewes by the raddled rams. The diet meets the nutrient requirements for sheep defined by the National Research Council (Committee on the Nutrient Requirements of Small Ruminants National Research Council, Nutrient Requirements of Small Ruminants, Sheep, Goats, Cervids, and New World Camelids, Washington, DC) (Manikkam, et al. 2004). Aureomycin crumbles (chlortetracycline: 250 mg per ewe per day) were administered to reduce abortion from diseases such as *Campylobacter* and *Chlamydia*. Breeder animals assigned to generate control, T-treated, and T plus AR antagonist (*i.e.* flutamide)-treated fetuses were blocked by maternal weight, body score, age, and animal providers. Details of T and flutamide treatments and descriptions of phenotypic effects on offspring (*i.e.* ovarian phenotype, neuroendocrine defects, intrauterine growth restriction, and masculinization of external genitalia) have been published previously (Manikkam, et al. 2004). Gestational T treatment consisted of twice-weekly im injections of 100 mg T propionate (Sigma-Aldrich Corp., St. Louis,MO) in cottonseed oil (2 ml volume) from d30 to d90 of gestation (term = 147 d) (Manikkam, et al. 2004). AR antagonist treatment consisted of daily sc injections of 15 mg/kg flutamide [Sigma-Aldrich]. Control ewes received the same volume of vehicle. The

concentrations of T achieved in the mother and female fetus during gestational T treatment are comparable with that of the adult males and the early T rise seen in male fetuses, respectively (Veiga-Lopez, et al. 2011). T treatment also increased fetal levels of estradiol (Veiga-Lopez, et al. 2011), suggestive of the potential for the involvement of both androgenic and estrogenic pathways in programming adult dysfunction.

Collection of fetal ovaries

Fetal ovaries were collected from control and T-treated dams on d 64–66 and d 87–90 (range) of gestation (referred as d 65 and d 90, respectively, hereafter). All dams were sedated with 20–30 ml pentobarbital iv (Nembutol Na solution; 50 mg/ml; Abbott Laboratories, Chicago, IL) and subsequently maintained under general anesthesia (1–2% halothane; Halocarbon Laboratories, River Edge, NJ). The gravid uterus was exposed through a midline incision and the uterine wall incised. Dams were administered a barbiturate overdose (Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI) and fetuses removed for tissue harvest. Fetal ovaries were removed from d65 and d90 control, prenatal T and T plus flutamide fetuses, weighed, washed with PBS, quick frozen, and stored at -80 C until processing. One ovary from one female offspring of each dam (the mother was the experimental unit) from d65 and d90 control (n = 5 and 4, respectively), prenatal T (d65, n = 4; d90, n = 5) and T plus flutamide (d65, n = 5; d90, n = 7)-treated animals were used for gene expression studies.

Quantitative RT-PCR

mRNA analysis

Fetal ovarian RNA was isolated with Trizol, per the manufacturer's instruction and assessed for quality using the Agilent Bioanalyzer Nano Chip (Agilent Technologies, Santa Clara, CA). All RNA samples had sharp 18S and 28S peaks and RNA integrity numbers greater than 8.0, indicating high-quality RNA. Total RNA(250 ng) isolated from whole ovarian fetal tissue was reverse transcribed (QIAGEN miScript System, Valencia, CA) per the manufacturer's instruction. The resulting cDNA was amplified by quantitative RT-PCR using forward and reverse primers designed to ovine or bovine sequences (Table XII-1, published on The Endocrine Society's Journals Online website at http://endo.endojournals.org) and Power Sybr Green (Applied Biosystems, Carlsbad, CA) with the Applied Biosystems 7900HT real-time system. Analysis of genes included enzymes in steroidogenic pathway [steroidogenic acute regulatory protein (*StAR*), cholesterol side chain cleavage enzyme (*Cyp11a1*), 3β1-hydroxysteroid dehydrogenase (*3β1HSD*), *3β2HSD, Cyp17, 17β-HSD, Cyp19,* steroid-5-α-reductase (*SRD5A1*), and ferredoxin- 1 (*FDX1*)], steroid receptors [*AR*, estrogen receptors [estrogen receptor α (ESR1); estrogen receptor β (ESR2)], progesterone receptor (*PGR*)], gonadotropin receptors [FSH receptor (*FSHR*) and LH receptor (*LHR*)], and key regulators of ovarian development and function [growth differentiation factor 9 (*GDF9*) and cyclin D2 (*CCND2*)]. Analysis of expression of insulin-related signaling genes included IGF-I (*IGFI*), IGF-I receptor (*IGFR1*), insulin receptor (*IR*), insulin receptor substrate 1 (*IRS1*), *IRS2*, mammalian target of rapamycin (*mTOR*), protein kinase B (*Akt*), phosphoionositide-3-kinase (*PI3K*), glucose transporter type 4 (*Glut4*), and peroxisome

proliferator-activated receptor γ (*PPARγ*). All assays used the standard cycling conditions (50C for 2 min, 95C for 10 min, followed by 40 cycles of 95C for 15 sec and 60 C for 1 min) in addition to a dissociation curve to determine product melting temperature insuring presence of a single amplicon. The small RNA U6 was used for normalization and a relative standard curve was analyzed for each specific gene target. All data were analyzed by comparing the relative amount of gene product to the relative amount of U6.

Table XII-1. Quantitative RT-PCR primers

Gene	Accession Number	Forward Sequence	Reverse Sequence
17βHSD	NM_001007809	CCTCACTTAATCAGGCTGCTTTG	AAGTTAGGATCAATGTGTAAGGGATTC
3β1HSD	NM_001135932	CATCATCCACACTGCCTCTATCA	ACAGAAGCTGGGTACCTTTCACA
3β2HSD	NM_174343	CCTGCTGGAAGGAGACATTCTG	GTGCTGGTGTGGATAAAGACCG
AKt	NM_173986.2	GACCACGCCCAGCCCCCACCAGT	GGACAAGGACGGCCACATCAAGA
AR	AF105713.1	CAATGAGTACCGCATGCACAA	GGTGCCTCATTCGGACACA
CCND2	EU626221.1	CAGAAGGACATTCAGCCCTACAT	CTCACAGACCTCCAGCATCCA
Cyp11a1	NM_176644	GCAGGGCTCCGGAAAGTT	GGTGATGGACTCAAAGGCAAA
Cyp17	M12547	TGATGATTGGACACCACCAGTTG	AGAGAGAGAGGCTCGGACAGATC
Cyp19	NM_174305	CCTCAATACCAGGTCCCAGCTA	GGAACCTGCAGTGGGAAATG
ESR1	AY033393.1	TCGTCTCGGTTCCGTATGATG	GACAGAAATGTGTACACCCCAGAAT
ESR2	NM_001009737.1	GCCGACAAGGAACTGGTACAC	CCACGAAGCCCGGAATCT
FSHR	NM_001009289.1	CGGTCGAACTGAGGTTTGTTC	GGTCTCCGAATCCTGAAAAGG
GDF9	NM_174681	TGGCGGCAGGAACCTTT	CAACAGTAACACGATCCAGGTTAAA
IGF1	NM_001009774.2	AGCGCCACACCGACATG	CCCTCTGCTTGTGTTCTTCAAA
IGFR1	AF025303.1	TTGAGCTGATGCGCATGTG	GAACGAGGGCCGCATCTT
IR	XM_590552.4	GACGCAGGCCGGAGATGACCA	GCTCCTGCCCGAAGACCGACTC
IRS1	XM_581382.3	TGGCACTGGGCGTAGAGGAGAAGG	CGCCCATCAGCTACGCCGACAT
IRS2	NM_003749.2	CCCGAGAAGGTGGCCCGCATCA	AGCAACACGCCCGAGTCCA
LHR	NM_174381	TGCCATCAAGAGAAAAATTTACCA	TCTGCTCTTTTGTTGGCAGGTT
mTOR	XM_001788228.1	ATCACCCTTGCTCTCCGAACTCTC	CCAGCTCCCGGATCTCAAACACCT
PGR	Z66555.1	CCAGATATGGTCTATGCAGGACAT	TGTCAGCAGAGAGCTGGAGGTA
PI3K	NM_174574	TTGTGCAACCTACGTGAATGTAAA	TCTCCTCCATGGTAGATACCTGTTC
PPARα	NM_001034036	TGAGGGCTGCAAGGGTTTC	CAACTACGGTCACATTTGTCATACAC
SRD5A1	NM_001099137	CTGGCCCAACTGCATCCT	AAGCTCCGTTGCGCATAGTG
StAR	NM_174189	AGCGCATGGAGGCTATGG	AATCTTCTGCAGGACCTTGATCTC

miRNA analysis

To identify miRNA expressed in fetal ovarian tissue, total RNA from fetal d 90 sheep was hybridized to the multispecies Affymetrix GeneChip miRNA Array (Affymetrix, Santa Clara, CA). The Affymetrix miRNA arrays were background corrected, normalized, and gene level summarized using the robust multichip average procedure (33). Fold change statistics for miRNA were calculated by taking the linear contrast between the least square means of the (log) treatment and (log) control groups and backtransforming the result to a linear scale (this is the ratio of the geometric mean of the treatment samples to the geometric mean of the control samples). Corresponding significance scores (*P* values) were calculated based on the t statistic of the linear contrast. The tissues were assayed in biological quadruplicates. For confirming changes in specific miRNA expression from array data, total RNA (25 ng) was reverse transcribed (miRCURY LNA universal reverse transcriptase; Exiqon, Vedback, Denmark) per the manufacturer's instruction. The resulting cDNA was amplified and quantified for miR-497, miR-10a, miR-150, miR-29a, and miR-15b using LNA primer assays (Exiqon) and Sybr Green (Exiqon). All assays were performed on the Applied Biosystems 7900HT real-time system with the following cycling conditions: 95 C for 10 min, 40 cycles of 95 C for 10 sec, and 60 Cf or 1 min and a dissociation curve to determine product melting temperature. The U6 primer assay (Exiqon) was used for normalization and a relative standard curve was analyzed for each miRNA. All data were analyzed by comparing the relative amount of gene product to the relative amount of U6.

Bioinformatic analysis was conducted on all miRNA differentially expressed between control and T or control and T+flutamide treated animals using TargetScan 5.1 (www. targetscan.org) to identify putative mRNA targets. Analysis of mRNA targets focused on insulin signaling molecules, steroid receptors/enzymes, and lipid metabolic hormones as well as key known ovarian regulatory genes. A comprehensive literature based analysis was also conducted on all differentially expressed miRNA. Those miRNA that were linked to diabetes, insulin signaling, steroid receptor action, steroidogenesis, ovarian function, sexual differentiation, and lipid metabolism were selected from the inclusive lists.

Statistical analysis

All hormone receptor and steroidogenic enzyme mRNA expression data generated by quantitative RT-PCR was log transformed and analyzed by two-way ANOVA followed by Bonferroni *post hoc* test (Prism, version 4; GraphPad Software, La Jolla, CA). Quantitative RT-PCR data relating to expression of miRNA was log transformed and analyzed by ANOVA followed by Dunnett's *post hoc* test (Prism). A $P < 0.05$ was considered significant, unless otherwise noted.

4. Results

Steroidogenic enzymes and related genes

An age-dependent increase in *3β1HSD* ($P < 0.05$) and *FDX-1*, and a decrease in *17β1HSD* ($P<0.001$) was evident between fetal d65 and d90 (Figure. XII-1). There was no age effect in mRNA expression levels for *3β2HSD*, *Cyp17*, *Cyp19*, 5α-reductase, or *StAR* (data not shown). Gestational T treatment had a fetal age-specific effect on expression of *Cyp11a1* mRNA, the enzymatic rate-limiting step of steroidogenesis (Figure XII-2). Prenatal T treatment decreased *Cyp11a1* expression in fetal d90 but not fetal d65. In contrast, co-treatment with T plus AR antagonist increased expression of *Cyp11a1* in fetal d 65 ovaries, relative to age-matched T-treated and control fetuses. Furthermore, the AR antagonist cotreatment prevented the decrease in expression associated with T treatment alone in the d 90 fetal ovaries. Maternal T as well as T plus AR antagonist treatment increased expression of mRNA for Cyp19 in d65 but not d90 ovaries ($P < 0.05$; Figure XII-2), relative to controls. Maternal T and T plus AR antagonist treatment also tended ($P<0.0785$) to increase expression of mRNA for 5α-reductase, which converts T to DHT, on fetal d65 but not d90. Prenatal T or T plus AR antagonist did not alter the expression of mRNA for *StAR* and other steroidogenic enzymes (*3β1HSD, 3β2HSD, 17β1HSD,* or *Cyp17*) at either time points.

Figure XII-1. Fetal ovarian genes (mRNA) that exhibit changes in expression between d65 (*white bars*) and d90 (*black bars*) of gestation as determined by quantitative RT-PCR of mRNA from whole ovarian tissue of control, T-treated, and T plus AR antagonist-treated (TF), female fetuses. Because no treatment effects were observed, the data for all treatment groups were pooled by age. Results are expressed as mean ± SEM. *Asterisks* denotes an age effect (*, $P < 0.05$; **, $P < 0.01$). with respect to age (not shown). Maternal T treatment, with and without AR antagonist treatment, did not alter expression of any of the insulin-related regulatory genes.

Figure XII-1

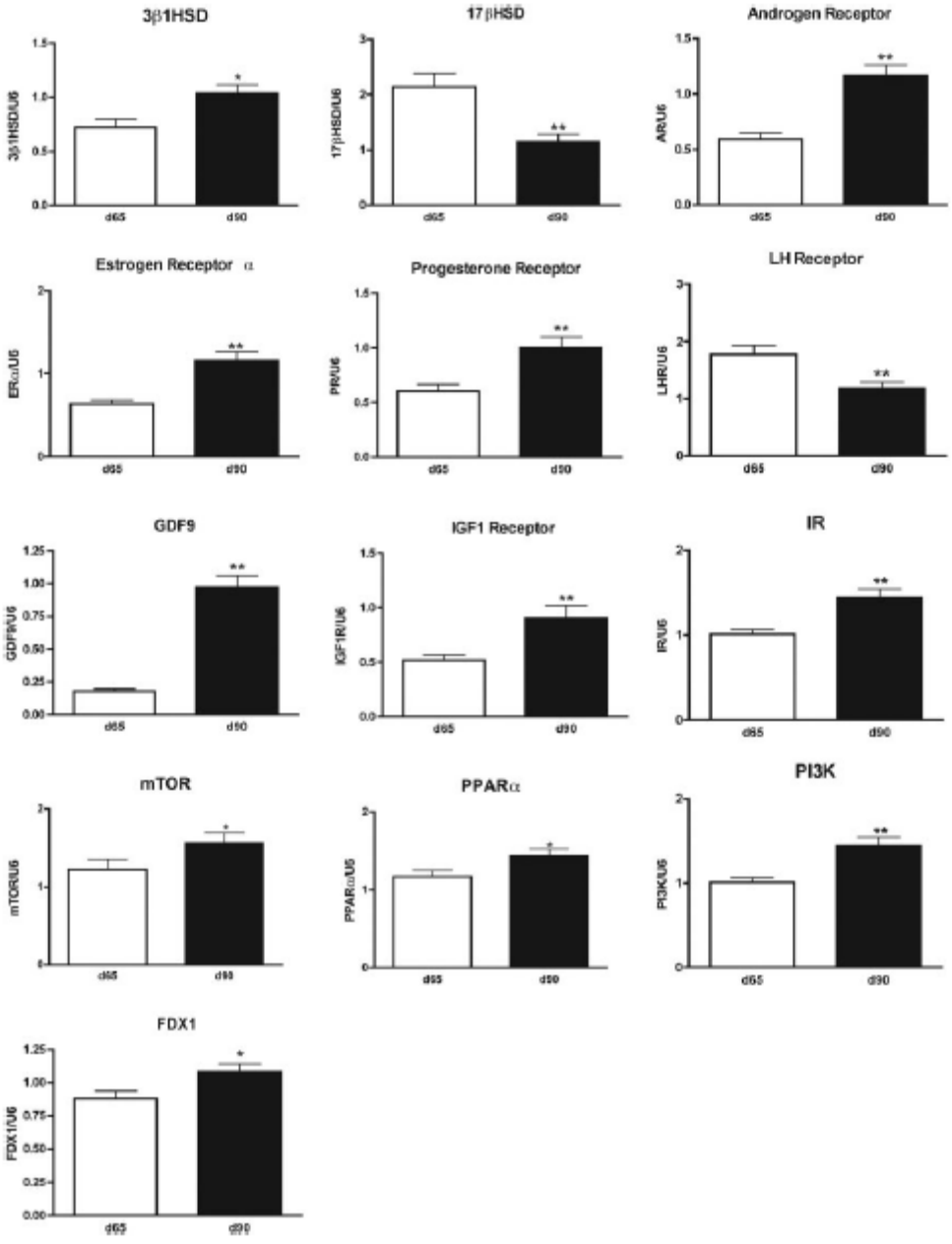
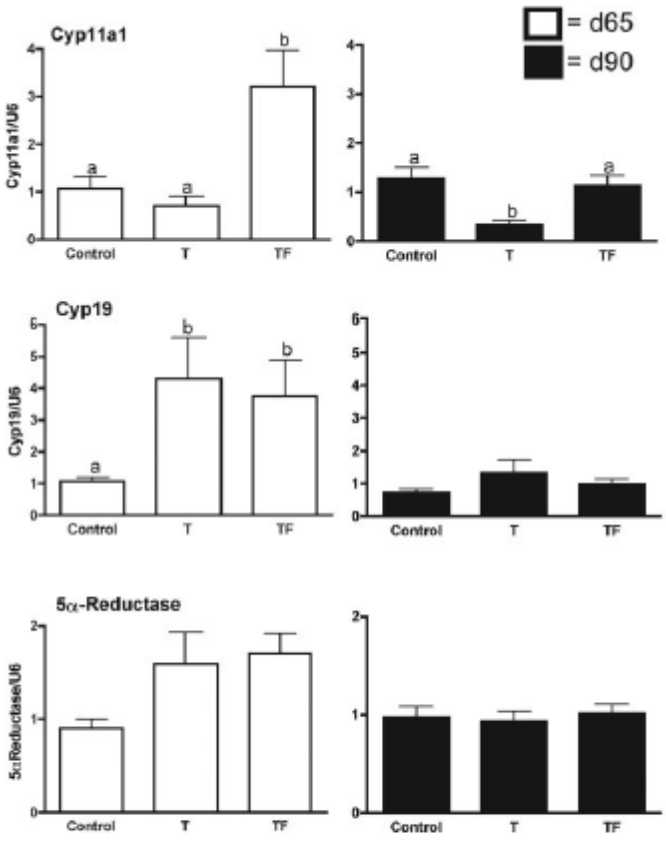

3β1HSD
17βHSD
Androgen Receptor
Estrogen Receptor α
Progesterone Receptor
LH Receptor
GDF9
IGF1 Receptor
IR
mTOR
PPARα
PI3K
FDX1
d65
d90

Figure XII-2. Fetal ovarian steroidogenic enzyme genes (mRNA) that are altered by prenatal T (T) and T plus AR antagonist (TF) treatment as determined by quantitative RT-PCR of mRNA from fetal d65 (*white bars*) and fetal d90 (*black bars*) whole ovarian tissue. Treatment effects on mRNA expression were determined by two-way ANOVA followed by Bonferroni *post hoc* tests. *Differing superscripts* indicate significant differences ($P < 0.05$). Note for 5α-reductase, the T and TF females showed a tendency to be higher compared with controls ($P = 0.0795$).

Figure XII-2


Cyp11a1
Cyp11a1/U6
= d65
= d90
a
a
b
a
b
a
Control
T
TF
Cyp19
Cyp19/U6
a
b
b
5α-Reductase
5αReductase/U6

Hormone receptors

An age-related increase ($P < 0.01$; ~2-fold) in expression of *AR*, *ESR1* (*ER*α), and *PGR* mRNA was evident between d65 and d90 (Figure XII-1). In contrast, *ESR2* expression was similar between fetal d 65 and d 90. Maternal T or T plus AR antagonist failed to alter expression of any of the steroid hormone receptor mRNA (*AR, PGR, ESR1*, and *ESR2*). In terms of the gonadotropin receptors, there was a decrease in expression of *LHR* ($P<0.01$) from fetal d65 to d90 (Figure XIII-1), whereas *FSHR* levels tended ($P <0.057$, 1.74-fold) to increase between d65 and d90 (data not shown). Gestational T or T plus AR antagonist treatment failed to alter expression pattern of the gonadotropin receptors, *LHR*, and *FSHR*.

Ovarian regulatory factors

Increased expression between fetal d 65 and d 90 was observed for *GDF9* ($P<0.01$, Figure.XII-1), whereas cyclin D2 did not change in response to age (not shown). Maternal T treatment, with and without AR antagonist treatment, did not alter expression of any of the ovarian regulatory genes examined.

Insulin-related genes

Age-dependent increase in expression of *IGF-I* receptor ($P < 0.01$), IR, mammalian target of rapamycin, and PPARγ, and PI3K were observed between d65 and d90 of gestation (Figure XII-1). Conversely, *IGF-I*, *IRS1*, *IRS2*, *Akt*, and glucose transporter exhibited no change in expression

MicroRNA

Comparison of control *vs.* T-treated ovaries using a multispecies miRNA microarray identified 31 up-regulated (>1.5-fold) and 18 down-regulated (>1.5-fold) miRNA (Table XII-2) in d90 fetal ovaries. Comparison of control *vs.*T plus AR antagonist treated ovaries identified 21 miRNA up-regulated (>1.5-fold) and 16 miRNA down-regulated (>1.5-fold; Table XIII-2). A total of 35 miRNA were differentially expressed between T and T plus AR antagonist-treated fetal d 90 ovaries (Table XII-2) compared with controls. Examination of the mRNA predicted (TargetScan) to be targets of the differentially expressed miRNA indicated a large number (25) of miRNA had putative insulin-signaling target transcripts (Table XII-3). Of these 25 miRNA with predicted insulin-signaling target mRNAs, 15 have been associated with insulin signaling/diabetes in cited papers (Table XII-3). Four additional miRNA were included in Table XII-3, based on their prior association (literature based) with ovarian tissue. Interestingly, of the 29 miRNA included in Table 1 that were differentially expressed in control, T and T+F treated animals, 20 miRNA have previously been linked (literature) to steroid expression, ovarian regulation, sexual differentiation, or fetal programming (Table XII-3). Increased expression of miR-15b and miR-497 in fetal d90 ovaries in response to gestational T treatment was validated by quantitative RT-PCR (Figure XII-3). Additionally, miR-29a tended to increase ($P = 0.0613$) in the T (2.38 ± 0.71, mean ± SEM) compared with control (0.54 ± 0.20) ovaries.

Table XII-2. microRNA differentially expressed in control vs testosterone, control vs testosterone plus flutamide, and testosterone vs testosterone plus flutamide treated fetal ovine ovaries.

miRNA	C vs. T FC	C vs TF FC	T vs TF FC
miR-497	3.83	3.71	–
miR-29a	2.98	–	–
miR-193	2.68	–	–
miR-128	2.44	1.91	-2.67
miR-495	2.39	–	–
miR-452	2.34	–	–
miR-141*	2.29	–	–
miR-329	2.27	–	–
miR-192	2.19	–	–
miR-150	2.14	–	–
miR-24-2*	2.12	–	-2.06
miR-15b	2.06	2.93	–
miR-135b	1.96	–	–
miR-455	1.90	2.23	–
miR-101	1.90	–	–
miR-212	1.87	–	–
miR-451	1.85	–	–
miR-186	1.82	–	–
miR-672	1.82	–	–
miR-661	1.80	–	-1.77
miR-376c	1.75	–	–
miR-7	1.67	–	–
miR-376b	1.65	1.71	–
miR-712	1.63	–	–
miR-30b-5p	1.58	–	–
miR-381	1.57	–	–
miR-380-5p	1.55	–	-1.84
miR-496	1.54	–	-1.65
miR-540-3p	1.53	–	–
miR-557	1.53	–	-1.65
miR-554	1.53	–	–
miR-142b-5p	1.53	–	–
miR-22*	1.52	–	–
miR-460	1.52	–	–
miR-206	1.52	-1.56	-1.73
miR-203	1.51	–	–
miR-548f	1.51	–	-1.56
miR-934	1.51	–	-1.61
miR-378	-4.13	–	4.29
miR-193a-5p	-2.86	–	–
miR-760	-2.80	–	–
miR-10a	-2.76	-3.36	–
miR-541*	-2.71	–	–
miR-182	-2.71	–	–

miRNA	C vs. T FC	C vs TF FC	T vs TF FC
miR-132	-2.49	–	–
miR-129*	-2.22	–	–
miR-727*	-2.12	–	–
miR-532-3p	-1.99	–	–
miR-1247	-1.93	–	–
miR-138	-1.85	–	–
miR-325	-1.76	–	–
miR-138*	-1.75	–	–
miR-1224*	-1.69	–	–
miR-223	-1.69	–	–
miR-667	-1.66	–	–
miR-759	-1.64	–	–
miR-423-5p	-1.60	–	–
miR-939	-1.58	–	–
miR-513b	-1.51	–	–
miR-219-5p	-1.50	–	–
miR-363	–	4.50	–
miR-20b	–	3.24	–
miR-374b	–	2.07	–
miR-736	–	1.98	–
miR-27e	–	1.94	–
miR-365	–	1.88	–
miR-124	–	1.78	–
miR-1181	–	1.74	–
miR-330	–	1.71	–
miR-27c	–	1.71	–
miR-29c	–	1.64	–
miR-29b	–	1.62	–
miR-519*	–	1.61	–
miR-891b	–	1.60	1.54
miR-338-3p	–	1.59	–
miR-886-3p	–	1.59	–
miR-454	–	1.57	–
miR-191*	–	1.56	1.69
miR-218-1*	–	1.56	–
miR-101a*	–	1.53	–
miR-467d	–	1.53	–
miR-105	–	1.52	–
miR-1308	–	-2.89	–
miR-182	–	-2.82	–
miR-1230	–	-2.48	-2.41
miR-450a	–	-1.90	–
miR-133a	–	-1.85	–
miR-1235	–	-1.75	–
miR-1202	–	-1.66	–
miR-471	–	-1.65	–
miR-431	–	-1.63	–
miR-183	–	-1.62	–
miR-568	–	-1.60	–

miR-135a	–	-1.57	–
miRNA	**C vs. T FC**	**C vs TF FC**	**T vs TF FC**
miR-93*	–	-1.55	–
miR-144	–	-1.54	–
miR-23a*	–	-1.51	–
miR-383	–	–	3.27
miR-467e*	–	–	2.05
miR-21	–	–	1.91
miR-302b*	–	–	1.88
miR-518d	–	–	1.63
miR-721	–	–	1.62
miR-330-5p	–	–	1.58
miR-96	–	–	1.58
miR-502-5p	–	–	1.54
miR-208a	–	–	1.51
miR-714	–	–	-2.33
miR-451	–	–	-2.22
miR-148b*	–	–	-2.10
miR-200a*	–	–	-2.05
miR-715	–	–	-2.01
miR-1305	–	–	-1.85
miR-196b	–	–	-1.75
miR-466j	–	–	-1.66
miR-1191	–	–	-1.65
miR-660	–	–	-1.58
miR-379	–	–	-1.53
miR-146a*	–	–	-1.51

Table XII-3. microRNA differentially expressed in control vs testosterone or testosterone plus flutamide treated fetal ovine ovaries

miRNA	C vs. T FC	C vs TF FC	Functional Analysis-Literature Based[$]	Predicted Targets (Targetscan)
miR-497	3.83	3.71	Type 2 diabetes rat [miR-15b/497 family (1)	PAPPA, INSR, GHR, IGF2R, IRS2, IGF1R, Furin
miR-29a	2.98	–	Diabetes/insulin signaling (1-5), FSH regulated (6)	IGF1, INSIG1, Leptin
miR-192	2.19	–	Diabetic neuropathy (4, 7, 8)	IGF1
miR-24-2*	2.12	–	Diabetes (9), Insulin signaling (10), bovine ovary (11)	Insig1, PPARa, Furin, IGFBP5, IGF2BP2
miR-15b	2.06	2.93	Type 2 diabetes rat FSH (1), regulated in ovary (6)	PAPPA, INSR, GHR, IGF2R, IRS2, IGF1R, Furin
miR-101	1.90	–	AR regulated (12)	PGRMC2, PPARa
miR-212	1.87	–	LH regulated in ovary (13)	
miR-451	1.85	–	Sex dependent in liver (14)	
miR-186	1.82	–	Type 1 diabetes (15)	INSM1, LEPR, IGF1, IGF1R
miR-672	1.82	–	Mouse ovary (16)	IGF1R
miR-7	1.67	–	Insulin signaling (17, 18)	IRS2, IRS1, IGF1R, PAPPA
miR-30b-5p	1.58	–	Estrogen regulated (19)	LEPR, IGF2R, INSIG2, IRS1, IRS2, LDLR, IGF1R, IGF1
miR-22*	1.52	–	Fetal ovine gonad (20), Androgen regulated (21), Represses ERα (22)	ESR1, GHRHR, PTGS1, IGF2BP1, Furin
miR-378	-4.13	–	Lipid/fatty acid metabolism (23), Regulates estrogen production (24)	
miR-760	-2.80	–	Estrogen regulated (25)	INSR
miR-10a	-2.76	-3.36	Androgen regulated (26), Ovary (27)	PPARa
miR-182	-2.71	–	Insulin signaling (10), Ovary (27)	INSIG1, IGF2BP1
miR-129*	-2.22	–	Diabetes (28)	IGF1, GHR, ERalpha, INSIG2
miR-132	-1.82	–	LH regulated in ovary (13)	
miR-223	-1.69	–	Diabetes (9)	IGF1R
miR-363	–	4.50	Sex differentiation (29)	PTGER4, IRS2, INSIG1
miR-20b	–	3.24	Diabetes (9), ERα regulated (30)	PPARa, LDLR, IGF2BP1, ADIPOR2, PPARd
miR-330	–	1.71	Fetal programming (31)	IGF2BP1
miR-29c	–	1.64	Diabetic neuropathy (32), Diabetes (2)	IGF1, INSIG1, Leptin
miR-29b	–	1.62	Diabetes (2, 9), Sex dependent in liver (14)	IGF1, INSIG1, Leptin
miR-191*	–	1.56	Diabetes (9)	
miR-101a*	–	1.53	AR regulated (12)	PGRMC2, PPARa
miR-105	–	1.52	Human ovary (27)	
miR-133a	–	-1.85	Insulin signaling (33, 34)	INSR, IGF1R

[$] References for Table are included in numbered format below.

Table XIII-3 References

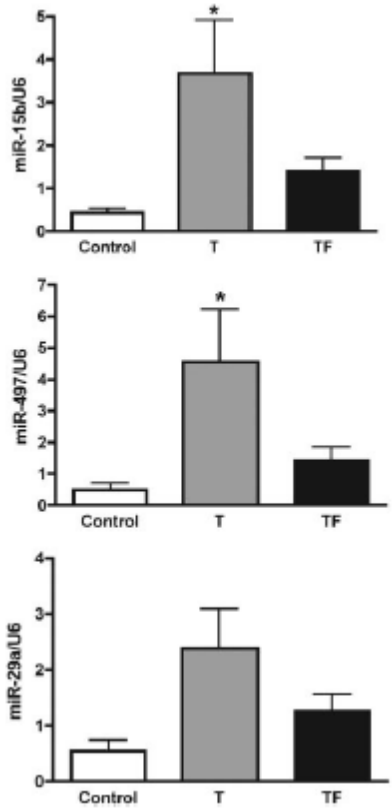
1. **Herrera BM, Lockstone HE, Taylor JM, Ria M, Barrett A, Collins S, Kaisaki P, Argoud K, Fernandez C, Travers ME, Grew JP, Randall JC, Gloyn AL, Gauguier D, McCarthy MI, Lindgren CM** 2010 Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes. Diabetologia 53:1099-1109
2. **He A, Zhu L, Gupta N, Chang Y, Fang F** 2007 Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. Mol Endocrinol 21:2785-2794
3. **Herrera BM, Lockstone HE, Taylor JM, Wills QF, Kaisaki PJ, Barrett A, Camps C, Fernandez C, Ragoussis J, Gauguier D, McCarthy MI, Lindgren CM** 2009 MicroRNA-125a is over-expressed in insulin target tissues in a spontaneous rat model of Type 2 Diabetes. BMC Med Genomics 2:54
4. **Kong L, Zhu J, Han W, Jiang X, Xu M, Zhao Y, Dong Q, Pang Z, Guan Q, Gao L, Zhao J, Zhao L** 2011 Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. Acta Diabetol 48:61-69
5. **Pandey AK, Verma G, Vig S, Srivastava S, Srivastava AK, Datta M** 2011 miR-29a levels are elevated in the db/db mice liver and its overexpression leads to attenuation of insulin action on PEPCK gene expression in HepG2 cells. Mol Cell Endocrinol 332:125-133
6. **Yao N, Lu CL, Zhao JJ, Xia HF, Sun DG, Shi XQ, Wang C, Li D, Cui Y, Ma X** 2009 A network of miRNAs expressed in the ovary are regulated by FSH. Front Biosci 14:3239-3245
7. **Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ, Natarajan R** 2007 MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. Proc Natl Acad Sci U S A 104:3432-3437
8. **Krupa A, Jenkins R, Luo DD, Lewis A, Phillips A, Fraser D** 2010 Loss of MicroRNA-192 promotes fibrogenesis in diabetic nephropathy. J Am Soc Nephrol 21:438-447
9. **Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M** 2010 Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res 107:810-817
10. **Melkman-Zehavi T, Oren R, Kredo-Russo S, Shapira T, Mandelbaum AD, Rivkin N, Nir T, Lennox KA, Behlke MA, Dor Y, Hornstein E** 2011 miRNAs control insulin content in pancreatic beta-cells via downregulation of transcriptional repressors. EMBO J 30:835-845
11. **Hossain MM, Ghanem N, Hoelker M, Rings F, Phatsara C, Tholen E, Schellander K, Tesfaye D** 2009 Identification and characterization of miRNAs expressed in the bovine ovary. BMC Genomics 10:443
12. **Cao P, Deng Z, Wan M, Huang W, Cramer SD, Xu J, Lei M, Sui G** MicroRNA-101 negatively regulates Ezh2 and its expression is modulated by androgen receptor and HIF-1alpha/HIF-1beta. Mol Cancer 9:108
13. **Fiedler SD, Carletti MZ, Hong X, Christenson LK** 2008 Hormonal regulation of MicroRNA expression in periovulatory mouse mural granulosa cells. Biol Reprod 79:1030-1037

14. **Cheung L, Gustavsson C, Norstedt G, Tollet-Egnell P** 2009 Sex-different and growth hormone-regulated expression of microRNA in rat liver. BMC Mol Biol 10:13
15. **Chen BZ, Yu SL, Singh S, Kao LP, Tsai ZY, Yang PC, Chen BH, Shoei-Lung Li S** 2011 Identification of microRNAs expressed highly in pancreatic islet-like cell clusters differentiated from human embryonic stem cells. Cell Biol Int 35:29-37
16. **Ahn HW, Morin RD, Zhao H, Harris RA, Coarfa C, Chen ZJ, Milosavljevic A, Marra MA, Rajkovic A** 2010 MicroRNA transcriptome in the newborn mouse ovaries determined by massive parallel sequencing. Mol Hum Reprod 16:463-471
17. **Jiang L, Liu X, Chen Z, Jin Y, Heidbreder CE, Kolokythas A, Wang A, Dai Y, Zhou X** 2010 MicroRNA-7 targets IGF1R (insulin-like growth factor 1 receptor) in tongue squamous cell carcinoma cells. Biochem J 432:199-205
18. **Yu JY, Reynolds SH, Hatfield SD, Shcherbata HR, Fischer KA, Ward EJ, Long D, Ding Y, Ruohola-Baker H** 2009 Dicer-1-dependent Dacapo suppression acts downstream of Insulin receptor in regulating cell division of Drosophila germline stem cells. Development 136:1497-1507
19. **Mellios N, Galdzicka M, Ginns E, Baker SP, Rogaev E, Xu J, Akbarian S** 2010 Gender-Specific Reduction of Estrogen-Sensitive Small RNA, miR-30b, in Subjects With Schizophrenia. Schizophr Bull Epub
20. **Torley KJ, da Silveira JC, Smith P, Anthony RV, Veeramachaneni DN, Winger QA, Bouma GJ** 2011 Expression of miRNAs in ovine fetal gonads: potential role in gonadal differentiation. Reprod Biol Endocrinol 9:2
21. **Delic D, Grosser C, Dkhil M, Al-Quraishy S, Wunderlich F** 2010 Testosterone-induced upregulation of miRNAs in the female mouse liver. Steroids 75:998-1004
22. **Pandey DP, Picard D** 2009 miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. Mol Cell Biol 29:3783-3790
23. **Fernandez-Hernando C, Suarez Y, Rayner KJ, Moore KJ** 2011 MicroRNAs in lipid metabolism. Curr Opin Lipidol 22:86-92
24. **Xu S, Linher-Melville K, Yang B, Wu D, Li J** 2011 Micro-RNA378 (miR-378) Regulates Ovarian Estradiol Production by Targeting Aromatase. Endocrinology 152
25. **Cicatiello L, Mutarelli M, Grober OM, Paris O, Ferraro L, Ravo M, Tarallo R, Luo S, Schroth GP, Seifert M, Zinser C, Chiusano ML, Traini A, De Bortoli M, Weisz A** 2010 Estrogen receptor alpha controls a gene network in luminal-like breast cancer cells comprising multiple transcription factors and microRNAs. Am J Pathol 176:2113-2130
26. **Waltering KK, Porkka KP, Jalava SE, Urbanucci A, Kohonen PJ, Latonen LM, Kallioniemi OP, Jenster G, Visakorpi T** 2011 Androgen regulation of micro-RNAs in prostate cancer. Prostate 71:604-614
27. **Sirotkin AV, Laukova M, Ovcharenko D, Brenaut P, Mlyncek M** 2010 Identification of microRNAs controlling human ovarian cell proliferation and apoptosis. J Cell Physiol 223:49-56
28. **Wang XH, Qian RZ, Zhang W, Chen SF, Jin HM, Hu RM** 2009 MicroRNA-320 expression in myocardial microvascular endothelial cells and its relationship with insulin-like growth factor-1 in type 2 diabetic rats. Clin Exp Pharmacol Physiol 36:181-188
29. **Huang P, Gong Y, Peng X, Li S, Yang Y, Feng Y** 2010 Cloning, identification, and expression analysis at the stage of gonadal sex differentiation of chicken miR-363 and 363*. Acta Biochim Biophys Sin (Shanghai) 42:522-529
30. **Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, Thiruchelvam P, Barton G, Jiao LR, Wait R, Waxman J, Hannon GJ, Stebbing J** 2009 The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. Proc Natl Acad Sci U S A 106:15732-15737

31. **Goyal R, Goyal D, Leitzke A, Gheorghe CP, Longo LD** 2010 Brain renin-angiotensin system: fetal epigenetic programming by maternal protein restriction during pregnancy. Reprod Sci 17:227-238
32. **Long J, Wang Y, Wang W, Chang BH, Danesh FR** 2011 MicroRNA-29c is a signature MicroRNA under high glucose conditions which targets sprouty homolog 1, and its in vivo knockdown prevents progression of diabetic nephropathy. J Biol Chem 286:11837-11848
33. **Fred RG, Bang-Berthelsen CH, Mandrup-Poulsen T, Grunnet LG, Welsh N** 2010 High glucose suppresses human islet insulin biosynthesis by inducing miR-133a leading to decreased polypyrimidine tract binding protein-expression. PLoS One 5:e10843
34. **Granjon A, Gustin MP, Rieusset J, Lefai E, Meugnier E, Guller I, Cerutti C, Paultre C, Disse E, Rabasa-Lhoret R, Laville M, Vidal H, Rome S** 2009 The microRNA signature in response to insulin reveals its implication in the transcriptional action of insulin in human skeletal muscle and the role of a sterol regulatory element-binding protein-1c/myocyte enhancer factor 2C pathway. Diabetes 58:2555-2564

Figure XII-3. Fetal ovarian miRNA expression affected by prenatal T (T) and T plus AR antagonist (TF) treatment as determined by quantitative RT-PCR of miRNA from whole ovarian tissue from fetal d90. Treatment effects on expression of miRNA were determined by one-way ANOVA followed by Dunnett's *post hoc* tests. *Asterisks* indicate significant differences from control ($P < 0.05$). Note for miR-29a (*bottom panel*) the T group showed a tendency to be higher than controls ($P = 0.0613$).

Figure XII-3


miR-15b/U6
*
Control
T
TF
miR-497/U6
*
Control
T
TF
miR-29a/U6
Control
T
TF

5. Discussion

Findings from this study demonstrate that prenatal T treatment alters the developmental expression of key ovarian steroidogenic enzymes and miRNA during fetal life. These early developmental changes likely contribute toward the ovarian disruption and increased estradiol release seen in the adult prenatal T-treated females. Comparison of mRNA and miRNA expression data between prenatal T and prenatal T plus AR antagonist treated females provide evidence that some of the regulation is mediated via androgenic programming and others likely by estrogenic programming.

Key ovarian genes expressed by fetal d65 ovary

Our studies show that expression of *3*β*HSD*, essential for the biosynthesis of steroids, namely progesterone, androgens, and estrogens, is evident before primordial follicular differentiation (Sawyer, et al. 2002). Earlier studies have found cells within cell streams and rete cell tubules contain 3βHSD (Quirke, et al. 2001), suggesting that somatic cells destined to differentiate into granulosa/theca cells are likely the sites of this expression (Conley, et al. 1995). The findings that d65 fetal ovary expresses *Cyp19* (aromatase) and *Cyp11a1* are also in agreement with earlier findings (Quirke, et al. 2001). In addition, for the first time, our findings document that fetal d65 sheep ovary expresses 5α-reductase and *17*β*HSD*. Expression of gonadotropin receptors (*LHR* and *FSHR*), steroid receptors (*AR*,*ESR1*, and *ESR2*), and *GDF9* mRNA in the d65 fetal ovary is also consistent with previous findings (Hogg, et al. 2011, Juengel, et al. 2006, Mandon-

Pepin, et al. 2003). Expression of *PGR* in our study at d65 and not until d75 in an earlier study (Juengel, et al. 2006) may relate to differences in sensitivity of the approaches used.

Changes in expression of ovarian genes from fetal d65 to d90

The increase in expression of *3βHSD*, a new finding, and decrease in *17βHSD* in d90 fetal ovary relative to fetal d65 ovary corresponds to the time when primordial follicular differentiation occurs. The decrease in *17βHSD* seen in d90 ovary is expected because overexpression would lead to masculinization of external and internal genitalia in female fetuses (Saloniemi, et al. 2009). The direction of change in *AR*, *PGR*, and *ESR1* (increase) seen between d65 and d90 parallels previous findings (Saloniemi, et al. 2009). The dichotomy in *ESR1* and *ESR2* expression with *ESR1* increasing, but not *ESR2*, likely reflects the roles they play at this developmental stage. The increase in *ESR1* between d65 and d90 coincides with continued formation of ovigerous cords and ovarian tissue remodeling (Sawyer, et al. 2002). The increase in mRNA expression from d65 to d90 of *FDX1*, a key step in P450 enzymes activity including *Cyp11a1*, *Cyp17*, and *Cyp19* (Miller 2005), supports increased steroidogenic ability of the ovary. The increase in expression of *GDF9* and *IGF-I* receptor between d65 and d90 likely plays a role in advancing follicular differentiation and establishing oocyte somatic cell communication.

Effects of gestational T treatment on fetal ovarian gene expression

Prenatal T treatment had fetal age-specific but opposing effects on the expression of *Cyp11a1* and *Cyp19*, with the effect on *Cyp11a1* evident at fetal d90, and *Cyp19* at fetal d65. Prenatal T-induced increase in *Cyp19* is in line with increased estradiol levels seen in fetal circulation (Veiga-Lopez, et al. 2011). This suggests that the fetal ovary also contributes toward the aromatization of T to estradiol during the T treatment period. These changes differ from those of the Scottish Greyface sheep (Hogg, et al. 2011), which may reflect timing of exposure to T (starting d30 in present study and d60 in the Hogg *et al*. study) or breed differences. A trend for an increase in 5α-reductase was also evident in d65 T-treated fetuses, a time point when a significant increase in *Cyp19* was evident, supporting the possibility of conversion of T to both estradiol [increased in d 65 female fetuses (Veiga-Lopez, et al. 2011)] and DHT. These changes appear to be mediated by estrogenic actions stemming from aromatization of T because cotreatment with T and an AR antagonist resulted in similar changes in *Cyp19* (significant increase) and 5α-reductase (tendency for an increase) expression. The finding that such changes are evident only at d65 but not at d90 suggests vulnerable periods for susceptibility to reprogramming ovarian function; prenatal T treatment from d30 to d90 but not from d60 to d90 leads to polycystic ovarian phenotype (Padmanabhan, et al. 2010a). The decrease in *Cyp11a1* mRNA expression at d90 also occurred in Scottish Greyface sheep (d60–90 of gestation) (Hogg, et al. 2011). This reduction in *Cyp11a1* mRNA expression in the T group, if evident at protein level, would reduce conversion of cholesterol to the steroid precursor pregnenolone and consequent downsteam effect on steroid production. A recent

study found mutation in *Cyp11a1* gene resulted in phenotypes ranging from classic lipoid congenital adrenal hyperplasia to a nonclassic phenotype (Sahakitrungruang, et al. 2011). The paradoxical increase in *Cyp11a1* only in T plus AR antagonist-treated but not T-treated d65 ovaries suggests that the AR antagonist, flutamide, may have direct effects, such as previously reported for rats (Kubota, et al. 2003). Alternatively, these findings support the need for a threshold level of endogenous androgen action, which flutamide cotreatment overcomes. Failure of AR antagonist cotreatment to overcome the effects of T treatment on *Cyp11a1* at fetal d90 supports estrogenic mediation. The lack of effect of T treatment on mRNA expression of *AR* does not parallel our findings of increased *AR* protein expression in the stroma and granulosa cells of fetal d90 ovaries (Ortega, et al. 2009). Such differences may relate to differences in the impact of T at the mRNA and protein level or alternatively that monitoring changes in whole ovary dilutes detection of cell-specific changes. The lack of changes in *ESR2* and *PGR* mRNA expression parallel findings with immunocytochemical approaches (Ortega, et al. 2009).

Effects of gestational T treatment on fetal ovarian miRNA expression

MicroRNA, short noncoding RNA that mediate gene expression post-transcriptionally, regulate gene expression important in cellular differentiation and tissue development (Bernstein, et al. 2003, Hong, et al. 2008). They have been identified in the fetal ovary of the sheep (Torley, et al. 2011) and cow (Tripurani, et al. 2010) as well as shortly after birth in the mouse (Choi, et al. 2007, Ro, et al. 2007). Evidence exists in support of T-regulating expression of distinct miRNA via genotropic and nongenotropic mechanisms in the mouse liver (Delic, et al. 2010, Narayanan, et al. 2010). Our finding

of 31 up-regulated and 18 down-regulated miRNA in the d90 gestational T-treated ovaries is consistent with this premise. The finding that 35 miRNA were differentially expressed between T and T plus AR antagonist-treated fetal d90 ovaries indicate some of the mediation occurs via androgenic and others estrogenic pathways. A recent study found several miRNA to be differentially expressed in fetal d42 and d75 ovary and testes (Torley, et al. 2011). Importantly, several of these expressed miRNA are predicted to target genes such as *ESR1*, *CYP19*, and Sry-related-HMG Box (*SOX*), which are known to be important in gonadal development. Several of the miRNA differentially expressed in our gestational T-treated ovaries (see Table XIII.3) have been shown to be estrogen or androgen regulated in other studies (see references associated with Table XIII.3). In our study miR-378 exhibited the greatest decrease in expression in response to the prenatal T treatment; interestingly, this miRNA was recently shown to posttranscriptionally regulate granulosa cell aromatase levels (Xu, et al. 2011). Furthermore, down-regulation of miR-378 is consistent with the increase in aromatase mRNA expression observed in the d65 ovaries from T-treated dams.

A common theme identified from comprehensive literature and bioinformatic analysis was that many of the differentially expressed miRNA are linked to regulation of insulin signaling and metabolism (Table XIII.3). Interestingly, two miRNA up-regulated by T, miR-497 and miR-15b, share similar seed sequences, or bases 2–9 of the miRNA that are thought to be the primary bases that interact with the 3'untranslated region of the mRNA target. Bioinformatic analysis of putative targets for family members, miR-497 and miR-15b identified a number of members of the insulin signaling pathway including insulin receptor, IRS2, IGF1R, IGF2R, and pregnancy-associated plasma protein

A/pappalysin1, which cleaves IGF binding protein to regulate insulin signaling. miRNA-15b was previously identified as being regulated by FSH in the ovary (Yao, et al. 2009) and differentially expressed in rat models of diabetes (Herrera, et al. 2009). Thus far, none of the putative targets of miR-497 have been validated functionally, and further study is needed to elucidate whether it impacts insulin signaling. Prediction of potential targets for miR-29a also revealed members of the insulin signaling pathway (IGF-I, insulin induced gene 1, INSIG1), and miR-29a is up-regulated in patients with diabetes (Kong, et al. 2011). Moreover, miR-29a was recently shown to regulate the expression of p85 subunit of and PI3K, preventing insulin-mediated activation of Akt and downstream genes involved in gluconeogenesis (He, et al. 2007, Herrera, et al. 2009, Pandey, et al. 2011)).

Examination of mRNA expression of several of these genes in fetal ovarian tissues failed to detect loss or gain in mRNA expression for this entire class of insulin regulated genes. Our studies, however, examined only mRNA expression, and although evidence exists that miRNA decrease levels of their specific target transcripts approximately 20% (Guo, et al. 2010), a large number of studies implicate blockade of translation as the predominant miRNA mediated posttranscriptional regulatory mechanism in animals (Kiriakidou, et al. 2007, Petersen, et al. 2006). In-depth analyses of protein levels within fetal ovarian tissues of T-treated ewes will address whether any of the putative miRNA-regulated, insulin-related genes are regulated by the differentially expressed miRNA. Although the targets of the specific miRNA remains to be determined at the ovarian level, the predicted targets are consistent with steroidal and metabolic perturbations in the gestational T-treated fetuses.

Functional significance

Because the sheep genome is not completely characterized and annotated information is not available, a global screen using arrays is not optimal to get a more comprehensive assessment of changes in transcriptome. As such, we chose to target our investigation to several critical regulatory genes implicated in ovarian differentiation. Identified changes are biologically relevant and link well with subsequently observed functional changes. The increase in Cyp19 seen during fetal life and the increase in estradiol found in adult prenatal T-treated females substantiate programming of adult phenotype early in life. Similarly, the increase in 5α-reductase activity seen in T fetuses may form the basis for the increased expression in 5α-reductase in granulosa cells of polycystic ovarian syndrome ovaries (Jakimiuk, et al. 1999), the reproductive and metabolic phenotype of whom the prenatal T-treated sheep recapitulates. Because the mechanisms regulating ovarian differentiation and follicular activation/recruitment/persistence are poorly understood, defining the relative role of early changes in 5α-reductase, Cyp19, and Cyp11a1 in reprogramming ovarian dysfunction and identifying additional mediators is an exciting avenue for future research. Changes in miRNA expression after prenatal T exposure, in concert with their reported involvement in cellular differentiation and tissue development, suggest that miRNA are likely to play a role in ovarian remodeling. Similarly, linkage of several of the differentially regulated miRNA to insulin signaling and ovarian steroidogenesis bring functional relevance to these findings in view of the functional hyperandrogenism and insulin resistance manifested by the adult prenatal T-treated females (Padmanabhan, et al. 2010a). Furthermore, given the general importance of this gene regulatory system and its

disruption in type 2 diabetes (Ferland-McCollough, et al. 2010), these miRNA may be involved in long-term alteration of insulin sensitivity at the ovarian level in prenatal T-treated females due to reprogramming of the fetal ovary. Establishment of specific function attributed to each miRNA impacted by prenatal T excess would require site-specific targeted knockdown or functional testing *in vitro* using ovaries generated from control and prenatal T-treated females, a goal for the future. Nonetheless, considering that studies relative to miRNA are in their infancy, the findings that prenatal T excess modulates expression of miRNA implicated in insulin and steroidogenic pathway very early during fetal life are novel, in view of the modulatory role insulin and steroids play in establishing ovarian sensitivity and differentiation, respectively.

6. Acknowledgments

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XIII: CHAPTER 8

Expression of microRNA in human theca cells is altered in polycystic ovarian syndrome and by forskolin treatment.

1. Abstract

Polycystic ovarian syndrome (PCOS) is a heterogenous syndrome with origins that are poorly understood, yet appears to be a heritable disease. Numerous genetic studies have attempted to identify candidate genes that may be involved in the manifestation of this syndrome, yet have yielded no gene or loci that can definitely describe the underlying genetic cause. A potential alternative mechanism is microRNA (miRNA) mediated post-transcriptional gene regulation. The aim of this study was to identify miRNA expressed in human theca cells in basal and forskolin treated conditions and to then determine if miRNA expression is aberrantly expressed in PCOS. Using a qRT-PCR miRNA profiling platform, we identified 191 miRNA expressed in human theca cells. We further identified 13 miRNA differentially expressed in PCOS theca cells compared to normal theca cells. Two of these miRNA, mir-181a and -181b, are clustered together and share similar seed sequences. Bioinformatic analysis indicates that potential targets include members of the insulin/IGF signaling pathway and steroid hormone receptors. Future studies will inhibit and over-express these miRNA in normal and PCOS theca cells, as well as attempt to validate potential targets to determine what function they may play in this syndrome and determine if they can be used as non-invasive biomarkers for easier or more thorough diagnosis.

2. Introduction

Polycystic ovarian syndrome (PCOS) is the most prevalent endocrine disorder affecting women and is one of the leading causes of female infertility (Teede, et al. 2010). The most common and defining features of PCOS are unexplained, chronic hyperandrogenism, oligo/anovulation, and the presence of polycystic ovaries (Rotterdamn Criteria (Rotterdam 2004)). The increased production of androgens is believed to be due to ovarian thecal cell hyperplasia and sensitivity to gonadotropins, thus increasing cellular steroidogenic potential (Magoffin 2006). Ovaries of women suffering from PCOS often have the presence of multiple, persistent follicular cysts on the ovary that are unable to undergo ovulation, yet do not undergo atresia to allow the beginning of another ovarian cycle (Chang 2007). Women with PCOS often have increased serum levels of the gonadotropin luteinizing hormone (LH; (Rebar, et al. 1976)), which binds directly to the thecal cell membrane and promotes increased androgen production. Diagnosis of PCOS is often associated with obesity, type 2 diabetes, insulin resistance, and/or metabolic syndrome (Azziz 2006). The underlying cause of PCOS remains unknown, however numerous association studies suggest that it is an inherited disorder that is genetic in origin (Azziz, et al. 2011, Kosova and Urbanek 2012).

Excess steroid production in women with PCOS is largely attributed to the hyper-synthesis and secretion of androgens from ovarian theca cells (Gilling-Smith, et al. 1997). Freshly isolated primary and long-term passaged cultured human PCOS theca cells exhibit increased basal and LH/cAMP-stimulated steroidogenic capacity, producing elevated levels of testosterone, progesterone, and other intermediaries compared to normal theca cells on a per cell basis (Gilling-Smith, et al. 1994, Nelson, et al. 1999).

Expression of the steroidogenic enzymes necessary for androgen production, CYP17, CYP11A1, HSD3B2, and 20α-HSD, are elevated in cultured PCOS theca cells (Nelson, et al. 1999, Nelson, et al. 2001). Microarray expression profiling of messenger RNA (mRNA) has further verified the distinct molecular phenotype of cultured PCOS theca cells compared to cultured normal theca cells (Wood, et al. 2003). These stable and consistent changes in the transcriptome of theca cells obtained from women suffering from PCOS, even after long-term passage in the absence of any effectors, suggests an intrinsic change in the cellular programming that is responsible for the altered cellular function (Wood, et al. 2004).

One mechanism to change cellular programming is via post-transcriptional regulation of gene expression. Multiple mechanisms of post-transcriptional gene regulation have been identified and broadly include the regulation of mRNA degradation and subsequent translation (Halbeisen, et al. 2008). Previous studies have reported that post-transcriptional gene regulation is altered in PCOS theca cells (Wickenheisser, et al. 2005). Stability of CYP17 mRNA is increased in PCOS theca cells, exhibiting a two-fold longer half-life compared to normal theca cells (Wickenheisser, et al. 2005). This increase in mRNA stability appears to be specific to CYP17, as the half-life of steroidogenic acute regulatory protein (StAR) mRNA was not altered in thecal PCOS cells. The reason for this selective regulation of specific mRNA degradation following transcription, rather than a global change in mRNA half-lives (i.e. increased expression of mRNA endo- or exo-nucleases) remains unknown. One potential mechanism that could explain this transcript-specific regulation is microRNA (miRNA) mediated mRNA degradation. MicroRNA are short ~22 nucleotide long non-coding RNA molecules that

post-transcriptionally regulate gene expression by base-pairing with complementary sequences in the 3'untranslated region (3'UTR) and facilitating mRNA degradation or inhibition of translation (Filipowicz, et al. 2008). The majority of miRNA are transcribed by RNA polymerase II into primary transcripts that can be several kilobases long and share properties similar to protein coding RNAs (Lee, et al. 2004). A series of RNAse endonucleases, Drosha and Dicer, then cleave the primary miRNA into the mature miRNA (Hutvagner, et al. 2001, Lee, et al. 2004). To date, over 2000 mature miRNA have been identified in the human (mirBase V19) and it is believed that over 60% of protein coding genes are targeted by miRNA (Friedman, et al. 2009). Because most miRNA are transcribed by RNA polymerase II similar to mRNA encoding genes, they have canonical transcriptional start sites and promoter elements that allow for rapid and specific spatial-temporal induction of expression (Bushati and Cohen 2007). Functionally, miRNA are involved in the regulation of nearly every cellular and biological process, including cell proliferation and differentiation, apoptosis, steroid synthesis, and intra- and extra-cellular signaling (Bushati and Cohen 2007, Huang, et al. 2011). Aberrant miRNA expression and function has been implicated in numerous diseases including hormone dependent cancers of the prostrate and breast (Tessel, et al. 2010), ovarian cancer (Dahiya and Morin 2010), metabolic disorders (Rottiers and Naar 2012), type 2 diabetes mellitus (Ali, et al. 2011, Shantikumar, et al. 2012), and cardiac disease (Dorn 2011). MicroRNA are present in serum and other bodily fluids, thus allowing the systemic transport of these molecules and also providing clinicians valuable biomarkers (Reid, et al. 2011).

In the ovary, miRNA expression has previously been reported to be hormonally regulated by gonadotropins (Carletti, et al. 2010, Fiedler, et al. 2008, Yao, et al. 2009, Yao, et al. 2010) and steroid hormones (Klinge 2012, Luense, et al. 2011). Conditional loss of miRNA in murine ovarian granulosa cells resulted in altered folliculogenesis (Lei, et al. 2010), decreased ovulation rate (Carletti, et al. 2010, Hong, et al. 2008) and increased trapped oocytes in luteinized follicles (Carletti, et al. 2010, Hong, et al. 2008, Nagaraja, et al. 2008) thus suggesting an important role for miRNA in ovulation and other facets of ovarian function. Loss of miRNA in the murine ovary also prevented the formation of a functional corpus luteum due to impaired angiogenesis (Otsuka, et al. 2008). To date, no reported studies have examined the expression or possible function of miRNA in ovarian theca cells or their possible role in the pathobiology of PCOS. Therefore, this study compares miRNA expression in thecal cells from PCOS versus normal (control) patients. Given that miRNA can be hormonally regulated, this study additionally investigates whether expression of miRNA in human theca cells (both PCOS and normal) is regulated by the cAMP pathway (ie, forskolin) which mediates much of LH/LH receptor cell signaling activity within these cells.

3. Materials and Methods

Human theca interna tissue was obtained from follicles of women undergoing hysterectomy, after informed consent, under a protocol approved by the Institutional Review Board of the Pennsylvania State University College of Medicine. Individual follicles were dissected away from ovarian stroma. The isolated follicles were size selected for diameters ranging from 3 to 5 mm so that theca cells derived from follicles of similar size from normal and PCOS subjects could be compared. The dissected follicles were placed into serum-containing medium and bisected. Under a dissecting microscope, the theca interna was stripped from the follicle wall, and the granulosa cells were removed with a platinum loop. The cleaned theca cell layers were dispersed with 0.05% collagenase I, 0.05% collagenase IA, and 0.01% deoxyribonuclease in medium containing 10% fetal bovine serum (FBS; (McAllister, et al. 1994) . Dispersed cells were placed in culture dishes that had been precoated with fibronectin by incubation at 37 C with culture medium containing 5 μg/ml human fibronectin. The growth medium used was a 1:1 mixture of Dulbecco's Eagle's Medium (DME) and Ham's F-12 medium containing 10% FBS, 10% horse serum, 2% UltroSer G, 20 nm insulin, 20 nm selenium, 1 μm vitamin E, and antibiotics. From each follicle, 12 35-mm dishes of primary theca interna cells were grown until confluent, removed from the dish with neutral protease (pronase-E; protease type XXIV; Sigma, St. Louis, MO) in DME-F12 (1:1), frozen, and stored in liquid nitrogen (one 35-mm dish per vial) in culture medium that contained 20% FBS and 10% dimethylsulfoxide. In all experiments cells were thawed and propagated in the growth medium described above. To obtain successive passages of normal and PCOS theca cells, cells were thawed, propagated, and frozen at consecutive passages. The cells

were grown in 5% O_2, 90% N_2, and 5% CO_2. Reduced oxygen tension and supplemental antioxidants (vitamin E and selenium) were used to prevent oxidative damage.

The PCOS and normal ovarian tissue came from age-matched women, 38–40 yr old. The diagnosis of PCOS was made according to established guidelines (Rotterdam 2004)), including hyperandrogenemia; oligoovulation; and the exclusion of 21-hydroxylase deficiency, Cushing's syndrome, and hyperprolactinemia. All of the PCOS theca cell preparations studied came from ovaries of women with fewer than six menses per year and elevated serum total testosterone or bioavailable testosterone levels, as previously described (Legro, et al. 1998, Nelson, et al. 1999). Each of the PCOS ovaries contained multiple subcortical follicles of less than 10 mm in diameter. The control (normal) theca cell preparations came from ovaries of fertile women with normal menstrual histories, menstrual cycles of 21–35 d, and no clinical signs of hyperandrogenism. Neither PCOS nor normal subjects were receiving hormonal medications at the time of surgery. Indications for surgery were dysfunctional uterine bleeding, endometrial cancer, and pelvic pain. The passage conditions and split ratios for all normal and PCOS cells were identical. Experiments comparing PCOS and normal theca were performed using fourth-passage (31–38 population doublings) theca cells isolated from size-matched follicles obtained from age-matched subjects. The theca cells examined in these experiments included stocks of cells isolated and propagated from PCOS and normal women that we have previously examined (Nelson-DeGrave, et al. 2004)) as well as stocks of cells that we have recently generated from newly characterized patients. Sera and growth factors were obtained from the following sources: FBS and DME/F12 were obtained from Irvine Scientific (Irvine, CA); horse serum was

obtained from HyClone (Logan, UT); UltroSer G was from Reactifs IBF (Villeneuve-la-Garenne, France).

Isolation and analysis of miRNA

Total RNA was isolated with Trizol (Sigma-Aldrich, St. Louis, MO) per manufacturer's instruction from fourth passaged theca cells that were grown to subconfluence, transferred into serum-free media, and treated with 20μM forskolin (Sigma-Aldrich) or vehicle control. MicroRNA were reverse transcribed from 50ng of total RNA using the miRCURY LNA universal RT kit (Exiqon, Denmark). The reverse transcribed miRNA products were diluted 1:110, added to an equal volume of SybrGreen MasterMix (Exiqon, Denmark) and loaded onto miRCURY LNA microRNA PCR panels containing a total of 742 miRNA based on human miRBase 16 (Human Panels I and II, V2.M, Exiqon, Denmark). Quantitative RT-PCR was performed using the ABI 7900HT Fast Real Time System with an initial polymerase activation/denaturation step at 95 °C for 10 min followed by 40 cycles at 95 °C for 10 sec and 60 °C for 60 sec. Following product amplification, a melting curve analysis was performed to insure amplification of a sole miRNA product.

Statistical Analysis

To analyze data obtained from qRT-PCR panels the threshold (Ct) levels for each sample was set at log1 in the SDS V4.0 software (Applied Biosystems, Foster City, CA). Threshold values greater than 37 were considered non-detectable and miRNA with Cts greater than 37 in more than 1 patient per group were omitted from further analysis. Data

were analyzed by the ΔΔ Ct method using the global mean of each plate as the normalizer (ΔCt). Comparisons were then made between patient and treatment groups (ΔΔCt) followed by calculation of the fold change (2^ΔΔCt). To determine if any expression changes occurred, a nested ANOVA statistical test was performed (Matlab, MathWorks, Natick, MA).

Bioinformatic analysis of predicted miRNA targets

To identify putative mRNA targets, bioinformatic analysis was conducted on all differentially expressed miRNA using TargetScan 6.2 (www.targetscan.org). The focus of these analyses was on steroid receptors/enzymes, ovarian regulatory molecules, insulin signaling molecules, and lipid metabolic hormones. A comprehensive literature based analysis was also undertaken for all differentially expressed miRNA linked to steroid receptor action, steroidogenesis, ovarian function, sexual differentiation, insulin-signaling, diabetes, and lipid metabolism (Luense, et al. 2011). Gene ontology analysis of putative mRNA targets of differentially expressed miRNA was conducted by using miRGator Functional Analysis (http://genome.ewha.ac.kr/miRGator).

4. **Results**

Expression of miRNA in human thecal cells

To identify and quantify human theca cell miRNA, quantitative RT-PCR panels pre-loaded with locked nucleic acid (LNA) primers for 742 human miRNA sequences obtained from miRbase V.16 were used. A total of 191 miRNA were found to be expressed (Ct < 37) in normal, untreated human theca cells (Table XIII-1). A total of 184 miRNA were found to be expressed (Ct < 37) in un-stimulated, PCOS theca cells (Table XIII-1).

Table XIII-1. MicroRNA expressed in human theca cells

miRNA expressed in normal and PCOS theca cells			
hsa-let-7a	hsa-mir-140-3p	hsa-mir-193a-5p	hsa-mir-27a
hsa-let-7b	hsa-mir-140-5p	hsa-mir-193b	hsa-mir-27b
hsa-let-7c	hsa-mir-142-3p	hsa-mir-195	hsa-mir-28-5p
hsa-let-7d	hsa-mir-143	hsa-mir-197	hsa-mir-299-5p
hsa-let-7d*	hsa-mir-145	hsa-mir-1979	hsa-mir-29a
hsa-let-7e	hsa-mir-146b-5p	hsa-mir-199a-3p	hsa-mir-29b
hsa-let-7f	hsa-mir-148b	hsa-mir-199a-5p	hsa-mir-29c
hsa-let-7g	hsa-mir-149	hsa-mir-199b-5p	hsa-mir-301a
hsa-let-7i	hsa-mir-151-3p	hsa-mir-19a	hsa-mir-30a
hsa-mir-100	hsa-mir-151-5p	hsa-mir-19b	hsa-mir-30b
hsa-mir-103	hsa-mir-152	hsa-mir-20a	hsa-mir-30c
hsa-mir-106a	hsa-mir-154	hsa-mir-20b	hsa-mir-30d
hsa-mir-106b	hsa-mir-154*	hsa-mir-21	hsa-mir-30e
hsa-mir-107	hsa-mir-155	hsa-mir-21*	hsa-mir-30e*
hsa-mir-10a	hsa-mir-15a	hsa-mir-210	hsa-mir-31
hsa-mir-10b	hsa-mir-15b	hsa-mir-212	hsa-mir-31*
hsa-mir-125a-5p	hsa-mir-16	hsa-mir-214	hsa-mir-320a
hsa-mir-125b	hsa-mir-17	hsa-mir-218	hsa-mir-323-3p
hsa-mir-126	hsa-mir-181a	hsa-mir-22	hsa-mir-324-3p
hsa-mir-126*	hsa-mir-181b	hsa-mir-22*	hsa-mir-324-5p
hsa-mir-127-3p	hsa-mir-181d	hsa-mir-221	hsa-mir-328
hsa-mir-128	hsa-mir-185	hsa-mir-222	hsa-mir-329
hsa-mir-130a	hsa-mir-186	hsa-mir-23a	hsa-mir-331-3p
hsa-mir-130b	hsa-mir-18a	hsa-mir-23b	hsa-mir-335
hsa-mir-132	hsa-mir-18a*	hsa-mir-24	hsa-mir-337-3p
hsa-mir-134	hsa-mir-18b	hsa-mir-25	hsa-mir-338-3p
hsa-mir-135a	hsa-mir-191	hsa-mir-26a	hsa-mir-339-5p

miRNA expressed in normal and PCOS theca cells

hsa-mir-135b	hsa-mir-192	hsa-mir-26b	hsa-mir-33a
hsa-mir-342-3p	hsa-mir-382	hsa-mir-490-3p	hsa-mir-671-5p
hsa-mir-345	hsa-mir-409-3p	hsa-mir-491-5p	hsa-mir-708
hsa-mir-346	hsa-mir-410	hsa-mir-493	hsa-mir-720
hsa-mir-34a	hsa-mir-411	hsa-mir-494	hsa-mir-744
hsa-mir-34c-3p	hsa-mir-421	hsa-mir-495	hsa-mir-886-3p
hsa-mir-361-3p	hsa-mir-423-3p	hsa-mir-497	hsa-mir-886-5p
hsa-mir-365	hsa-mir-423-5p	hsa-mir-501-5p	hsa-mir-92a
hsa-mir-369-5p	hsa-mir-424	hsa-mir-502-5p	hsa-mir-92b
hsa-mir-370	hsa-mir-425	hsa-mir-503	hsa-mir-93
hsa-mir-374b	hsa-mir-425*	hsa-mir-505	hsa-mir-934
hsa-mir-342-3p	hsa-mir-431	hsa-mir-532-5p	hsa-mir-940
hsa-mir-347	hsa-mir-433	hsa-mir-542-5p	hsa-mir-98
hsa-mir-348	hsa-mir-450a	hsa-mir-574-3p	hsa-mir-99a
hsa-mir-34a	hsa-mir-455-5p	hsa-mir-590-5p	hsa-mir-99b
hsa-mir-34c-3p	hsa-mir-483-3p	hsa-mir-615-3p	snord38b
hsa-mir-379	hsa-mir-484	hsa-mir-652	snord49a
hsa-mir-381	hsa-mir-487b	hsa-mir-660	u6

Only Normal			**Only PCOS**
hsa-mir-137	hsa-mir-330-3p	hsa-mir-454	hsa-mir-148b
hsa-mir-181a*	hsa-mir-337-5p	hsa-mir-625*	hsa-mir-500
hsa-mir-204	hsa-mir-34c-5p	hsa-mir-99a*	hsa-mir-539
hsa-mir-301b	hsa-mir-362-5p		hsa-mir-654-5p

Differential expression of thecal miRNA in normal versus PCOS ovaries

To identify miRNA that are aberrantly expressed in PCOS theca cells, expression levels of miRNA in normal thecal cells were compared to PCOS theca cells. A total of 13 miRNA were found to exhibit altered expression in PCOS theca cells compared to theca cells from normal ovaries (Table XIII-2). The three miRNA that were up regulated in PCOS theca cells ranged from 1.36 to 2.84-fold increased expression, while the 10 miRNA down-regulated in cultured theca cells from polycystic ovaries ranged from -1.40 to -5.06. Bioinformatic analysis revealed multiple differentially expressed miRNA had complementary sequences in the 3'UTR of mRNA coding for numerous receptors and factors involved in insulin signaling, lipid metabolism, steroid receptors, and other factors important to ovarian function and development (i.e. β-catenin, matrix-metalloproteinase, etc) (Table XIII-2).

Interestingly, two miRNA, which exhibited decreased expression in PCOS theca cells (miR-181a and -181b) are co-transcribed as one primary transcript and share a similar seed sequence (i.e., likely target similar mRNA targets; Figure XIII-2). A thorough review of the literature identified numerous published studies linking miR-181a/b to obesity, metabolic syndrome, coronary artery disease insulin sensitivity, endometrial cancer, as well as many others.

Table XIII-2. miRNA differentially expressed in PCOS theca cells compared to normal theca cells.

miRNA	Fold Change	p-value	Genomic Location	Targetscan Targets
hsa-mir-337-3p	2.8359	0.020979	chr14: 101340830-101340922 [+]	PDE10A, PTEN, SP5, LDLRAD3, INSIG1, SP1, CREBBP, CREB1
hsa-mir-31	1.5445	0.004806	chr9: 21512114-21512184 [-]	IDE, NR5A2, StARD13, ADCY6, AK4, PTGFRN, SOX11, CTNND2, LRP10, PDE4D
hsa-let-7c	1.3639	0.05243	chr21: 17912148-17912231 [+]	
hsa-mir-30a	-1.3955	0.035004	chr6: 72113254-72113324 [-]	
hsa-mir-30e*	-1.4135	0.010114	chr1: 41220027-41220118 [+]	
hsa-mir-185	-1.4852	0.039367	chr22: 20020662-20020743 [+]	
hsa-mir-708	-1.4924	0.041569	chr11: 79113066-79113153 [-]	
hsa-mir-181a	-1.7036	0.019361	chr1: 198828173-198828282 [-] chr9: 127454721-127454830[+]	TGFBRAP1, PDE5A, SPRY4, PPARA, PARM1, ADCY1, CREB1, IRS2, ADCY9, LRP12, protocadherins, BMPR2, RUNX1, EPS15, NR4A3, PDE10A, KITL, PGR, IGF2BP3, LRP4, ESR1, SP1, LRP6, CYP26B1,
hsa-mir-33a	-1.8044	0.027328	chr22: 42296948-42297016 [+]	
hsa-mir-331-3p	-1.8244	0.045723	chr12: 95702196-95702289 [+]	
hsa-mir-130b	-2.017	0.047158	chr22: 22007593-22007674 [+]	
hsa-mir-181b	-2.6567	0.012991	chr1: 198828002-198828111 [-] chr9: 127455989-127456077[+]	TGFBRAP1, PDE5A, SPRY4, PPARA, PARM1, ADCY1, CREB1, IRS2, ADCY9, LRP12, protocadherins, BMPR2, RUNX1, EPS15, NR4A3, PDE10A, KITL, PGR, IGF2BP3, LRP4, ESR1, SP1, LRP6, CYP26B1,
hsa-mir-204	-5.0555	0.048868	chr9: 73424891-73425000 [-]	SOX4, ANGPT1, IGFBP5, ESRRG, PPARGC1A, AK4, HSD17B2, IGF2R, CREB5, ADCY6, SOX11, SPRY3, NPTX1, LRP2, NRG3, EPS8, BDNF, WNT4, CCND1, ESR1, TGFPR1, NRP1, IGF2BP3, CREB1, LRP8, RUNX1T1, SP1, PTGIS,

Figure XIII-1. Sequence alignment of mature hsa-miR-181a and b. Bases highlighted in red are different between 181a and 181b.

Figure XIII-1

hsa-mir-181a-5p	aacauucaacgcugucggugagu	chr1: 198828173-198828282 [-]
hsa-mir-181a-5p	aacauucaacgcugucggugagu	chr9: 127454721-127454830 [+]
hsa-mir-181b-5p	aacauucauugcugucgguggguu	chr1: 198828002-198828111 [-]
hsa-mir-181b-5p	aacauucauugcugucgguggguu	chr9: 127455989-127456077 [+]

Another miRNA, miR-204, which exhibited the greatest decrease expression in PCOS thecal cells (-5.1) also has a number of interesting putative target genes (Table XIII-2). Several factors involved in insulin signaling (IGF2R, IGFBP5, PPARGC1A, IGF2BP3), estrogen signaling and steroidogenesis (ESR1, ESRRG, HSD17B2, SP1), cAMP/CREB signaling (CREB1, CREB5, ADCY6), and a number of other ovarian factors or related family members (SPRY3, NPTX1, NRG3, BDNF, WNT4, CCND1, TGFPR1, NRP1). A literature search for miR-204 provided no previous publications for a role in ovarian function, however there appears to be a possible link to obesity and diabetes.

Hormonal stimulation of microRNA in normal and PCOS theca cells

Upon treatment with forskolin, the adenylate cyclase activator used to mimic LH and thus cAMP signaling, 13 miRNA were found to be differentially expressed in normal thecal cells when using a paired t-test (Table XIII-3). Nine miRNA exhibited increased expression in response to forskolin, ranging from 1.27 to 2.0 fold greater expression than in non-treated theca cells. The expression of the four miRNA that decreased expression following forskolin treatment ranged from -1.34 to -3.07.

Table XIII-3. Forskolin regulated miRNA in normal human theca cells

miRNA	Fold Change	p-value	Genomic Location
hsa-mir-34a	2.0052	0.006033	chr1: 9211727-9211836 [-]
hsa-mir-542-5p	1.7726	0.04827	chrX: 133675371-133675467 [-]
hsa-mir-134	1.7522	0.043736	chr14: 101521024-101521096 [+]
hsa-mir-148b	1.5407	0.03327	chr12: 54731000-54731098 [+]
hsa-mir-484	1.5072	0.000502	chr16: 15737151-15737229 [+]
hsa-mir-365	1.3638	0.047706	chr16: 14403142-14403228 [+]
hsa-mir-382	1.3493	0.028936	chr14: 101520643-101520718 [+]
hsa-mir-320a	1.3049	0.000333	chr8: 22102475-22102556 [-]
hsa-mir-125a-5p	1.2718	0.023999	chr19: 52196507-52196592 [+]
hsa-mir-27a	-1.3383	0.012155	chr19: 13947254-13947331 [-]
hsa-mir-27b	-1.8868	0.010257	chr9: 97847727-97847823 [+]
hsa-mir-483-3p	-1.9863	0.037736	chr11: 2155364-2155439 [-]
hsa-mir-337-3p	-3.0654	0.00339	chr14: 101340830-101340922 [+]

Analysis of forskolin effects on miRNA expression in PCOS theca cells indicated that, a total of 25 miRNA were differentially expressed in PCOS thecal cells (Table XIII-4). Expression of 11 miRNA increased in response to forskolin treatment, ranging from 1.20 to 5.24-fold greater, while 14 miRNA had decreased expression, which ranged from -1.19 to -2.49-fold. Surprisingly only two miRNA, miR-125a-5p and miR-34a exhibited increased expression following forskolin treatment in both normal and PCOS thecal cells.

Table XIII-4. Forskolin regulated miRNA in human PCOS theca cells

miRNA	Fold Change	p-value	Genomic location
hsa-mir-99a*	5.2418	0.023291	chr21: 17911409-17911489 [+]
hsa-mir-132	1.7082	0.026774	chr17: 1953202-1953302 [-]
hsa-mir-328	1.5031	0.003344	chr16: 67236224-67236298 [-]
hsa-mir-154	1.4296	0.022894	chr14: 101526092-101526175 [+]
hsa-mir-28-5p	1.4157	0.033191	chr3: 188406569-188406654 [+]
hsa-mir-409-3p	1.3952	0.043925	chr14: 101531637-101531715 [+]
hsa-mir-34a	1.3812	0.043305	chr1: 9211727-9211836 [-]
hsa-mir-125a-5p	1.3148	0.028831	chr19: 52196507-52196592 [+]
hsa-mir-99a	1.2747	0.033282	chr21: 17911409-17911489 [+]
hsa-mir-652	1.2666	0.019034	chrX: 109298557-109298654 [+]
hsa-mir-30b	1.1992	0.025256	chr8: 135812763-135812850 [-]
hsa-mir-431	-1.1867	0.025844	chr14: 101347344-101347457 [+]
hsa-mir-16	-1.2381	0.039249	chr13: 50623109-50623197 [-]
hsa-mir-199a-5p	-1.2823	0.035704	chr19: 10928102-10928172 [-]
hsa-mir-379	-1.2861	0.022787	chr14: 101488403-101488469 [+]
hsa-mir-25	-1.3453	0.048742	chr7: 99691183-99691266 [-]
hsa-mir-425	-1.356	0.037014	chr3: 49057581-49057667 [-]
hsa-mir-100	-1.4464	0.021109	chr11: 122022937-122023016 [-] chr1: 198828173-198828282 [-]
hsa-mir-181b	-1.6284	0.008198	chr9: 127455989-127456077 [+]
hsa-mir-503	-1.6607	0.047851	chrX: 133680358-133680428 [-]
hsa-mir-199a-3p	-1.6684	0.030844	chr19: 10928102-10928172 [-]
hsa-mir-376b	-1.9075	0.020373	chr14: 101506773-101506872 [+]
hsa-mir-130b	-1.937	0.030724	chr22: 22007593-22007674 [+]
hsa-mir-214	-2.1399	0.000421	chr1: 172107938-172108047 [-]
hsa-mir-20b	-2.492	0.030058	chrX: 133303839-133303907 [-]

5. Discussion

These experiments characterize the expression profile of miRNA in human theca cells. Additionally, it identifies miRNA aberrantly expressed in PCOS theca cells and miRNA that are regulated by cAMP (forskolin treatment) in theca cells. To the best of our knowledge, this is the first report of a human theca cell miRNA profile and also the first study to look at expression of miRNA in cells from women with PCOS. Numerous genetic studies have identified candidate genes responsible for development of PCOS, however none of these candidates fully explains the heterogenous nature of this disorder (Kosova and Urbanek 2012). The aim of this study was to characterize miRNA, a mechanism of post-transcriptional gene regulation, in normal and PCOS theca cells in an attempt to determine if a factor other than transcriptional regulation of protein-coding genes is involved in the PCOS etiology.

Of the 191 miRNA identified as expressed in normal theca cells, 13 (6.8%) were found to be differentially expressed in theca cells from PCOS ovaries. This low percentage of miRNA changing could reflect the ability of a single miRNA to be able to regulate a large number of mRNA targets (Lewis, et al. 2005). MicroRNA post-transcriptionally regulate gene expression by base-pairing with complementary sequences in the 3'UTR of the target mRNA and initiating RNA degradation or inhibition of translation (Filipowicz, et al. 2008). Bases 2 through 7 of the miRNA, known as the seed sequence, are critical for targeting the RNA-induced silencing complex (RISC) to the target mRNA and regulating expression levels (Lewis, et al. 2005). The shortness of this sequence allows for miRNA to potentially regulate the protein expression of thousands of mRNA targets (Selbach, et al. 2008). Likewise, an individual mRNA may be targeted

by multiple miRNA, thus allowing for the downstream effects of miRNA regulation to be the culmination of multiple small changes (less than 4-fold decrease in protein expression) rather than an individual miRNA:mRNA interaction (Selbach, et al. 2008). Using a bioinformatics algorithm (Targetscan) thousands of putative mRNA targets were identified for the miRNA differentially expressed in this study. While it is unlikely that the majority of these putative targets will be bonafide targets, even if only a few hundred genes are targeted the overall cumulative effect is likely to alter theca cell function. Interestingly, the percentage of miRNA changing in PCOS theca cells compared to normal cells is greater than the number of mRNA transcripts differentially expressed using the same experimental paradigm (2.3% (Wood, et al. 2003). The overall low percentage of change in transcriptionally regulated genes (mRNA and miRNA) may suggest the importance of mechanisms such as post-transcriptional gene regulation.

Of the miRNA differentially expressed in PCOS theca cells compared to normal cells, miR-181a and b are of particular interest. The co-transcription and identical seed sequence of these miRNA make them a particularly interesting family of miRNA as they share a similar set of mRNA targets. Furthermore, they have been reported to be regulated by estrogen and progesterone in the uterus (Pan, et al. 2008) and by estrogen in breast cancer (Maillot, et al. 2009). Expression of miR-181a has been identified in bovine oocytes ((Lingenfelter, et al. 2011) and has been identified in women suffering from preeclampsia (Mayor-Lynn, et al. 2011, Wu, et al. 2012). Micro-RNA-181a has previously been reported to be linked to obesity, metabolic syndrome, and coronary artery disease (Hulsmans, et al. 2012). Multiple studies have also validated the anti-apoptotic factor Bcl-2 as a direct target of both miR-181a and b (Chen, et al. 2010, Li, et

al. 2012, Ouyang, et al. 2012, Zhu, et al. 2012). This could be of particular interest in PCOS theca cells as persistent follicles in polycystic ovaries tend to not undergo atresia as follicles from normal ovaries do. Another interesting validated target of miR-181b is CREB1, a major component in the LH signaling pathway that is critical to regulation of gene programming in thecal cells (Chen, et al. 2012).

Bioinformatic analysis of miR-204 also resulted in the identification of numerous targets of interest to ovarian function. Several members of the insulin signaling pathway were identified as putative targets and a previous study found that expression of miR-204 was down in adipose tissue after exposure to a high fat diet (Chartoumpekis, et al. 2012). While insulin resistance and type 2 diabetes are not necessary for diagnosis of PCOS, they are often observed in women suffering from PCOS (Azziz 2006).

In summary, PCOS is a condition that affects 5 to 7% of women of reproductive age (Guo 2012) and is a major factor in infertility and a diminished quality of life for those affected (Cronin 1998). Even though the cause of this disorder remains unknown, numerous research groups are investigating the molecular changes local to the ovary as well as those of a more systemic origin. The aim of this study was to characterize the expression of miRNA in human theca cells from both normal and PCOS ovaries in basal and hormonally stimulated conditions and identify miRNA that are aberrantly expressed in PCOS theca cells. Future experiments will increase and decrease levels of miR-181a and -181b in our thecal cell culture and attempt to determine what functional significance they have with regards to this syndrome. Putative targets will attempt to be validated. There is much potential for the use of these differentially expressed miRNA to be used as

non-invasive biomarkers and to ideally be used as a therapeutic approach to help treat and understand the basis of PCOS.

SECTION IV

Concluding Remarks

CHAPTER 12

Concluding Remarks

Until recently, our understanding of regulation of ovarian gene expression and how this relates to its ultimate function has largely been focused on a transcriptional or translational level. With the recent identification of miRNA, a new avenue of potential regulatory networks has opened. The focus of the studies presented here begin to investigate what role, if any, miRNA play in ovarian function and development, and to further determine if they are hormonally regulated. We have approached these questions in a multi-species approach, beginning with the mouse to perform conditional genetic deletion experiments to allow us to narrow our focus on miRNA expression and function specifically to the somatic cells of the ovary. Next, we investigated hormonal regulation of miRNA expression and subsequent function in sheep, a model with many similarities to the human. These experiments were designed to allow us to look at the regulation and role of miRNA in a physiologically relevant model and question (pre-natal androgen exposure). Finally, we used our previous experimental findings and techniques to study miRNA expression and potential function in human theca cell in both normal and diseased conditions.

Through these experiments we determined the following:

1. **Dicer, and thus miRNA, are essential for female fertility, as loss of these factors lead to complete female sterility**. Although the primary reason for this loss of female fertility is believed to be due to the formation of large, fluid filled cysts in the oviduct, thus preventing entry of fertilized embryos into the uterus, a drastic decrease in ovulation rate was observed. Use of conditional

deletion mice suggested that ovarian development was perturbed due to the smaller sized ovaries. However, as no defect in folliculogenesis was observed, this suggests that the functional relevance of these miRNA occurs during the LH-regulated peri-ovulatory period.

2. **The LH regulated miRNA-212/132 do not appear to be have a functional role in ovulation or follicular development.** Although these two miRNA are co-transcribed in response to LH and share a similar seed sequence, thus presumably targeting the same mRNA, conditional deletion of these miRNA in ovarian granulosa cells does not appear to have any effect on ovarian function. To ensure that we deleted miR-212/132 at the correct time point, we used two different Cre-driven promoters that would delete miR-212/132 expression during early follicular development or in the antral stage of development.

3. **Expression of steroidogenic enzymes in the fetal sheep ovary are altered in response to prenatal androgen exposure.** The prenatal androgenized sheep model has been proposed as an excellent mechanism to study the possible developmental origins of PCOS. Although much is known about the phenotypes of sheep exposed to excess androgens during gestation, little is known about the changes in fetal ovarian gene expression. This study, for the first time, identified changes in fetal ovarian mRNA gene expression—both with regards to age (d65 vs. d90) and with hormone. The increased

expression of Cyp19 and 5α-reductase corresponds with the need to convert testosterone to subsequent metabolites estradiol or DHT. This finding further supports the theory that the effects of excess testosterone observed in fetal ovaries may also be due to estrogenic, as well as androgenic effects.

4. **Fetal ovarian miRNA are hormonally regulated.** Use of the prenatal androgenized sheep model allowed us to create a profile of miRNA expressed in fetal development—something that would be nearly impossible to acquire in human. Although fetal mouse miRNA profiles have been generated, use of the sheep provides us with the first look at a system more physiological similar to the human. Furthermore, we identified miRNA that were hormonally regulated by excess androgen exposure. Several of these miRNA appear to putatively target genes involved in sex differentiation and the insulin/IGF pathway.

5. **Generation of a profile of miRNA expressed in human theca cells under basal and hormone stimulated conditions.** To our knowledge, this is the first time miRNA have been isolated or characterized from human theca cells. We have generated a profile of miRNA that were expressed in human theca cells under normal conditions (non-treated) and LH stimulated (forskolin treated). These miRNA will provide us important information and insight into potential post-transcriptional regulatory networks in human theca cells.

Comparison of expression profiles of non-treated and forskolin treated cells again provides evidence of hormone mediate miRNA regulation.

6. **miRNA are aberrantly expressed in PCOS theca cells.** Identification of 13 miRNA differentially expressed in human PCOS theca cells compared to normal theca cells provides evidence that miRNA expression are altered in this diseased state. While the actual functions of these miRNA remain to be elucidated, the identification of these miRNA provide us an opportunity to determine if post-transcriptional gene regulation plays a role in the PCOS etiology. Because PCOS remains an enigma, these findings may be a crucial link in us understanding the causes of this condition.

Numerous studies have investigated RNA and protein expression in the ovary, however, these findings, for the first time, implicate miRNA as being important functional mediators of post-transcriptional gene regulation in the somatic cells of ovary. Previous work has established that Dicer is critical for proper oocyte function (Murchison, et al. 2007, Watanabe, et al. 2006), however our conditional deletion of Dicer in the female reproductive tract has established that miRNA are necessary for proper development and function of the oviduct (Hong, et al. 2008). Furthermore, we have established for the first time that miRNA are necessary for ovulation (Hong, et al. 2008). These findings have been the basis for further study of miRNA in relation to female fertility (Carletti and Christenson 2009, Luense, et al. 2009, Nothnick 2012). Combined with a parallel study from our laboratory that identified miRNA up-regulated

by the LH surge and the study by Otsuka et al. that established miRNA are necessary for luteal formation and function, it is clear that miRNA play an important role in modulating gene regulation during the peri-ovulatory period.

We have also demonstrated that beginning with fetal ovarian development, the expression of miRNA are regulated temporally and by steroid hormone receptors (Luense, et al. 2011). These findings correspond with other studies that have identified miRNA in the fetal ovary (Torley, et al. 2011, Tripurani, et al. 2010) and the regulation of miRNA by steroid hormones (Cochrane, et al. 2011), but is the first to establish their aberrant expression in response to an abnormal hormone environment. It remains unclear if altered expression of miRNA during fetal development potentiates a change in the developmental trajectory of the ovary that results in an adult disease state. It is conceivable, however, to hypothesize that the altered miRNA expression from such an early time point can trigger slight alterations in gene expression that perpetuate throughout development and into adulthood.

Perhaps most importantly, the study involving PCOS theca cells suggests a potentially game changing role for miRNA in this disease state. This is the first study, to our knowledge, to identify miRNA aberrantly expressed in theca cells from polycystic ovaries. These findings complement the identification of the transcriptome from PCOS theca cells (Wood, et al. 2004, Wood, et al. 2003) and provides a potential mechanism for what is causing the mis-expression of these genes. Furthermore, the abnormal expression of these miRNA provides further evidence that the molecular programming of PCOS theca cells are intrinsically altered (Gilling-Smith, et al. 1997). Further study is needed to determine how exactly these miRNA identified in PCOS theca cells may be regulating

gene expression. Specifically, identification of validated mRNA targets are necessary so that we can further understand the role they play in this disease etiology. If specific miRNA with bonafide targets are identified, great potential exists for the development of assays to identify non-invasive biomarkers or therapeutic targets.

XV: CHAPTER 9 – REFERENCES

Abbott, DH, DK Barnett, CM Bruns, and DA Dumesic 2005 Androgen excess fetal programming of female reproduction: a developmental aetiology for polycystic ovary syndrome? *Hum Reprod Update* **11** 357-374.

Abel, MH, AN Wootton, V Wilkins, I Huhtaniemi, PG Knight, and HM Charlton 2000 The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology* **141** 1795-1803.

Ahn, HW, RD Morin, H Zhao, RA Harris, C Coarfa, ZJ Chen, A Milosavljevic, MA Marra, and A Rajkovic 2010 MicroRNA transcriptome in the newborn mouse ovaries determined by massive parallel sequencing. *Mol Hum Reprod* **16** 463-471.

Ajit, SK 2012 Circulating microRNAs as biomarkers, therapeutic targets, and signaling molecules. *Sensors (Basel)* **12** 3359-3369.

Ali, AS, S Ali, A Ahmad, B Bao, PA Philip, and FH Sarkar 2011 Expression of microRNAs: potential molecular link between obesity, diabetes and cancer. *Obes Rev* **12** 1050-1062.

Andl, T, EP Murchison, F Liu, Y Zhang, M Yunta-Gonzalez, JW Tobias, CD Andl, JT Seykora, GJ Hannon, and SE Millar 2006 The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. *Curr Biol* **16** 1041-1049.

Arango, NA, A Kobayashi, Y Wang, SP Jamin, HH Lee, GD Orvis, and RR Behringer 2008 A mesenchymal perspective of Mullerian duct differentiation and regression in Amhr2-lacZ mice. *Mol Reprod Dev* **75** 1154-1162.

Arango, NA, PP Szotek, TF Manganaro, E Oliva, PK Donahoe, and J Teixeira 2005 Conditional deletion of beta-catenin in the mesenchyme of the developing mouse uterus results in a switch to adipogenesis in the myometrium. *Dev Biol* **288** 276-283.

Azziz, R 2004 Androgen Excess in Women: Experience with Over 1000 Consecutive Patients. *Journal of Clinical Endocrinology & Metabolism* **89** 453-462.

Azziz, R 2006 Controversy in clinical endocrinology: diagnosis of polycystic ovarian syndrome: the Rotterdam criteria are premature. *J Clin Endocrinol Metab* **91** 781-785.

Azziz, R, DA Dumesic, and MO Goodarzi 2011 Polycystic ovary syndrome: an ancient disorder? *Fertil Steril* **95** 1544-1548.

Azziz, R, KS Woods, R Reyna, TJ Key, ES Knochenhauer, and BO Yildiz 2004 The prevalence and features of the polycystic ovary syndrome in an unselected population. *J Clin Endocrinol Metab* **89** 2745-2749.

Barker, DJ 1990 The fetal and infant origins of adult disease. *BMJ* **301** 1111.

Bartel, DP 2004 MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116** 281-297.

Berezikov, E, WJ Chung, J Willis, E Cuppen, and EC Lai 2007 Mammalian mirtron genes. *Mol Cell* **28** 328-336.

Bernstein, E, AA Caudy, SM Hammond, and GJ Hannon 2001 Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409** 363-366.

Bernstein, E, SY Kim, MA Carmell, EP Murchison, H Alcorn, MZ Li, AA Mills, SJ Elledge, KV Anderson, and GJ Hannon 2003 Dicer is essential for mouse development. *Nat Genet* **35** 215-217.

Betel, D, M Wilson, A Gabow, DS Marks, and C Sander 2008 The microRNA.org resource: targets and expression. *Nucleic Acids Res* **36** D149-153.

Biggers, JD, LK McGinnis, and M Raffin 2000 Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. *Biol Reprod* **63** 281-293.

Billig, H, I Furuta, and AJ Hsueh 1993 Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology* **133** 2204-2212.

Blaszczyk, J, JE Tropea, M Bubunenko, KM Routzahn, DS Waugh, DL Court, and X Ji 2001 Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. *Structure* **9** 1225-1236.

Boerboom, D, M Paquet, M Hsieh, J Liu, SP Jamin, RR Behringer, J Sirois, MM Taketo, and JS Richards 2005 Misregulated Wnt/beta-catenin signaling leads to ovarian granulosa cell tumor development. *Cancer Res* **65** 9206-9215.

Boerboom, D, LD White, S Dalle, J Courty, and JS Richards 2006 Dominant-stable beta-catenin expression causes cell fate alterations and Wnt signaling antagonist expression in a murine granulosa cell tumor model. *Cancer Res* **66** 1964-1973.

Bohnsack, MT, 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, GM, W Lanier, and BL Davidson 2006 RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* **13** 1097-1101.

Borum, K 1967 Oogenesis in the mouse. A study of the origin of the mature ova. *Exp Cell Res* **45** 39-47.

Branford, WW, GV Benson, L Ma, RL Maas, and SS Potter 2000 Characterization of Hoxa-10/Hoxa-11 transheterozygotes reveals functional redundancy and regulatory interactions. *Dev Biol* **224** 373-387.

Brennan, J, and B Capel 2004 One tissue, two fates: molecular genetic events that underlie testis versus ovary development. *Nat Rev Genet* **5** 509-521.

Britt, KL, and JK Findlay 2003 Regulation of the phenotype of ovarian somatic cells by estrogen. *Mol Cell Endocrinol* **202** 11-17.

Brockmann, R, A Beyer, JJ Heinisch, and T Wilhelm 2007 Posttranscriptional expression regulation: what determines translation rates? *PLoS Comput Biol* **3** e57.

Brodie, AM, WC Schwarzel, and HJ Brodie 1976 Studies on the mechanism of estrogen biosynthesis in the rat ovary--I. *J Steroid Biochem* **7** 787-793.

Burghen, GA, JR Givens, and AE Kitabchi 1980 Correlation of hyperandrogenism with hyperinsulinism in polycystic ovarian disease. *J Clin Endocrinol Metab* **50** 113-116.

Bushati, N, and SM Cohen 2007 microRNA functions. *Annu Rev Cell Dev Biol* **23** 175-205.

Cai, X, CH Hagedorn, and BR Cullen 2004 Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA* **10** 1957-1966.

Carletti, MZ, and LK Christenson 2009 MicroRNA in the ovary and female reproductive tract. *J Anim Sci* **87** E29-38.

Carletti, MZ, SD Fiedler, and LK Christenson 2010 MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells. *Biol Reprod* **83** 286-295.

Carlsen, SM, and E Vanky 2010 Metformin influence on hormone levels at birth, in PCOS mothers and their newborns. *Hum Reprod* **25** 786-790.

Carta, L, and D Sassoon 2004 Wnt7a is a suppressor of cell death in the female reproductive tract and is required for postnatal and estrogen-mediated growth. *Biol Reprod* **71** 444-454.

Chakrabarty, A, S Tranguch, T Daikoku, K Jensen, H Furneaux, and SK Dey 2007 MicroRNA regulation of cyclooxygenase-2 during embryo implantation. *Proc Natl Acad Sci U S A* **104** 15144-15149.

Chang, RJ 2007 The reproductive phenotype in polycystic ovary syndrome. *Nat Clin Pract Endocrinol Metab* **3** 688-695.

Chartoumpekis, DV, A Zaravinos, PG Ziros, RP Iskrenova, AI Psyrogiannis, VE Kyriazopoulou, and IG Habeos 2012 Differential expression of microRNAs in adipose tissue after long-term high-fat diet-induced obesity in mice. *PLoS One* **7** e34872.

Cheloufi, S, CO Dos Santos, MM Chong, and GJ Hannon 2010 A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature* **465** 584-589.

Chen, G, W Zhu, D Shi, L Lv, C Zhang, P Liu, and W Hu 2010 MicroRNA-181a sensitizes human malignant glioma U87MG cells to radiation by targeting Bcl-2. *Oncol Rep* **23** 997-1003.

Chen, L, Q Yang, WQ Kong, T Liu, M Liu, X Li, and H Tang 2012 MicroRNA-181b targets cAMP responsive element binding protein 1 in gastric adenocarcinomas. *IUBMB Life* **64** 628-635.

Chendrimada, TP, KJ Finn, X Ji, D Baillat, RI Gregory, SA Liebhaber, AE Pasquinelli, and R Shiekhattar 2007 MicroRNA silencing through RISC recruitment of eIF6. *Nature* **447** 823-828.

Choi, Y, DJ Ballow, Y Xin, and A Rajkovic 2008 Lim homeobox gene, lhx8, is essential for mouse oocyte differentiation and survival. *Biol Reprod* **79** 442-449.

Choi, Y, Y Qin, MF Berger, DJ Ballow, ML Bulyk, and A Rajkovic 2007 Microarray analyses of newborn mouse ovaries lacking Nobox. *Biol Reprod* **77** 312-319.

Chong, MM, G Zhang, S Cheloufi, TA Neubert, GJ Hannon, and DR Littman 2010 Canonical and alternate functions of the microRNA biogenesis machinery. *Genes Dev* **24** 1951-1960.

Christenson, LK, and L Devoto 2003 Cholesterol transport and steroidogenesis by the corpus luteum. *Reprod Biol Endocrinol* **1** 90.

Cifuentes, D, H Xue, DW Taylor, H Patnode, Y Mishima, S Cheloufi, E Ma, S Mane, GJ Hannon, ND Lawson, SA Wolfe, and AJ Giraldez 2010 A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science* **328** 1694-1698.

Clarke, IJ, RJ Scaramuzzi, and RV Short 1976 Effects of testosterone implants in pregnant ewes on their female offspring. *J Embryol Exp Morphol* **36** 87-99.

Clarke, IJ, RJ Scaramuzzi, and RV Short 1977 Ovulation in prenatally androgenized ewes. *J Endocrinol* **73** 385-389.

Cochrane, DR, DM Cittelly, and JK Richer 2011 Steroid receptors and microRNAs: relationships revealed. *Steroids* **76** 1-10.

Conley, AJ, and IM Bird 1997 The role of cytochrome P450 17 alpha-hydroxylase and 3 beta-hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the delta 5 and delta 4 pathways of steroidogenesis in mammals. *Biol Reprod* **56** 789-799.

Conley, AJ, MA Kaminski, SA Dubowsky, A Jablonka-Shariff, DA Redmer, and LP Reynolds 1995 Immunohistochemical localization of 3 beta-hydroxysteroid dehydrogenase and P450 17 alpha-hydroxylase during follicular and luteal development in pigs, sheep, and cows. *Biol Reprod* **52** 1081-1094.

Couse, JF, and KS Korach 1999 Reproductive phenotypes in the estrogen receptor-alpha knockout mouse. *Ann Endocrinol (Paris)* **60** 143-148.

Cuellar, TL, TH Davis, PT Nelson, GB Loeb, BD Harfe, E Ullian, and MT McManus 2008 Dicer loss in striatal neurons produces behavioral and neuroanatomical phenotypes in the absence of neurodegeneration. *Proc Natl Acad Sci U S A* **105** 5614-5619.

Cui, XS, XH Shen, and NH Kim 2007 Dicer1 expression in preimplantation mouse embryos: Involvement of Oct3/4 transcription at the blastocyst stage. *Biochem Biophys Res Commun* **352** 231-236.

da Costa Martins, PA, M Bourajjaj, M Gladka, M Kortland, RJ van Oort, YM Pinto, JD Molkentin, and LJ De Windt 2008 Conditional dicer gene deletion in the postnatal myocardium provokes spontaneous cardiac remodeling. *Circulation* **118** 1567-1576.

Dahiya, N, and PJ Morin 2010 MicroRNAs in ovarian carcinomas. *Endocr Relat Cancer* **17** F77-89.

Dammer, EB, and MB 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.

Davis, TH, TL Cuellar, SM Koch, AJ Barker, BD Harfe, MT McManus, and EM Ullian 2008 Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus. *J Neurosci* **28** 4322-4330.

de Boo, HA, and JE Harding 2006 The developmental origins of adult disease (Barker) hypothesis. *Aust N Z J Obstet Gynaecol* **46** 4-14.

Delic, D, C Grosser, M Dkhil, S Al-Quraishy, and F Wunderlich 2010 Testosterone-induced upregulation of miRNAs in the female mouse liver. *Steroids* **75** 998-1004.

Dell'Aquila, ME, M Caillaud, F Maritato, A Martoriati, N Gerard, G Aiudi, P Minoia, and G Goudet 2004 Cumulus expansion, nuclear maturation and connexin 43, cyclooxygenase-2 and FSH receptor mRNA expression in equine cumulus-oocyte complexes cultured in vitro in the presence of FSH and precursors for hyaluronic acid synthesis. *Reprod Biol Endocrinol* **2** 44.

Demissie, M, M Lazic, EM Foecking, F Aird, A Dunaif, and JE Levine 2008 Transient prenatal androgen exposure produces metabolic syndrome in adult female rats. *Am J Physiol Endocrinol Metab* **295** E262-268.

Denli, AM, BB Tops, RH Plasterk, RF Ketting, and GJ Hannon 2004 Processing of primary microRNAs by the Microprocessor complex. *Nature* **432** 231-235.

Deutscher, E, and H Hung-Chang Yao 2007 Essential roles of mesenchyme-derived beta-catenin in mouse Mullerian duct morphogenesis. *Dev Biol* **307** 227-236.

Diaz, FJ, MJ O'Brien, K Wigglesworth, and JJ Eppig 2006 The preantral granulosa cell to cumulus cell transition in the mouse ovary: development of competence to undergo expansion. *Dev Biol* **299** 91-104.

Diaz, FJ, K Wigglesworth, and JJ Eppig 2007 Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice. *Dev Biol* **305** 300-311.

Dong, J, DF Albertini, K Nishimori, TR Kumar, N Lu, and MM Matzuk 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383** 531-535.

Doody, KJ, MC Lorence, JI Mason, and ER Simpson 1990 Expression of messenger ribonucleic acid species encoding steroidogenic enzymes in human follicles and corpora lutea throughout the menstrual cycle. *J Clin Endocrinol Metab* **70** 1041-1045.

Dorn, GW, 2nd 2011 MicroRNAs in cardiac disease. *Transl Res* **157** 226-235.

Dumesic, DA, DH Abbott, and V Padmanabhan 2007 Polycystic ovary syndrome and its developmental origins. *Rev Endocr Metab Disord* **8** 127-141.

Dumesic, DA, RD Schramm, and DH Abbott 2005 Early origins of polycystic ovary syndrome. *Reprod Fertil Dev* **17** 349-360.

Elmen, J, M Lindow, S Schutz, M Lawrence, A Petri, S Obad, M Lindholm, M Hedtjarn, HF Hansen, U Berger, S Gullans, P Kearney, P Sarnow, EM Straarup, and S Kauppinen 2008 LNA-mediated microRNA silencing in non-human primates. *Nature* **452** 896-899.

Elvin, JA, C Yan, P Wang, K Nishimori, and MM Matzuk 1999 Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. *Mol Endocrinol* **13** 1018-1034.

Ender, C, and G Meister 2010 Argonaute proteins at a glance. *J Cell Sci* **123** 1819-1823.

Eppig, JJ, and MJ O'Brien 1996 Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* **54** 197-207.

Erickson, GF, C Wang, and AJ Hsueh 1979 FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium. *Nature* **279** 336-338.

Espey, LL, AS Bellinger, and JA Healy 2004 Ovulation: An Inflammatory Cascade of Gene Expression. *In* PC Leung and EY Adashi (ed.), The Ovary, edn Second, pp. 145-165.

Fabian, MR, G Mathonnet, T Sundermeier, H Mathys, JT Zipprich, YV Svitkin, F Rivas, M Jinek, J Wohlschlegel, JA Doudna, CY Chen, AB Shyu, JR Yates, 3rd, GJ Hannon, W Filipowicz, TF Duchaine, and N Sonenberg 2009 Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol Cell* **35** 868-880.

Falck, B 1959 Site of production of oestrogen in the ovary of the rat. *Nature* **184(Suppl 14)** 1082.

Fan, HY, Z Liu, N Cahill, and JS Richards 2008a Targeted disruption of Pten in ovarian granulosa cells enhances ovulation and extends the life span of luteal cells. *Mol Endocrinol* **22** 2128-2140.

Fan, HY, A O'Connor, M Shitanaka, M Shimada, Z Liu, and JS Richards 2010 Beta-catenin (CTNNB1) promotes preovulatory follicular development but represses LH-mediated ovulation and luteinization. *Mol Endocrinol* **24** 1529-1542.

Fan, HY, M Shimada, Z Liu, N Cahill, N Noma, Y Wu, J Gossen, and JS Richards 2008b Selective expression of KrasG12D in granulosa cells of the mouse ovary causes defects in follicle development and ovulation. *Development* **135** 2127-2137.

Ferland-McCollough, D, SE Ozanne, K Siddle, AE Willis, and M Bushell 2010 The involvement of microRNAs in Type 2 diabetes. *Biochem Soc Trans* **38** 1565-1570.

Fiedler, SD, MZ Carletti, X Hong, and LK Christenson 2008 Hormonal regulation of MicroRNA expression in periovulatory mouse mural granulosa cells. *Biol Reprod* **79** 1030-1037.

Filipowicz, W, SN Bhattacharyya, and N Sonenberg 2008 Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9** 102-114.

Fortin, KR, RH Nicholson, and AW Nicholson 2002 Mouse ribonuclease III. cDNA structure, expression analysis, and chromosomal location. *BMC Genomics* **3** 26.

Fortune, JE 1994 Ovarian follicular growth and development in mammals. *Biol Reprod* **50** 225-232.

Fortune, JE 2003 The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Animal Reproduction Science* **78** 135-163.

Fortune, JE, and W Hansel 1979 The effects of 17 beta-estradiol on progesterone secretion by bovine theca and granulosa cells. *Endocrinology* **104** 1834-1838.

Fortune, JE, GM Rivera, AC Evans, and AM Turzillo 2001 Differentiation of dominant versus subordinate follicles in cattle. *Biol Reprod* **65** 648-654.

Friedman, RC, KK Farh, CB Burge, and DP Bartel 2009 Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* **19** 92-105.

Fukuda, T, K Yamagata, S Fujiyama, T Matsumoto, I Koshida, K Yoshimura, M Mihara, M Naitou, H Endoh, T Nakamura, C Akimoto, Y Yamamoto, T Katagiri, C Foulds, S Takezawa, H Kitagawa, K Takeyama, BW O'Malley, and S Kato 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.

Gilling-Smith, C, H Story, V Rogers, and S Franks 1997 Evidence for a primary abnormality of thecal cell steroidogenesis in the polycystic ovary syndrome. *Clin Endocrinol (Oxf)* **47** 93-99.

Gilling-Smith, C, DS Willis, RW Beard, and S Franks 1994 Hypersecretion of androstenedione by isolated thecal cells from polycystic ovaries. *J Clin Endocrinol Metab* **79** 1158-1165.

Ginsburg, M, MH Snow, and A McLaren 1990 Primordial germ cells in the mouse embryo during gastrulation. *Development* **110** 521-528.

Ginther, OJ, MC Wiltbank, PM Fricke, JR Gibbons, and K Kot 1996 Selection of the dominant follicle in cattle. *Biol Reprod* **55** 1187-1194.

Goldenberg, RL, JL Vaitukaitis, and GT Ross 1972 Estrogen and follicle stimulation hormone interactions on follicle growth in rats. *Endocrinology* **90** 1492-1498.

Gonzalez, G, and RR Behringer 2009 Dicer is required for female reproductive tract development and fertility in the mouse. *Mol Reprod Dev* **76** 678-688.

Gregory, RI, KP Yan, G Amuthan, T Chendrimada, B Doratotaj, N Cooch, and R Shiekhattar 2004 The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432** 235-240.

Griffiths-Jones, S, HK Saini, S van Dongen, and AJ Enright 2008 miRBase: tools for microRNA genomics. *Nucleic Acids Res* **36** D154-158.

Gubbay, J, J Collignon, P Koopman, B Capel, A Economou, A Munsterberg, N Vivian, P Goodfellow, and R Lovell-Badge 1990 A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* **346** 245-250.

Guo, H, NT Ingolia, JS Weissman, and DP Bartel 2010 Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* **466** 835-840.

Halbeisen, RE, A Galgano, T Scherrer, and AP Gerber 2008 Post-transcriptional gene regulation: from genome-wide studies to principles. *Cell Mol Life Sci* **65** 798-813.

Hall, PF 1986 Cytochromes P-450 and the regulation of steroid synthesis. *Steroids* **48** 131-196.

Han, J, Y Lee, KH Yeom, YK Kim, H Jin, and VN Kim 2004 The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* **18** 3016-3027.

Harfe, BD, MT McManus, JH Mansfield, E Hornstein, and CJ 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.

Harris, KS, Z Zhang, MT McManus, BD Harfe, and X Sun 2006 Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci U S A* **103** 2208-2213.

Harrison, RJ, and BJ Weir 1977 Structure of the mammalian ovary. *In* L Zuckerman (ed.), The Ovary, edn 2, pp. 113-217.

He, A, L Zhu, N Gupta, Y Chang, and F Fang 2007 Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. *Mol Endocrinol* **21** 2785-2794.

Hermeking, H 2012 MicroRNAs in the p53 network: micromanagement of tumour suppression. *Nat Rev Cancer* **12** 613-626.

Hernandez Gifford, JA, ME Hunzicker-Dunn, and JH Nilson 2009 Conditional deletion of beta-catenin mediated by Amhr2cre in mice causes female infertility. *Biol Reprod* **80** 1282-1292.

Herrera, BM, HE Lockstone, JM Taylor, QF Wills, PJ Kaisaki, A Barrett, C Camps, C Fernandez, J Ragoussis, D Gauguier, MI McCarthy, and CM Lindgren 2009 MicroRNA-125a is over-expressed in insulin target tissues in a spontaneous rat model of Type 2 Diabetes. *BMC Med Genomics* **2** 54.

Hirshfield, AN 1991 Theca cells may be present at the outset of follicular growth. *Biol Reprod* **44** 1157-1162.

Ho, C, LK Christenson, and JF Strauss 2004 Intracellular Cholesterol Dynamics in Steroidogenic Cells. *In* PC Leung and EY Adashi (ed.), The Ovary, pp. 93-110.

Hogg, K, AS McNeilly, and WC Duncan 2011 Prenatal androgen exposure leads to alterations in gene and protein expression in the ovine fetal ovary. *Endocrinology* **152** 2048-2059.

Hollander, JA, HI Im, AL Amelio, J Kocerha, P Bali, Q Lu, D Willoughby, C Wahlestedt, MD Conkright, and PJ Kenny 2010 Striatal microRNA controls cocaine intake through CREB signalling. *Nature* **466** 197-202.

Hong, X, LJ Luense, LK McGinnis, WB Nothnick, and LK Christenson 2008 Dicer1 is essential for female fertility and normal development of the female reproductive system. *Endocrinology* **149** 6207-6212.

Hossain, MM, N Ghanem, M Hoelker, F Rings, C Phatsara, E Tholen, K Schellander, and D Tesfaye 2009 Identification and characterization of miRNAs expressed in the bovine ovary. *BMC Genomics* **10** 443.

Hsieh, M, K Thao, and M Conti 2011 Genetic dissection of epidermal growth factor receptor signaling during luteinizing hormone-induced oocyte maturation. *PLoS One* **6** e21574.

Hu, SJ, G Ren, JL Liu, ZA Zhao, YS Yu, RW Su, XH Ma, H Ni, W Lei, and ZM Yang 2008 MicroRNA expression and regulation in mouse uterus during embryo implantation. *J Biol Chem* **283** 23473-23484.

Huang, Y, XJ Shen, Q Zou, SP Wang, SM Tang, and GZ Zhang 2011 Biological functions of microRNAs: a review. *J Physiol Biochem* **67** 129-139.

Hulsmans, M, P Sinnaeve, B Van der Schueren, C Mathieu, S Janssens, and P Holvoet 2012 Decreased miR-181a expression in monocytes of obese patients is associated with the occurrence of metabolic syndrome and coronary artery disease. *J Clin Endocrinol Metab* **97** E1213-1218.

Hutvagner, G, J McLachlan, AE Pasquinelli, E Balint, T Tuschl, and PD Zamore 2001 A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293** 834-838.

Im, HI, JA Hollander, P Bali, and PJ Kenny 2010 MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. *Nat Neurosci* **13** 1120-1127.

Iorio, MV, and CM Croce 2012 MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med* **4** 143-159.

Jakimiuk, AJ, SR Weitsman, and DA Magoffin 1999 5alpha-reductase activity in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **84** 2414-2418.

Jamin, SP, NA Arango, Y Mishina, and RR Behringer 2002a Genetic studies of MIS signalling in sexual development. *Novartis Found Symp* **244** 157-164; discussion 164-158, 203-156, 253-157.

Jamin, SP, NA Arango, Y Mishina, MC Hanks, and RR Behringer 2002b Requirement of Bmpr1a for Mullerian duct regression during male sexual development. *Nat Genet* **32** 408-410.

Jegga, AG, J Chen, S Gowrisankar, MA Deshmukh, R Gudivada, S Kong, V Kaimal, and BJ Aronow 2007 GenomeTrafac: a whole genome resource for the detection of transcription factor binding site clusters associated with conventional and microRNA

encoding genes conserved between mouse and human gene orthologs. *Nucleic Acids Res* **35** D116-121.

Jerome, T, P Laurie, B Louis, and C Pierre 2007 Enjoy the Silence: The Story of let-7 MicroRNA and Cancer. *Curr Genomics* **8** 229-233.

Jeyasuria, P, Y Ikeda, SP Jamin, L Zhao, DG De Rooij, AP Themmen, RR Behringer, and KL Parker 2004 Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Mol Endocrinol* **18** 1610-1619.

John, GB, TD Gallardo, LJ Shirley, and DH Castrillon 2008 Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. *Dev Biol* **321** 197-204.

John, GB, LJ Shirley, TD Gallardo, and DH Castrillon 2007 Specificity of the requirement for Foxo3 in primordial follicle activation. *Reproduction* **133** 855-863.

Jorgez, CJ, M Klysik, SP Jamin, RR Behringer, and MM Matzuk 2004 Granulosa cell-specific inactivation of follistatin causes female fertility defects. *Mol Endocrinol* **18** 953-967.

Juengel, JL, DA Heath, LD Quirke, and KP McNatty 2006 Oestrogen receptor alpha and beta, androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep. *Reproduction* **131** 81-92.

Kash, JC, and KM Menon 1999 Sequence-specific binding of a hormonally regulated mRNA binding protein to cytidine-rich sequences in the lutropin receptor open reading frame. *Biochemistry* **38** 16889-16897.

Kawamata, T, and Y Tomari 2010 Making RISC. *Trends Biochem Sci* **35** 368-376.

Kezele, P, and MK Skinner 2003 Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. *Endocrinology* **144** 3329-3337.

King, AJ, NB Olivier, PS Mohankumar, JS Lee, V Padmanabhan, and GD Fink 2007 Hypertension caused by prenatal testosterone excess in female sheep. *Am J Physiol Endocrinol Metab* **292** E1837-1841.

Kiriakidou, M, GS Tan, S Lamprinaki, M De Planell-Saguer, PT Nelson, and Z Mourelatos 2007 An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell* **129** 1141-1151.

Klinge, CM 2012 miRNAs and estrogen action. *Trends Endocrinol Metab* **23** 223-233.

Kong, L, J Zhu, W Han, X Jiang, M Xu, Y Zhao, Q Dong, Z Pang, Q Guan, L Gao, J Zhao, and L Zhao 2011 Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. *Acta Diabetol* **48** 61-69.

Koopman, P, J Gubbay, N Vivian, P Goodfellow, and R Lovell-Badge 1991 Male development of chromosomally female mice transgenic for Sry. *Nature* **351** 117-121.

Kosova, G, and M Urbanek 2012 Genetics of the polycystic ovary syndrome. *Mol Cell Endocrinol*.

Kozomara, A, and S Griffiths-Jones 2011 miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* **39** D152-157.

Krek, A, D Grun, MN Poy, R Wolf, L Rosenberg, EJ Epstein, P MacMenamin, I da Piedade, KC Gunsalus, M Stoffel, and N Rajewsky 2005 Combinatorial microRNA target predictions. *Nat Genet* **37** 495-500.

Krutzfeldt, J, N Rajewsky, R Braich, KG Rajeev, T Tuschl, M Manoharan, and M Stoffel 2005 Silencing of microRNAs in vivo with 'antagomirs'. *Nature* **438** 685-689.

Kubota, K, S Ohsako, S Kurosawa, K Takeda, W Qing, M Sakaue, T Kawakami, R Ishimura, and C Tohyama 2003 Effects of vinclozolin administration on sperm production and testosterone biosynthetic pathway in adult male rat. *J Reprod Dev* **49** 403-412.

Kumar, TR, Y Wang, N Lu, and MM Matzuk 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* **15** 201-204.

Labrie, F, V Luu-The, SX Lin, C Labrie, J Simard, R Breton, and A Belanger 1997 The key role of 17 beta-hydroxysteroid dehydrogenases in sex steroid biology. *Steroids* **62** 148-158.

Lala, DS, DA Rice, and KL Parker 1992 Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. *Mol Endocrinol* **6** 1249-1258.

Lee, I, SS Ajay, JI Yook, HS Kim, SH Hong, NH Kim, SM Dhanasekaran, AM Chinnaiyan, and BD Athey 2009 New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites. *Genome Res* **19** 1175-1183.

Lee, RC, and V Ambros 2001 An extensive class of small RNAs in Caenorhabditis elegans. *Science* **294** 862-864.

Lee, RC, RL 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, C Ahn, J Han, H Choi, J Kim, J Yim, J Lee, P Provost, O Radmark, S Kim, and VN Kim 2003 The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425** 415-419.

Lee, Y, M Kim, J Han, KH Yeom, S Lee, SH Baek, and VN Kim 2004 MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* **23** 4051-4060.

Legro, RS, R Spielman, M Urbanek, D Driscoll, JF Strauss, 3rd, and A Dunaif 1998 Phenotype and genotype in polycystic ovary syndrome. *Recent Prog Horm Res* **53** 217-256.

Lei, L, S Jin, G Gonzalez, RR Behringer, and TK Woodruff 2010 The regulatory role of Dicer in folliculogenesis in mice. *Mol Cell Endocrinol* **315** 63-73.

Leventhal, ML, and MR Cohen 1951 Bilateral polycystic ovaries, the Stein syndrome. *Am J Obstet Gynecol* **61** 1034-1046.

Lewis, BP, CB Burge, and DP Bartel 2005 Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120** 15-20.

Li, H, L Hui, and W Xu 2012 miR-181a sensitizes a multidrug-resistant leukemia cell line K562/A02 to daunorubicin by targeting BCL-2. *Acta Biochim Biophys Sin (Shanghai)* **44** 269-277.

Lingenfelter, BM, SK Tripurani, J Tejomurtula, GW Smith, and J Yao 2011 Molecular cloning and expression of bovine nucleoplasmin 2 (NPM2): a maternal effect gene regulated by miR-181a. *Reprod Biol Endocrinol* **9** 40.

Liu, YX, and AJ Hsueh 1986 Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera. *Biol Reprod* **35** 27-36.

Lovell-Badge, R, and E Robertson 1990 XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. *Development* **109** 635-646.

Lu, DL, H Peegel, SM Mosier, and KM Menon 1993 Loss of lutropin/human choriogonadotropin receptor messenger ribonucleic acid during ligand-induced down-regulation occurs post transcriptionally. *Endocrinology* **132** 235-240.

Luense, LJ, MZ Carletti, and LK Christenson 2009 Role of Dicer in female fertility. *Trends Endocrinol Metab* **20** 265-272.

Luense, LJ, A Veiga-Lopez, V Padmanabhan, and LK Christenson 2011 Developmental programming: gestational testosterone treatment alters fetal ovarian gene expression. *Endocrinology* **152** 4974-4983.

Lun, S, P Smith, T Lundy, A O'Connell, N Hudson, and KP McNatty 1998 Steroid contents of and steroidogenesis in vitro by the developing gonad and mesonephros around sexual differentiation in fetal sheep. *J Reprod Fertil* **114** 131-139.

Lund, E, S Guttinger, A Calado, JE Dahlberg, and U Kutay 2004 Nuclear export of microRNA precursors. *Science* **303** 95-98.

Luo, X, and N Chegini 2008 The expression and potential regulatory function of microRNAs in the pathogenesis of leiomyoma. *Semin Reprod Med* **26** 500-514.

Lydon, JP, FJ DeMayo, OM Conneely, and BW O'Malley 1996 Reproductive phenotpes of the progesterone receptor null mutant mouse. *J Steroid Biochem Mol Biol* **56** 67-77.

Macrae, IJ, K Zhou, F Li, A Repic, AN Brooks, WZ Cande, PD Adams, and JA Doudna 2006 Structural basis for double-stranded RNA processing by Dicer. *Science* **311** 195-198.

Magill, ST, XA Cambronne, BW Luikart, DT Lioy, BH Leighton, GL Westbrook, G Mandel, and RH Goodman 2010 microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus. *Proc Natl Acad Sci U S A* **107** 20382-20387.

Magoffin, DA 2005 Ovarian theca cell. *Int J Biochem Cell Biol* **37** 1344-1349.

Magoffin, DA 2006 Ovarian enzyme activities in women with polycystic ovary syndrome. *Fertil Steril* **86 Suppl 1** S9-S11.

Maillot, G, M Lacroix-Triki, S Pierredon, L Gratadou, S Schmidt, V Benes, H Roche, F Dalenc, D Auboeuf, S Millevoi, and S Vagner 2009 Widespread estrogen-dependent repression of micrornas involved in breast tumor cell growth. *Cancer Res* **69** 8332-8340.

Mandon-Pepin, B, A Oustry-Vaiman, B Vigier, F Piumi, E Cribiu, and C Cotinot 2003 Expression profiles and chromosomal localization of genes controlling meiosis and follicular development in the sheep ovary. *Biol Reprod* **68** 985-995.

Manikkam, M, EJ Crespi, DD Doop, C Herkimer, JS Lee, S Yu, MB Brown, DL Foster, and V Padmanabhan 2004 Fetal programming: prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. *Endocrinology* **145** 790-798.

Manikkam, M, TL Steckler, KB Welch, EK Inskeep, and V Padmanabhan 2006 Fetal programming: prenatal testosterone treatment leads to follicular persistence/luteal defects; partial restoration of ovarian function by cyclic progesterone treatment. *Endocrinology* **147** 1997-2007.

Matsuda, F, N Inoue, N Manabe, and S Ohkura 2012 Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. *J Reprod Dev* **58** 44-50.

Mattiske, DM, L Han, and JR Mann 2009 Meiotic maturation failure induced by DICER1 deficiency is derived from primary oocyte ooplasm. *Reproduction* **137** 625-632.

Mayor-Lynn, K, T Toloubeydokhti, AC Cruz, and N Chegini 2011 Expression profile of microRNAs and mRNAs in human placentas from pregnancies complicated by preeclampsia and preterm labor. *Reprod Sci* **18** 46-56.

McAllister, JM, W Byrd, and ER Simpson 1994 The effects of growth factors and phorbol esters on steroid biosynthesis in isolated human theca interna and granulosa-lutein cells in long term culture. *J Clin Endocrinol Metab* **79** 106-112.

Menon, B, H Peegel, and KM Menon 2009 Evidence for the association of luteinizing hormone receptor mRNA-binding protein with the translating ribosomes during receptor downregulation. *Biochim Biophys Acta* **1793** 1787-1794.

Menon, KM, AK Nair, and L Wang 2006 A novel post-transcriptional mechanism of regulation of luteinizing hormone receptor expression by an RNA binding protein from the ovary. *Mol Cell Endocrinol* **246** 135-141.

Merritt, WM, YG Lin, LY Han, AA Kamat, WA Spannuth, R Schmandt, D Urbauer, LA Pennacchio, JF Cheng, AM Nick, MT Deavers, A Mourad-Zeidan, H Wang, P Mueller, ME Lenburg, JW Gray, S Mok, MJ Birrer, G Lopez-Berestein, RL Coleman, M Bar-Eli, and AK Sood 2008 Dicer, Drosha, and outcomes in patients with ovarian cancer. *N Engl J Med* **359** 2641-2650.

Miller, C, A Pavlova, and DA Sassoon 1998 Differential expression patterns of Wnt genes in the murine female reproductive tract during development and the estrous cycle. *Mech Dev* **76** 91-99.

Miller, C, and DA Sassoon 1998 Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. *Development* **125** 3201-3211.

Miller, WL 2005 Minireview: regulation of steroidogenesis by electron transfer. *Endocrinology* **146** 2544-2550.

Mishima, T, T Takizawa, SS Luo, O Ishibashi, Y Kawahigashi, Y Mizuguchi, T Ishikawa, M Mori, T Kanda, T Goto, and T Takizawa 2008 MicroRNA (miRNA) cloning analysis reveals sex differences in miRNA expression profiles between adult mouse testis and ovary. *Reproduction* **136** 811-822.

Miyoshi, K, H Tsukumo, T Nagami, H Siomi, and MC Siomi 2005 Slicer function of Drosophila Argonautes and its involvement in RISC formation. *Genes Dev* **19** 2837-2848.

Mudhasani, R, Z Zhu, G Hutvagner, CM Eischen, S Lyle, LL Hall, JB Lawrence, AN Imbalzano, and SN Jones 2008 Loss of miRNA biogenesis induces p19Arf-p53 signaling and senescence in primary cells. *J Cell Biol* **181** 1055-1063.

Murchison, EP, and GJ Hannon 2004 miRNAs on the move: miRNA biogenesis and the RNAi machinery. *Curr Opin Cell Biol* **16** 223-229.

Murchison, EP, P Stein, Z Xuan, H Pan, MQ Zhang, RM Schultz, and GJ Hannon 2007 Critical roles for Dicer in the female germline. *Genes Dev* **21** 682-693.

Nagaraja, AK, C Andreu-Vieyra, HL Franco, L Ma, R Chen, DY Han, H Zhu, JE Agno, PH Gunaratne, FJ DeMayo, and MM Matzuk 2008 Deletion of Dicer in somatic cells of the female reproductive tract causes sterility. *Mol Endocrinol* **22** 2336-2352.

Nair, AK, JC Kash, H Peegel, and KM Menon 2002 Post-transcriptional regulation of luteinizing hormone receptor mRNA in the ovary by a novel mRNA-binding protein. *J Biol Chem* **277** 21468-21473.

Nair, AK, and KM Menon 2004 Isolation and characterization of a novel trans-factor for luteinizing hormone receptor mRNA from ovary. *J Biol Chem* **279** 14937-14944.

Nam, S, B Kim, S Shin, and S Lee 2008 miRGator: an integrated system for functional annotation of microRNAs. *Nucleic Acids Res* **36** D159-164.

Narayanan, R, J Jiang, Y Gusev, A Jones, JD Kearbey, DD Miller, TD Schmittgen, and JT Dalton 2010 MicroRNAs are mediators of androgen action in prostate and muscle. *PLoS One* **5** e13637.

Nelson-DeGrave, VL, JK Wickenheisser, JE Cockrell, JR Wood, RS Legro, JF Strauss, 3rd, and JM McAllister 2004 Valproate potentiates androgen biosynthesis in human ovarian theca cells. *Endocrinology* **145** 799-808.

Nelson, VL, RS Legro, JF Strauss, 3rd, and JM McAllister 1999 Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. *Mol Endocrinol* **13** 946-957.

Nelson, VL, KN Qin, RL Rosenfield, JR Wood, TM Penning, RS Legro, JF Strauss, 3rd, and JM McAllister 2001 The biochemical basis for increased testosterone production in theca cells propagated from patients with polycystic ovary syndrome. *J Clin Endocrinol Metab* **86** 5925-5933.

Nicholson, RH, and AW Nicholson 2002 Molecular characterization of a mouse cDNA encoding Dicer, a ribonuclease III ortholog involved in RNA interference. *Mamm Genome* **13** 67-73.

Nijland, MJ, SP Ford, and PW Nathanielsz 2008 Prenatal origins of adult disease. *Curr Opin Obstet Gynecol* **20** 132-138.

Nilsson, EE, and MK Skinner 2009 Progesterone regulation of primordial follicle assembly in bovine fetal ovaries. *Mol Cell Endocrinol* **313** 9-16.

Niswender, GD, JL Juengel, WJ McGuire, CJ Belfiore, and MC Wiltbank 1994 Luteal function: the estrous cycle and early pregnancy. *Biol Reprod* **50** 239-247.

Niswender, GD, JL Juengel, PJ Silva, MK Rollyson, and EW McIntush 2000 Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* **80** 1-29.

Nothnick, WB 2008 Regulation of uterine matrix metalloproteinase-9 and the role of microRNAs. *Semin Reprod Med* **26** 494-499.

Nothnick, WB 2012 The role of micro-RNAs in the female reproductive tract. *Reproduction* **143** 559-576.

Nudelman, AS, DP DiRocco, TJ Lambert, MG Garelick, J Le, NM Nathanson, and DR Storm 2010 Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo. *Hippocampus* **20** 492-498.

O'Rourke, JR, SA Georges, HR Seay, SJ Tapscott, MT McManus, DJ Goldhamer, MS Swanson, and BD Harfe 2007 Essential role for Dicer during skeletal muscle development. *Dev Biol* **311** 359-368.

O'Shaughnessy, PJ, D McLelland, and MW McBride 1997 Regulation of luteinizing hormone-receptor and follicle-stimulating hormone-receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary. *Biol Reprod* **57** 602-608.

Ortega, HH, F Rey, MM Velazquez, and V Padmanabhan 2010 Developmental programming: effect of prenatal steroid excess on intraovarian components of insulin signaling pathway and related proteins in sheep. *Biol Reprod* **82** 1065-1075.

Ortega, HH, NR Salvetti, and V Padmanabhan 2009 Developmental programming: prenatal androgen excess disrupts ovarian steroid receptor balance. *Reproduction* **137** 865-877.

Osman, A 2012 MicroRNAs in health and disease--basic science and clinical applications. *Clin Lab* **58** 393-402.

Otsuka, M, M Zheng, M Hayashi, JD Lee, O Yoshino, S Lin, and J Han 2008 Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. *J Clin Invest* **118** 1944-1954.

Ouyang, YB, Y Lu, S Yue, and RG Giffard 2012 miR-181 targets multiple Bcl-2 family members and influences apoptosis and mitochondrial function in astrocytes. *Mitochondrion* **12** 213-219.

Padmanabhan, V 2007 Environment and origin of disease. *Rev Endocr Metab Disord* **8** 67-69.

Padmanabhan, V, M Manikkam, S Recabarren, and D Foster 2006 Prenatal testosterone excess programs reproductive and metabolic dysfunction in the female. *Mol Cell Endocrinol* **246** 165-174.

Padmanabhan, V, HN Sarma, M Savabieasfahani, TL Steckler, and A Veiga-Lopez 2010a Developmental reprogramming of reproductive and metabolic dysfunction in sheep: native steroids vs. environmental steroid receptor modulators. *Int J Androl* **33** 394-404.

Padmanabhan, V, A Veiga-Lopez, DH Abbott, SE Recabarren, and C Herkimer 2010b Developmental programming: impact of prenatal testosterone excess and postnatal weight gain on insulin sensitivity index and transfer of traits to offspring of overweight females. *Endocrinology* **151** 595-605.

Pan, Q, and N Chegini 2008 MicroRNA signature and regulatory functions in the endometrium during normal and disease states. *Semin Reprod Med* **26** 479-493.

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.

Pan, Q, X Luo, T Toloubeydokhti, and N Chegini 2007 The expression profile of micro-RNA in endometrium and endometriosis and the influence of ovarian steroids on their expression. *Mol Hum Reprod* **13** 797-806.

Pan, Y, L Balazs, G Tigyi, and J Yue 2011 Conditional deletion of Dicer in vascular smooth muscle cells leads to the developmental delay and embryonic mortality. *Biochem Biophys Res Commun* **408** 369-374.

Pandey, AK, G Verma, S Vig, S Srivastava, AK Srivastava, and M Datta 2011 miR-29a levels are elevated in the db/db mice liver and its overexpression leads to attenuation of insulin action on PEPCK gene expression in HepG2 cells. *Mol Cell Endocrinol* **332** 125-133.

Pangas, SA, Y Choi, DJ Ballow, Y Zhao, H Westphal, MM Matzuk, and A Rajkovic 2006 Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8. *Proc Natl Acad Sci U S A* **103** 8090-8095.

Papaioannou, MD, JL Pitetti, S Ro, C Park, F Aubry, O Schaad, CE Vejnar, F Kuhne, P Descombes, EM Zdobnov, MT McManus, F Guillou, BD Harfe, W Yan, B Jegou, and S Nef 2009 Sertoli cell Dicer is essential for spermatogenesis in mice. *Dev Biol* **326** 250-259.

Paria, BC, YM Huet-Hudson, and SK Dey 1993 Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. *Proc Natl Acad Sci U S A* **90** 10159-10162.

Parrott, E, M Butterworth, A Green, IN White, and P Greaves 2001 Adenomyosis--a result of disordered stromal differentiation. *Am J Pathol* **159** 623-630.

Parrott, JA, and MK Skinner 1999 Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. *Endocrinology* **140** 4262-4271.

Pasquinelli, AE, BJ Reinhart, F Slack, MQ Martindale, MI Kuroda, B Maller, DC Hayward, EE Ball, B Degnan, P Muller, J Spring, A Srinivasan, M Fishman, J Finnerty, J Corbo, M Levine, P Leahy, E Davidson, and G Ruvkun 2000 Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408** 86-89.

Pastorelli, LM, S Wells, M Fray, A Smith, T Hough, BD Harfe, MT McManus, L Smith, AS Woolf, M Cheeseman, and A Greenfield 2009 Genetic analyses reveal a requirement for Dicer1 in the mouse urogenital tract. *Mamm Genome* **20** 140-151.

Payne, AH, and DB Hales 2004 Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* **25** 947-970.

Pedersen, T, and H Peters 1968 Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil* **17** 555-557.

Pepling, ME 2012 Follicular assembly: mechanisms of action. *Reproduction* **143** 139-149.

Pepling, ME, and AC Spradling 2001 Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev Biol* **234** 339-351.

Petersen, CP, ME Bordeleau, J Pelletier, and PA Sharp 2006 Short RNAs repress translation after initiation in mammalian cells. *Mol Cell* **21** 533-542.

Pillai, RS, CG Artus, and W Filipowicz 2004 Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA* **10** 1518-1525.

Post, LC, and JW Innis 1999 Infertility in adult hypodactyly mice is associated with hypoplasia of distal reproductive structures. *Biol Reprod* **61** 1402-1408.

Provost, P, RA Silverstein, D Dishart, J Walfridsson, I Djupedal, B Kniola, A Wright, B Samuelsson, O Radmark, and K Ekwall 2002 Dicer is required for chromosome segregation and gene silencing in fission yeast cells. *Proc Natl Acad Sci U S A* **99** 16648-16653.

Qin, Y, Y Choi, H Zhao, JL Simpson, ZJ Chen, and A Rajkovic 2007 NOBOX homeobox mutation causes premature ovarian failure. *Am J Hum Genet* **81** 576-581.

Quirk, SM, RG Cowan, RM Harman, CL Hu, and DA Porter 2004 Ovarian follicular growth and atresia: the relationship between cell proliferation and survival. *J Anim Sci* **82 E-Suppl** E40-52.

Quirke, LD, JL Juengel, DJ Tisdall, S Lun, DA Heath, and KP McNatty 2001 Ontogeny of steroidogenesis in the fetal sheep gonad. *Biol Reprod* **65** 216-228.

Rajkovic, A, SA Pangas, and MM Matzuk 2006 Follicular Development: Mouse, Sheep, and Human Models. *In* JD Neill (ed.), Knobil and Neill's Physiology of Reproduction, edn 3, pp. 383-424.

Rebar, R, HL Judd, SS Yen, J Rakoff, G Vandenberg, and F Naftolin 1976 Characterization of the inappropriate gonadotropin secretion in polycystic ovary syndrome. *J Clin Invest* **57** 1320-1329.

Recabarren, SE, V Padmanabhan, E Codner, A Lobos, C Duran, M Vidal, DL Foster, and T Sir-Petermann 2005 Postnatal developmental consequences of altered insulin sensitivity in female sheep treated prenatally with testosterone. *Am J Physiol Endocrinol Metab* **289** E801-806.

Redmer, DA, and LP Reynolds 1996 Angiogenesis in the ovary. *Rev Reprod* **1** 182-192.

Reid, G, MB Kirschner, and N van Zandwijk 2011 Circulating microRNAs: Association with disease and potential use as biomarkers. *Crit Rev Oncol Hematol* **80** 193-208.

Remenyi, J, CJ Hunter, C Cole, H Ando, S Impey, CE Monk, KJ Martin, GJ Barton, G Hutvagner, and JS Arthur 2010 Regulation of the miR-212/132 locus by MSK1 and CREB in response to neurotrophins. *Biochem J* **428** 281-291.

Revelli, A, L Delle Piane, S Casano, E Molinari, M Massobrio, and P Rinaudo 2009 Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol* **7** 40.

Rice, BF, and K Savard 1966 Steroid hormone formation in the human ovary. IV. Ovarian stromal compartment; formation of radioactive steroids from acetate-1-14C and action of gonadotropins. *J Clin Endocrinol Metab* **26** 593-609.

Richards, JS 1994 Hormonal control of gene expression in the ovary. *Endocr Rev* **15** 725-751.

Richards, JS 2001 New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Mol Endocrinol* **15** 209-218.

Richards, JS 2005 Ovulation: new factors that prepare the oocyte for fertilization. *Mol Cell Endocrinol* **234** 75-79.

Richards, JS, DL Russell, S Ochsner, and LL Espey 2002 Ovulation: new dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol* **64** 69-92.

Ro, S, R Song, C Park, H Zheng, KM Sanders, and W Yan 2007 Cloning and expression profiling of small RNAs expressed in the mouse ovary. *RNA* **13** 2366-2380.

Robinson, JE, RA Forsdike, and JA Taylor 1999 In utero exposure of female lambs to testosterone reduces the sensitivity of the gonadotropin-releasing hormone neuronal network to inhibition by progesterone. *Endocrinology* **140** 5797-5805.

Rotterdam 2004 Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril* **81** 19-25.

Rottiers, V, and AM Naar 2012 MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol* **13** 239-250.

Runner, MN, and A Gates 1954 Conception in prepuberal mice following artificially induced ovulation and mating. *Nature* **174** 222-223.

Ruvkun, G 2008 The perfect storm of tiny RNAs. *Nat Med* **14** 1041-1045.

Sahakitrungruang, T, MK Tee, PR Blackett, and WL Miller 2011 Partial defect in the cholesterol side-chain cleavage enzyme P450scc (CYP11A1) resembling nonclassic congenital lipoid adrenal hyperplasia. *J Clin Endocrinol Metab* **96** 792-798.

Saloniemi, T, M Welsh, T Lamminen, P Saunders, S Makela, T Streng, and M Poutanen 2009 Human HSD17B1 expression masculinizes transgenic female mice. *Mol Cell Endocrinol* **301** 163-168.

Sarma, HN, M Manikkam, C Herkimer, J Dell'Orco, KB Welch, DL Foster, and V Padmanabhan 2005 Fetal programming: excess prenatal testosterone reduces postnatal luteinizing hormone, but not follicle-stimulating hormone responsiveness, to estradiol negative feedback in the female. *Endocrinology* **146** 4281-4291.

Sawyer, HR, P Smith, DA Heath, JL Juengel, SJ Wakefield, and KP McNatty 2002 Formation of ovarian follicles during fetal development in sheep. *Biol Reprod* **66** 1134-1150.

Sayed, D, and M Abdellatif 2011 MicroRNAs in development and disease. *Physiol Rev* **91** 827-887.

Schams, D, and B Berisha 2004 Regulation of corpus luteum function in cattle--an overview. *Reprod Domest Anim* **39** 241-251.

Selbach, M, B Schwanhausser, N Thierfelder, Z Fang, R Khanin, and N Rajewsky 2008 Widespread changes in protein synthesis induced by microRNAs. *Nature* **455** 58-63.

Seliger, B, SP Dressler, E Wang, R Kellner, CV Recktenwald, F Lottspeich, FM Marincola, M Baumgartner, D Atkins, and R Lichtenfels 2009 Combined analysis of transcriptome and proteome data as a tool for the identification of candidate biomarkers in renal cell carcinoma. *Proteomics* **9** 1567-1581.

Sequeira-Lopez, ML, ET Weatherford, GR Borges, MC Monteagudo, ES Pentz, BD Harfe, O Carretero, CD Sigmund, and RA Gomez 2010 The microRNA-processing enzyme dicer maintains juxtaglomerular cells. *J Am Soc Nephrol* **21** 460-467.

Shantikumar, S, A Caporali, and C Emanueli 2012 Role of microRNAs in diabetes and its cardiovascular complications. *Cardiovasc Res* **93** 583-593.

Sharma, TP, C Herkimer, C West, W Ye, R Birch, JE Robinson, DL Foster, and V Padmanabhan 2002 Fetal programming: prenatal androgen disrupts positive feedback actions of estradiol but does not affect timing of puberty in female sheep. *Biol Reprod* **66** 924-933.

Simmons, RA 2007 Developmental origins of diabetes: the role of epigenetic mechanisms. *Curr Opin Endocrinol Diabetes Obes* **14** 13-16.

Simoncini, T, and AR Genazzani 2003 Non-genomic actions of sex steroid hormones. *Eur J Endocrinol* **148** 281-292.

Sirotkin, AV, D Ovcharenko, A Benco, and M Mlyncek 2009 Protein kinases controlling PCNA and p53 expression in human ovarian cells. *Funct Integr Genomics* **9** 185-195.

Skinner, MK 2005 Regulation of primordial follicle assembly and development. *Hum Reprod Update* **11** 461-471.

Sloboda, DM, GJ Howie, A Pleasants, PD Gluckman, and MH Vickers 2009 Pre- and postnatal nutritional histories influence reproductive maturation and ovarian function in the rat. *PLoS One* **4** e6744.

Smith, MF, EW McIntush, and GW Smith 1994 Mechanisms associated with corpus luteum development. *J Anim Sci* **72** 1857-1872.

Smith, P, TL Steckler, A Veiga-Lopez, and V Padmanabhan 2009 Developmental programming: differential effects of prenatal testosterone and dihydrotestosterone on follicular recruitment, depletion of follicular reserve, and ovarian morphology in sheep. *Biol Reprod* **80** 726-736.

Sokka, T, and I Huhtaniemi 1990 Ontogeny of gonadotrophin receptors and gonadotrophin-stimulated cyclic AMP production in the neonatal rat ovary. *J Endocrinol* **127** 297-303.

Soukup, GA, B Fritzsch, ML Pierce, MD Weston, I Jahan, MT McManus, and BD Harfe 2009 Residual microRNA expression dictates the extent of inner ear development in conditional Dicer knockout mice. *Dev Biol* **328** 328-341.

Steckler, T, M Manikkam, EK Inskeep, and V Padmanabhan 2007 Developmental programming: follicular persistence in prenatal testosterone-treated sheep is not programmed by androgenic actions of testosterone. *Endocrinology* **148** 3532-3540.

Steckler, T, J Wang, FF Bartol, SK Roy, and V Padmanabhan 2005 Fetal programming: prenatal testosterone treatment causes intrauterine growth retardation, reduces ovarian reserve and increases ovarian follicular recruitment. *Endocrinology* **146** 3185-3193.

Stefani, G, and FJ Slack 2008 Small non-coding RNAs in animal development. *Nat Rev Mol Cell Biol* **9** 219-230.

Strauss, JF, 3rd, CB Kallen, LK Christenson, H Watari, L Devoto, F Arakane, M Kiriakidou, and T Sugawara 1999 The steroidogenic acute regulatory protein (StAR): a window into the complexities of intracellular cholesterol trafficking. *Recent Prog Horm Res* **54** 369-394; discussion 394-365.

Strauss, JF, 3rd, JM McAllister, and M Urbanek 2012 Persistence pays off for PCOS gene prospectors. *J Clin Endocrinol Metab* **97** 2286-2288.

Strauss, JF, and WL Miller 1991 Molecular Basis of Ovarian Steroid Synthesis. *In* SG Hillier (ed.), Ovarian Endocrinology, pp. 25-72.

Su, AI, MP Cooke, KA Ching, Y Hakak, JR Walker, T Wiltshire, AP Orth, RG Vega, LM Sapinoso, A Moqrich, A Patapoutian, GM Hampton, PG Schultz, and JB Hogenesch 2002 Large-scale analysis of the human and mouse transcriptomes. *Proc Natl Acad Sci U S A* **99** 4465-4470.

Summers, MC, LK McGinnis, JA Lawitts, and JD Biggers 2005 Mouse embryo development following IVF in media containing either L-glutamine or glycyl-L-glutamine. *Hum Reprod* **20** 1364-1371.

Svoboda, P 2008 RNA silencing in mammalian oocytes and early embryos. *Curr Top Microbiol Immunol* **320** 225-256.

Tagatz, GE, and E Curpide 1973 Hormone secretion by the normal human ovary. *In* RO Greep and EB Astwood (ed.), Handbook of Physiology, pp. 603-613.

Tam, OH, AA Aravin, P Stein, A Girard, EP Murchison, S Cheloufi, E Hodges, M Anger, R Sachidanandam, RM Schultz, and GJ Hannon 2008 Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* **453** 534-538.

Tang, F, M Kaneda, D O'Carroll, P Hajkova, SC Barton, YA Sun, C Lee, A Tarakhovsky, K Lao, and MA Surani 2007 Maternal microRNAs are essential for mouse zygotic development. *Genes Dev* **21** 644-648.

Teede, H, A Deeks, and L Moran 2010 Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. *BMC Med* **8** 41.

Terranova, PF, and F Garza 1983 Relationship between the preovulatory luteinizing hormone (LH) surge and androstenedione synthesis of preantral follicles in the cyclic hamster: detection by in vitro responses to LH. *Biol Reprod* **29** 630-636.

Tessel, MA, NL Krett, and ST Rosen 2010 Steroid receptor and microRNA regulation in cancer. *Curr Opin Oncol* **22** 592-597.

Torley, KJ, JC da Silveira, P Smith, RV Anthony, DN Veeramachaneni, QA Winger, and GJ Bouma 2011 Expression of miRNAs in ovine fetal gonads: potential role in gonadal differentiation. *Reprod Biol Endocrinol* **9** 2.

Tripurani, SK, KB Lee, L Wang, G Wee, GW Smith, YS Lee, KE Latham, and J Yao 2011 A novel functional role for the oocyte-specific transcription factor newborn ovary homeobox (NOBOX) during early embryonic development in cattle. *Endocrinology* **152** 1013-1023.

Tripurani, SK, C Xiao, M Salem, and J Yao 2010 Cloning and analysis of fetal ovary microRNAs in cattle. *Anim Reprod Sci* **120** 16-22.

Vainio, S, M Heikkila, A Kispert, N Chin, and AP McMahon 1999 Female development in mammals is regulated by Wnt-4 signalling. *Nature* **397** 405-409.

van den Berg, A, J Mols, and J Han 2008 RISC-target interaction: cleavage and translational suppression. *Biochim Biophys Acta* **1779** 668-677.

Vasudevan, S, Y Tong, and JA Steitz 2007 Switching from repression to activation: microRNAs can up-regulate translation. *Science* **318** 1931-1934.

Veiga-Lopez, A, OI Astapova, EF Aizenberg, JS Lee, and V Padmanabhan 2009 Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. *Biol Reprod* **80** 718-725.

Veiga-Lopez, A, TL Steckler, DH Abbott, KB Welch, PS MohanKumar, DJ Phillips, K Refsal, and V Padmanabhan 2011 Developmental programming: impact of excess

prenatal testosterone on intrauterine fetal endocrine milieu and growth in sheep. *Biol Reprod* **84** 87-96.

Veiga-Lopez, A, W Ye, DJ Phillips, C Herkimer, PG Knight, and V Padmanabhan 2008 Developmental programming: deficits in reproductive hormone dynamics and ovulatory outcomes in prenatal, testosterone-treated sheep. *Biol Reprod* **78** 636-647.

Walters, KA, CM Allan, and DJ Handelsman 2008 Androgen actions and the ovary. *Biol Reprod* **78** 380-389.

Wandji, SA, V Srsen, AK Voss, JJ Eppig, and JE Fortune 1996 Initiation in vitro of growth of bovine primordial follicles. *Biol Reprod* **55** 942-948.

Wang, Y, R Medvid, C Melton, R Jaenisch, and R Blelloch 2007 DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet* **39** 380-385.

Watanabe, T, A Takeda, T Tsukiyama, K Mise, T Okuno, H Sasaki, N Minami, and H Imai 2006 Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. *Genes Dev* **20** 1732-1743.

Watanabe, T, Y Totoki, A Toyoda, M Kaneda, S Kuramochi-Miyagawa, Y Obata, H Chiba, Y Kohara, T Kono, T Nakano, MA Surani, Y Sakaki, and H Sasaki 2008 Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature* **453** 539-543.

Waters, KM, JG Pounds, and BD Thrall 2006 Data merging for integrated microarray and proteomic analysis. *Brief Funct Genomic Proteomic* **5** 261-272.

Wei, JJ, and P Soteropoulos 2008 MicroRNA: a new tool for biomedical risk assessment and target identification in human uterine leiomyomas. *Semin Reprod Med* **26** 515-521.

West, C, DL Foster, NP Evans, J Robinson, and V Padmanabhan 2001 Intra-follicular activin availability is altered in prenatally-androgenized lambs. *Mol Cell Endocrinol* **185** 51-59.

Whitfield, GK, PW Jurutka, CA Haussler, and MR Haussler 1999 Steroid hormone receptors: evolution, ligands, and molecular basis of biologic function. *J Cell Biochem* **Suppl 32-33** 110-122.

Wickenheisser, JK, VL Nelson-DeGrave, KL Hendricks, RS Legro, JF Strauss, 3rd, and JM McAllister 2005 Retinoids and retinol differentially regulate steroid biosynthesis in ovarian theca cells isolated from normal cycling women and women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **90** 4858-4865.

Wienholds, E, and RH Plasterk 2005 MicroRNA function in animal development. *FEBS Lett* **579** 5911-5922.

Wightman, B, TR Burglin, J Gatto, P Arasu, and G Ruvkun 1991 Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during Caenorhabditis elegans development. *Genes Dev* **5** 1813-1824.

Wightman, B, I Ha, and G Ruvkun 1993 Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* **75** 855-862.

Williams, AE 2008 Functional aspects of animal microRNAs. *Cell Mol Life Sci* **65** 545-562.

Wood, JR, CK Ho, VL Nelson-Degrave, JM McAllister, and JF Strauss, 3rd 2004 The molecular signature of polycystic ovary syndrome (PCOS) theca cells defined by gene expression profiling. *J Reprod Immunol* **63** 51-60.

Wood, JR, VL Nelson, C Ho, E Jansen, CY Wang, M Urbanek, JM McAllister, S Mosselman, and JF Strauss, 3rd 2003 The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. *J Biol Chem* **278** 26380-26390.

Wood, RI, FJ Ebling, H I'Anson, DC Bucholtz, SM Yellon, and DL Foster 1991 Prenatal androgens time neuroendocrine sexual maturation. *Endocrinology* **128** 2457-2468.

Wood, RI, and DL Foster 1998 Sexual differentiation of reproductive neuroendocrine function in sheep. *Rev Reprod* **3** 130-140.

Wu, CF, FD Huang, RF Sui, and JX Sun 2012 Preeclampsia serum upregulates CD40/CD40L expression and induces apoptosis in human umbilical cord endothelial cells. *Reprod Biol Endocrinol* **10** 28.

Xu, S, K Linher-Melville, BB Yang, D Wu, and J Li 2011 Micro-RNA378 (miR-378) regulates ovarian estradiol production by targeting aromatase. *Endocrinology* **152** 3941-3951.

Yang, JS, and EC Lai 2011 Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. *Mol Cell* **43** 892-903.

Yang, JS, T Maurin, N Robine, KD Rasmussen, KL Jeffrey, R Chandwani, EP Papapetrou, M Sadelain, D O'Carroll, and EC Lai 2010 Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. *Proc Natl Acad Sci U S A* **107** 15163-15168.

Yao, HH 2005 The pathway to femaleness: current knowledge on embryonic development of the ovary. *Mol Cell Endocrinol* **230** 87-93.

Yao, HH, MM Matzuk, CJ Jorgez, DB Menke, DC Page, A Swain, and B Capel 2004 Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. *Dev Dyn* **230** 210-215.

Yao, N, CL Lu, JJ Zhao, HF Xia, DG Sun, XQ Shi, C Wang, D Li, Y Cui, and X Ma 2009 A network of miRNAs expressed in the ovary are regulated by FSH. *Front Biosci* **14** 3239-3245.

Yao, N, BQ Yang, Y Liu, XY Tan, CL Lu, XH Yuan, and X Ma 2010 Follicle-stimulating hormone regulation of microRNA expression on progesterone production in cultured rat granulosa cells. *Endocrine* **38** 158-166.

Yi, R, BP Doehle, Y Qin, IG Macara, and BR Cullen 2005 Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *RNA* **11** 220-226.

Yi, R, D O'Carroll, HA Pasolli, Z Zhang, FS Dietrich, A Tarakhovsky, and E Fuchs 2006 Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. *Nat Genet* **38** 356-362.

Younger, ST, A Pertsemlidis, and DR Corey 2009 Predicting potential miRNA target sites within gene promoters. *Bioorg Med Chem Lett* **19** 3791-3794.

Zawadski, JK, and A Dunaif 1992 Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. *In* A Dunaif (ed.), Polycystic Ovary Syndrome, pp. 377-384.

Zhang, Z, JR O'Rourke, MT McManus, M Lewandoski, BD Harfe, and X Sun 2011 The microRNA-processing enzyme Dicer is dispensable for somite segmentation but essential for limb bud positioning. *Dev Biol* **351** 254-265.

Zhu, DX, W Zhu, C Fang, L Fan, ZJ Zou, YH Wang, P Liu, M Hong, KR Miao, P Liu, W Xu, and JY Li 2012 miR-181a/b significantly enhances drug sensitivity in chronic lymphocytic leukemia cells via targeting multiple anti-apoptosis genes. *Carcinogenesis* **33** 1294-1301.

Zuckerman, L, and TG Baker 1977 The Development of the Ovary and the Process of Oogenesis. *In* L Zuckerman and BJ Weir (ed.), The Ovary, edn 1, pp. 41-67.