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New Insights into Ovarian Function

JoAnne S. Richards and Stephanie A. Pangas

Departments of Molecular and Cellular Biology and Pathology, Baylor College of Medicine, BCM130, One Baylor Plaza, Houston, TX 77030, USA

JoAnne S. Richards: joanner@bcm.tmc.edu; Stephanie A. Pangas: spangas@bcm.tmc.edu

Abstract

Infertility adversely affects many couples worldwide. Conversely, the exponential increase in world population threatens our planet and its resources. Therefore, a greater understanding of the fundamental cellular and molecular events that control the size of the primordial follicle pool and follicular development is of utmost importance to develop improved in vitro fertilization as well as to design novel approaches to regulate fertility. In this review we attempt to highlight some new advances in basic research of the mammalian ovary that have occurred in recent years focusing primarily on mouse models that have contributed to our understanding of ovarian follicle formation, development, and ovulation. We hope that these new insights into ovarian function will trigger more research and translation to clinically relevant problems.

Keywords

Follicle development; FOXO; Luteinization; MAPK; Oocytes; Ovary; Ovulation; SMADS; TGFbeta family; WNT4

1 Introduction

Based on the theme provided by the Editors of this book *Fertility Control – Today and in the Future*, the mission of this chapter is to focus on new advances in basic research of the mammalian ovary that have occurred in recent years. This is a daunting task because of the vast number of novel studies and mouse models that have contributed to our understanding of ovarian follicle formation, development, and ovulation. Therefore, we will highlight those areas that seem to us to have provided the most impact. We hope that these personal choices are not overly biased and that any oversights and omissions are minimal.

Much of the reproductive lifespan of most mammals and women is determined ultimately on the size of the primordial follicle pool and the quality of eggs derived from them. However, oocytes within the pool of quiescent primordial follicles form during embryonic and postnatal ages, long before the onset of puberty. For this period of oogenesis, key questions still remain regarding the input of endogenous factors that impact the proliferation of oogonia, onset of meiosis, arrest of meiosis at metaphase I, the breakdown of oocyte nests, and finally, the formation of primordial follicles. Even more murky is knowledge regarding

fundamental mechanisms that regulate primordial follicle activation, as well as specification and development of the somatic cells surrounding the oocyte (i.e., the granulosa and thecal cells), which are essential for subsequent oocyte development, ovulation, and fertilization. Modern technologies have opened many new and exciting approaches by which investigators can explore the molecular, cellular, and physiologic mechanisms controlling follicle formation and growth.

2 Novel Aspects of Gonadal Development, Primordial Follicle Formation, and Early Follicle Growth

The mammalian gonad first develops adjacent to the urogenital ridges as a thickening of the coelomic epithelium and is devoid of germ cells. Migrating primordial germ cells (PGCs) that were specified outside the embryo colonize the indifferent gonad, then undergo a period of proliferation. In females, the PGCs then enter meiosis and arrest in the first meiotic prophase. Many of the underlying signaling events that control ovary specification during this time are still being analyzed, but several key pathways have been identified. One of these is the WNT pathway. The WNT family is comprised of secreted glycoproteins that bind to, and signal through, the FRIZZLED (FZD) receptors. Mice null for *Wnt4* exhibit abnormal ovarian morphology in which structures similar to testicular chords are observed (Vainio et al. 1999), indicating that WNT4 might be a specific determinant of the female gonad. Mutations in the human *RSPOL1* gene, a WNT pathway adapter molecule, indicate that this molecule is also a candidate female sex determining factor (Parma et al. 2006) and female mice null for *Rspo1* demonstrate partial sex reversal and oocyte loss (Tomizuka et al. 2008). Quite remarkably, mice null for both *Wnt4* and *Foxl2*, a forkhead box transcription factor, exhibit complete and functional sex reversal of the ovary to a testis in the XX genotype (Ottolenghi et al. 2007). These intriguing and novel results document unequivocally that there are organizers of ovarian vs. testis development. By contrast, XY male mice expressing stable beta catenin (CTNNB1), a downstream target of WNT signaling, using *Sfl-Cre* mice (Maatouk et al. 2008) or *Amhr2Cre* mice (Chang et al. 2008) exhibit partial male to female sex reversal with ovarian structures totally lacking germ cells or that exhibit seminiferous tubule demise and germ cell loss, respectively. Thus, proper WNT signaling, likely involving a critical role for *Rspo1* as well as a FZD receptor, is essential for normal gonad development.

Proper expression of CTNNB1 in the adult ovary is also essential for normal tissue maintenance because overexpression of a constitutively active form of *Ctnnb1* (*Ctnnb1^{flox(exon3)}*) can lead to abnormal follicle development and eventually to granulosa cell tumors (GCTs) (Boerboom et al. 2005). Moreover, the tumor phenotype can be enhanced when the tumor suppressor *Pten* is simultaneously disrupted in the *Ctnnb1^{flox(exon3)}; Amhr2-Cre* mouse strain. In these mice, abnormal lesions are observed in the embryonic gonad, aggressive tumors form before puberty, and the mice die within 6 weeks of age (Lague et al. 2008). Because FSH has been shown to phosphorylate and inactive GSK3 β , a downstream component of the WNT/FZD signaling pathways that regulates CTNNB1, FSH also has the potential to enhance the transcriptional activation of

CTNNB1 and its target genes but the physiological relevance of this pathway remains to be determined (Cross et al. 1995).

After their proliferative period, PGCs eventually divide to form syncytia of oocytes (termed germ cell nests or cysts) that are connected by intracellular bridges. These bridges are not essential for fertility in females (Greenbaum et al. 2006). Germ cell cysts break down during formation of primordial follicles, when individual oocytes become surrounded by somatic ("pre-granulosa") cells, putatively derived from the coelomic epithelium. The breakdown occurs prenatally in humans or shortly after birth in mice. Germ cell cyst breakdown is associated with massive germ cell loss, such that oocyte numbers are reduced from approximately six million in the fetal human ovary to one million at birth. These numbers further decline to puberty into adulthood (Faddy et al. 1992; Block 1952; Baker 1963). Inappropriate germ cell cyst breakdown may result in ovarian follicles with more than one oocyte, often called polyovular follicles or multiple oocyte follicles. Some inbred mouse strains are known to have increased incidence of polyovular follicles (Engle 1927; Jagiello and Ducayen 1973), and many mouse knockouts have been made that demonstrate this phenotype as well, included several in the TGF β family. These include mice that overexpress the inhibin α subunit (McMullen et al. 2001), mice conditionally null for ovarian activins (Pangas et al. 2007) or follistatin (Jorgez et al. 2004), and mice null for *Bmp15* (Yan et al. 2001). Exposure of neonatal mice to estrogen also increases polyovular follicle formation (Kipp et al. 2007; Iguchi et al. 1986, 1990; Iguchi and Takasugi 1986; Chen et al. 2007). This occurs in conjunction with loss of the activin β subunits (Kipp et al. 2007). These data along with the polyovular phenotype displayed in ovaries of activin β A conditional knockout (cKO) mice (see below) suggest a direct role for activin signaling in the appropriate organization of primordial follicles (Pangas et al. 2007). The effects of estrogen on primordial follicle formation have important implications regarding estrogen-like environmental contaminants that act as endocrine disruptors and may impact early follicle formation and eventually the ability to reproduce.

In theory, increasing the size of the primordial follicle pool may be one way to extend reproductive lifespan and prevent diseases associated with menopause or reproductive senescence, such as increased cardiovascular disease and osteoporosis. For example, adult female mice null for the proapoptotic gene *Bax* have increases in primordial follicle numbers, an extended period of folliculogenesis, and decreases in age-related health defects (i.e., *Bax* knockout mice demonstrate decreased bone and muscle loss, adiposity, alopecia, and some behavioral changes, amongst other measured parameters) (Perez et al. 1999, 2007), although some of these changes may not be directly related to ovarian function. However, recent studies have suggested that *Bax* deficient ovaries have an increase in follicular endowment that is due to increased embryonic oogonia proliferation and not a rescue of oocytes from apoptosis (Greenfeld et al. 2007). The factor(s) that govern oogonia proliferation and germ cell survival during germ cell cyst breakdown during embryogenesis and gonadogenesis are not known, and thus remain a key research focus.

3 Transcription Factors That Regulate Early Postnatal Follicle Growth

Recent studies have identified a number of transcription factors whose expression, at least in adult tissues, appears to be restricted to germ cells or oocytes, and which are necessary for early folliculogenesis (Pangas and Rajkovic 2006). These transcription factors control, in part, the coordinated expression of genes necessary for early follicle growth, including growth and differentiation factor 9 (*Gdf9*) (see below) and the zona pellucida genes (*Zp1-3*). Factor in the germline alpha (FIGLA) was the first of these transcription factors to be identified (Liang et al. 1997), and mice null for *Figla* are sterile and primordial follicles do not form in the ovary (Soyal et al. 2000). *Figla* encodes a basic helix–loop–helix (bHLH) transcription factor that regulates expression of the zona pellucida genes, which encode the egg coat (Liang et al. 1997). Subsequent to the discovery of *Figla*, several other germ-line expressed bHLH transcription factors have been identified, including spermatogenesis and oogenesis bHLH transcription factors 1 and 2 (*Sohlh1* and *Sohlh2*). SOHLH1 and SOHLH2 are approximately 47% identical in the bHLH sequence, have a similar expression pattern in oocytes, and mice null for either gene have a similar female phenotype: postnatal oocyte loss leading to female sterility (Choi et al. 2008; Pangas et al. 2006a). Gene expression changes are similar in the mutant mice, with alterations in expression of genes known to be critical in folliculogenesis. Both knockout mouse models have deficiencies in ovarian expression of several homeobox transcription factors, *Lhx8*, *Pou5f1* (Oct4), and *Nobox*; in *Figla* and the zona pellucida genes *Zp1* and *Zp3*, in growth factor *Gdf9* and the kit ligand receptor, *Kit*. In addition, deletion of *Sohlh2* results in a more than 90% decrease in *Sohlh1*, while deletion of *Sohlh1* causes a 60% reduction in *Sohlh2* (Choi et al. 2008), i.e., *Sohlh2* mutant ovaries lack both *Sohlh1* and *Sohlh2* (are in effect doubly mutant), while *Sohlh1* ovaries are hypomorphic for *Sohlh2*. It is possible then that SOHLH2 regulates *Sohlh1* expression and much of the phenotype in both mouse models may be a direct consequence of loss of *Sohlh1*. Additional gene expression changes can be attributed to loss of *Nobox* (newborn ovary homeobox gene) expression. NOBOX has been shown to directly regulate expression of *Gdf9* and *Pou5f1* (Choi and Rajkovic 2006), and deletion of *Nobox* causes female sterility and postnatal oocyte loss (Rajkovic et al. 2004). Currently, it is unclear how these transcriptional networks intersect to control oocyte development, and which genes are direct targets of the various oocyte-expressed homeobox and bHLH transcription factors.

While deletion of oocyte-expressed genes is a straightforward approach with little to no embryonic or adult phenotypic consequences beyond those due to reproductive dysfunction, many genes expressed in oocytes are also expressed in other adult or embryonic tissues. This makes it necessary to develop conditional mouse models to study their intraovarian function, most commonly by using the Cre/lox site-specific recombination system. Generation of oocyte-specific gene deletion in mice has been facilitated by a number of mouse lines with oocyte-restricted promoters to express Cre recombinase [reviewed in (Pangas and Matzuk 2008)]. In particular, Cre recombinase expression from the *Zp3* promoter has been widely used (Lewandoski et al. 1997). More recently, the *Gdf9* promoter, which has a slightly earlier oocyte expression pattern than the *Zp3* promoter, has been used to express Cre recombinase in oocytes (Lan et al. 2004). Various oocyte conditional knockouts and knockdowns with female reproductive phenotypes include *Pten* (see below) (Reddy et al.

2008), *Cpeb* (cytoplasmic polyadenylation element binding protein) (Racki and Richter 2006), *Gcnf* (*Nr6a1*; an orphan nuclear receptor) (Lan et al. 2003), *Pig-a* (phosphatidylinositol glycan class-A) (Alfieri et al. 2003), and *Pou5f1* (POU-type homeodomain-containing DNA-binding protein, Oct4) (Kehler et al. 2004). Mouse models to study somatic cell function during primordial, primary, and secondary follicle formation are lacking, in part due to the paucity of mouse lines that direct efficient expression of Cre recombinase to the somatic cell compartments during primordial and primary cell stages (see below). However, some models mice expressing *Sfl-Cre* (Maatouk et al. 2008), *Amhr2-Cre* (Chang et al. 2008; Boerboom et al. 2005), and *Cyp19-Cre* (Fan et al. 2008a,b, 2009) have been useful.

Members of the forkhead family such as *Foxl2* and *Foxo3* also impact early follicle growth. Targeted disruption of *Foxl2* in mice leads to abnormal follicle development and premature ovarian failure (Uda et al. 2004a), and in humans is also associated with the craniofacial disease, blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES) (Crisponi et al. 2001). *Foxl2* is expressed in the early stages of gonadal development and has been shown to direct ovarian and oppose testis development. Specifically, genes that increase in early postnatal *Foxl2* null ovaries include *Dax1* (*NrOb1*) and *Wnt4*; genes that decrease include *Nr5a2*, *Cyp19*, *Fst*, and *Apoa1*. Additional genes regulated in the ovary by FOXL2 at later stages of follicle development include *Inhbb*, *Nr5a2*, *Srebf1*, *Pgc1a*, *Cyp11a1*, and *Star* (Pisarska et al. 2004; Uda et al. 2004b; Moumne et al. 2008). These results indicate that FOXL2 likely impacts not only embryonic ovarian formation but also specific basic metabolic aspects required for somatic cell proliferation and differentiation. Reduced levels of *Foxl2* have also been linked to aggressive progression of ovarian GCTs (Kalfa et al. 2008), indicating that FOXL2 may regulate multiple effects in granulosa cells that are context and stage specific. Another striking, recently published, ovarian phenotype occurs in mice in which the *Foxo3* gene has been disrupted (Castrillon et al. 2003). These mice exhibit premature ovarian failure due to inappropriate oocyte activation and the premature entry of primordial follicles into the growing pool. Upon exhaustion of the primordial pool, the ovaries become devoid of growing follicles and the mice are infertile. In line with these studies, forced overexpression of *Foxo3* selectively in oocytes reduces the number of follicles growing (Liu et al. 2007). Because the activity of FOXO3 is negatively regulated by the PI3kinase (PI3K) pathway, investigators also generated mice in which the *Pten* gene was conditionally disrupted in oocytes (Reddy et al. 2008). Because PTEN is a negative regulator of PI3K, its removal enhances PI3K activity leading to increased phosphorylation of downstream targets including AKT and FOXO3. As a consequence, the activity and levels of FOXO3 are dramatically reduced, leading to premature oocyte activation and release of primordial follicles into the growing pool thereby generating an ovarian phenotype identical to that of the FOXO3 null mice. Although microarray data have been generated from the *Foxo3* null ovaries, the specific targets of FOXO3 in the oocyte that impact the surrounding somatic cells remain to be defined (Gallardo et al. 2007).

In contrast to FOXO3, which is expressed in and impacts oocyte functions, FOXO1 is expressed preferentially and at high levels in granulosa cells of growing follicles. Because *Foxo1* null mice are embryonic lethal (Hosaka et al. 2004), an analysis of the role of this

transcription factor in the ovary has been precluded. However, mice in which *Foxo1*, *Foxo3*, and *Foxo4* alleles have been engineered to contain loxP sites (“floxed” alleles) for conditional deletion provide the opportunity to determine the cell specific disruption of these genes individually or collectively in the ovary (Paik et al. 2007; Tothova et al. 2007). These studies are now in progress and suggest that disruption of *Foxo1* impairs fertility. Although the mechanisms remain to be determined, FOXO1 may impact specific genes controlling proliferation (Park et al. 2005), differentiation (Park et al. 2005), or metabolic pathways (Liu et al. 2008a) in granulosa cells based on the expression of FOXO1 mutants in these cells. Specifically, expression of a constitutively active nuclear form of FOXO1 (FOXOA3 in which three serines have been substituted for alanines) in granulosa cells not only suppresses expression of *Ccnd2*, *Cyp19*, *Fshr*, and *Lhcgr* but also acts as a potent negative regulator of essentially all genes in the cholesterol biosynthetic pathway (Park et al. 2005; Liu et al. 2008a). The negative effects of FOXO1 appear to be mediated in part by the ability of the FOXO1 mutants to interact with other transcription factors including nuclear receptors, SP1 and SMADs (van der Vos and Coffey 2008; Rudd et al. 2007), and to reduce expression and activity of *Srebf1* and *Srebf2* in granulosa cells (Liu et al. 2008a). Because these two transcription factors regulate essentially all genes in the cholesterol pathway and some involved in fatty acid synthesis as well, reduction of these transcription factors impacts multiple genes that coordinate cholesterol and fatty acid biosynthesis. Likewise in liver (Zhang et al. 2006; Matsumoto et al. 2006) and pancreatic beta cells (Buteau et al. 2007), FOXO1 appears to play a major role in cholesterol and glucose homeostasis. Thus, drugs given to regulate cholesterol levels in humans or patients with diabetes will likely and potentially impact the function of ovarian cells as well.

4 Oocyte-Derived Growth Factors That Mediate Somatic Cell Function and Follicle Growth

Early follicle growth (i.e., after primordial follicle activation but before antrum formation) is considered to be largely driven by ovarian-derived growth regulatory factors independent of pituitary-derived follicle stimulating hormone (Kumar et al. 1997). The first of these intraovarian factors to be identified was oocyte-derived GDF9, a member of the transforming growth factor β superfamily (McPherron and Lee 1993; McGrath et al. 1995). In the mouse, *Gdf9* is first expressed in oocyte cysts and primordial follicles of newborn ovaries (Rajkovic et al. 2004), although the protein is undetected until follicle stage 3a (a class of primary follicles) and subsequently increases in level in all other follicles (Elvin et al. 1999a). Consistent with this, mice with a genetic disruption of *Gdf9* are infertile and demonstrate abnormal follicle development, with an arrest at the primary follicle stage (Dong et al. 1996). However, the primary follicles that form have abnormal oocytes and somatic cells. While granulosa cells initially organize around the oocyte, they are defective in their proliferation. In addition, the thecal cell layer fails to organize. These defects occur in concert with inappropriate and accelerated oocyte growth, leading to abnormally large and defective oocytes (Carabatsos et al. 1998). Primary follicle stage arrest can be partially rescued by removing expression of the inhibin alpha (*Inha*) gene, which is inappropriately upregulated in granulosa cells of *Gdf9* knockout (KO) ovaries (Elvin et al. 1999b), suggesting that suppression of *Inha* expression in granulosa cells is an important step in

early folliculogenesis to allow normal granulosa cells to grow and differentiate. Follicles from double mutant *Inha Gdf9* homozygous null mice are able to form multilayer follicles, but then arrest prior to antrum formation and do not develop a functional thecal cell layer (Wu et al. 2004). These data further highlight the importance of the TGF β family in multiple stages of follicle development, though many of these functions are still not understood.

Other members of the TGF β family that influence follicle physiology and growth include BMP15 and activin. Similar to GDF9, BMP15 is an oocyte-derived growth factor (Dube et al. 1998) that functions by regulating granulosa cell proliferation and differentiation. Mice with homozygous null mutations in *Bmp15* are subfertile on some genetic backgrounds, while mice deficient for both *Gdf9* and *Bmp15* phenocopy the *Gdf9* homozygous null mouse model (Yan et al. 2001). However, removal of one copy of *Gdf9* in a *Bmp15* null background results in additional decreases in fertility compared with *Bmp15*^{-/-} (Yan et al. 2001). It appears that BMP15 is not critical for early follicle development in mice, or alternatively, its loss may be compensated for at these early stages by GDF9. However, BMP15 appears to influence the development of the granulosa cell layer most closely associated with the oocyte, collectively called the cumulus cell layer. Studies on double mutant *Gdf9*^{+/-}*Bmp15*^{-/-} mice demonstrate that cumulus cells cannot appropriately respond to signals from wild type oocytes to undergo the process of cumulus expansion (see below), suggesting that the cumulus cells in double mutant *Gdf9*^{+/-}*Bmp15*^{-/-} follicles are developmentally compromised (Su et al. 2004). The nature of these molecular defects is currently unknown but may be related to changes in cumulus cell metabolism (Su et al. 2008). Mouse and human BMP15 are mitogens for granulosa cells (Otsuka et al. 2000; McNatty et al. 2005), and transgenic overexpression of mouse BMP15 in oocytes causes normal but accelerated follicle development and subsequently, an early onset of acyclicity (McMahon et al. 2008). Even though GDF9 and BMP15 are highly conserved, there appears to be species-specific differences regarding their function within the ovary (Juengel and McNatty 2005). Homozygous sheep mutations in BMP15 have an ovarian phenotype that appears similar to the mouse *Gdf9* knockout. The BMP15 mutations when carried as only a single copy in sheep result in an increased ovulation rate, while no phenotype has been associated with *Bmp15* or *Gdf9* heterozygous mutations in mice. In humans, mutations in BMP15 and GDF9 have been infrequently found to be associated with premature ovarian failure (Di Pasquale et al. 2004, 2006; Simpson 2008), though heterozygous mutations in BMP15 have not been reported for twinning in humans (Zhao et al. 2008). Because of their restricted expression pattern and ability to modulate fertility, BMP15 and GDF9 might be good candidates for contraceptive development. Initial experiments demonstrate that sheep immunized against BMP15 or GDF9 have abnormal folliculogenesis and ovulation rates (McNatty et al. 2007; Juengel et al. 2002). Targeting antibodies to N-terminal peptides appear to be the most efficient means to neutralize their bioactivity (McNatty et al. 2007).

5 Novel Regulatory Mechanisms That Control Follicle Growth and Differentiation

Although many early stages of follicle growth can occur independently of pituitary gonadotropins, ovarian follicles, and more specifically granulosa cells, rely on FSH for

follicular antrum formation and for continued growth and differentiation during the antral follicle stages. Moreover, recent studies provide new insights into the multiple signaling pathways that are stimulated in granulosa cells by FSH. This glycoprotein hormone is known to activate adenylyl cyclase, leading to the production of cAMP and the activation of protein kinase A (PKA). There is no doubt that activation of this classical pathway is essential for many aspects of granulosa cell differentiation. However, FSH can also activate the PI3K pathway (likely via a SRC tyrosine kinase) leading to the phosphorylation and activation of AKT, which phosphorylates and thereby inactivates FOXO1 (Gonzalez-Robayna et al. 2000). As mentioned above, FOXO1 has the potential to regulate cholesterol metabolism in granulosa cells, thereby preventing premature increases in precursors for steroidogenesis (Liu et al. 2008a). FOXO1 can also reduce the expression of genes regulating granulosa cell proliferation and differentiation (Park et al. 2005; Liu et al. 2008a). As mentioned, because of the embryonic lethality of the *Foxo1* null mutation, the effects of disrupting *Foxo1* in granulosa cells have not yet been analyzed in vivo. However, the disruption of *Pten* in granulosa cells leads to increased activation of the PI3K pathway, and therefore increased phosphorylation and degradation of FOXO1, resulting in enhanced proliferation, ovulation, and the formation of corpora lutea that persist for unusually prolonged periods of time (Fan et al. 2008a). Surprisingly, although FOXO1 is expressed at elevated levels in granulosa cells, PTEN protein levels are remarkably low. Therefore, factors other than, or in addition to, PTEN may serve to control the PI3K pathway in granulosa cells. These results indicate that the functions of PI3K pathway components in granulosa cells are complex and likely to be stage- and context-specific (Fan et al. 2008a). Thus, disruption of *Pten* in the somatic cells of the mouse ovary causes distinctly different effects from the disruption of this gene in oocytes, as described above (Castrillon et al. 2003; Liu et al. 2007). Furthermore, although natural mutations or disruption of *Pten* in other tissues leads to tumor formation, the disruption of *Pten* alone in granulosa cells did not lead to granulosa cell tumors (Fan et al. 2008a), perhaps because other factors impact the PI3K pathway in these cells.

FSH and LH have recently been shown to activate RAS via a SRC tyrosine kinase-mediated process (Wayne et al. 2007). Activated RAS then leads to the phosphorylation and activation of downstream kinases, MEK1 and MAPK3/1 (also known as ERK1/2) (Wayne et al. 2007). Strikingly, KRAS is expressed at high levels in granulosa cells of small and antral follicles but the role of KRAS in granulosa cells remains to be determined (Fan et al. 2008b). Expression in granulosa cells of a constitutively active form of KRAS, KRAS^{G12D}, which is frequently associated with various cancers including ovarian cancer and cell transformation, does not stimulate proliferation or tumor formation in these cells (Fan et al. 2008b). Rather, the KRAS^{G12D} expressing granulosa cells cease dividing, do not exhibit apoptosis, and fail to differentiate, i.e., they become senescent. As a consequence, the abnormal follicle-like structures persist and accumulate in the ovaries of the KRAS^{G12D} mutant mice. Even when *Pten* is disrupted in the *Kras*^{G12D} mutant strain, GCTs do not form (Fan et al. 2009). These results indicate that granulosa cells are extremely resistant to the oncogenic insults of mutant *Kras* and the loss of *Pten*. By contrast, if the *Kras* and *Pten* mutations are made in ovarian surface epithelial cells, aggressive tumors appear within 6 weeks of age (Fan et al. 2009).

6 The TGF β Family in Regulation of Granulosa Cell Growth and Differentiation

The TGF β family of growth factors has wide-ranging roles in female reproduction. Various family members are expressed from the major ovarian cell types (i.e., oocytes, granulosa cells, thecal cells), though many of the effects appear to center on control of granulosa cell growth and differentiation that then impact folliculogenesis and oocyte development. Many recent studies have analyzed the role of this family by cre/loxP-mediated conditional deletion in granulosa cells. Two Cre recombinase lines are particularly used for granulosa cell deletion: *Amhr2cre*, a knockin of Cre recombinase into the anti-Mullerian hormone receptor type II locus (Jamin et al. 2002), and *Cyp19-Cre*, a transgenic line that contains a portion of the aromatase gene that limits Cre expression to granulosa cells and luteal cells (Fan et al. 2008a). While both Cre lines are expressed in granulosa cells, subtle differences may exist in their expression pattern, with *Cyp19-Cre* being expressed in slightly later stage follicles than *Amhr2-Cre* (Fan et al. 2008a).

Follistatin, a BMP and activin antagonist, was the first gene to be conditionally deleted from granulosa cells (Jorgez et al. 2004). Ovaries from follistatin knockout mice have almost complete loss of germ cells prior to birth (Yao et al. 2004). *Fst* conditional knockout female mice (cKOs) demonstrate premature ovarian failure, with few remaining follicles found by 8 months of age (Jorgez et al. 2004). Fertility defects are accompanied by changes in the levels of serum hormones, including increases in follicle stimulating hormone (FSH), luteinizing hormone (LH), and decreases in serum testosterone. Loss of follistatin within the ovary likely results in increased activin activity and possibly, BMP activity. The loss of intraovarian activins results in a different phenotype. Activin is a homo or heterodimer of two related β subunits: β A and β B. Mice null for β A die shortly after birth (Matzuk et al. 1995), but mice deficient for β B have normal size litters but defects in nursing (Vassalli et al. 1994). Ovaries from β B deficient females overproduce the β A subunit (Vassalli et al. 1994), suggesting that any intraovarian reproductive phenotype that is caused by loss of β B may be masked by a compensatory gain in activin A. Thus, the stepwise removal of the activin subunits by conditional deletion in granulosa cells eventually culminates in female sterility when no activin subunits are expressed (Pangas et al. 2007). While there are multiple defects in folliculogenesis in the activin deficient ovary (Pangas et al. 2007), one of the most obvious defects is the progressive and abnormal accumulation of corpora lutea that is accompanied by increases in serum FSH and progesterone. Other defects include preantral follicles undergoing early luteinization and an increased number of antral follicles. There are likely additional defects in granulosa cells during ovulation because the increase in antral follicle numbers is not reflected in the number of ovulated oocytes, which is significantly decreased. Even though mutations in the activin signal transduction pathway have been implicated in cancer development, and activins have been shown to be critical for growth inhibition in some cell types (i.e., breast and prostate cancer cells) (Cocolakis et al. 2001; Zhang et al. 1997), no tumors develop in the activin-deficient mouse model. Thus activin, like TGF β , may have variable oncogenic or tumor suppressor properties that are cell-type or context-specific. For example, in granulosa cells, activin appears to play a predominant role as a growth promoter, and its role in the promotion of GCTs has been established in the

inhibin alpha knockout mouse. Deletion of inhibin α results in sex-cord stromal tumors in male and female mice and premature death due to development of a cancer cachexia like syndrome (Matzuk et al. 1992, 1994). Genetic removal of the activin type II receptor, deletion of the activin downstream transcription factor *Smad3*, or injection of a chimeric activin binding receptor-murine Fc protein, slows, though does not prevent, tumor growth in inhibin α -deficient mice (Matzuk et al. 1992, 1994; Coerver et al. 1996; Li et al. 2007a, b; Looyenga and Hammer 2007), demonstrating that activin signaling plays a growth promoting role.

The role of the TGF β family in ovarian follicles has also been investigated by deletion of the SMAD transcription factors, which are part of the TGF β family canonical signaling pathway. SMAD2 and SMAD3 signal for activin, GDF9, and TGF β , while SMAD1, SMAD5, and SMAD8 signal for the BMPs and AMH. An additional SMAD, SMAD4, is shared by all members of the TGF β family. Conditional mutations for these SMADs have been generated in granulosa cells (Li et al. 2008; Pangas et al. 2006b, 2008). Conditional deletion of *Smad4* results in age-dependent infertility, with defects in steroidogenesis, ovulation, cumulus cell function, and eventually premature ovarian failure (Pangas et al. 2006b). Unlike the activin-deficient mouse model, *Smad4* cKO ovaries show an increase in preantral follicle death, a decrease in the number of antral follicles, and no accumulation of CLs. Similar to the activin-deficient ovary, small follicles appeared to luteinize prematurely, and even though *SMAD4* is a known tumor suppressor gene, no tumors developed in *Smad4* cKO mice. Cumulus cells in the *Smad4* cKO are defective and undergo a disorganized or limited cumulus cell expansion. The defects in preantral follicle growth and cumulus cells may be attributable to the inability of GDF9 to fully function through the SMAD pathway when *Smad4* is deleted.

A similar phenotype to *Smad4* cKO female mice is seen in granulosa cell conditional knockouts of the activin/TGF β signaling SMADs (AR-SMADs), *Smad2* and *Smad3* (Li et al. 2008). SMAD2 and SMAD3 have both unique and redundant roles in various tissues, but appear to have redundant functions in granulosa cells because single conditional knockouts of *Smad2* or *Smad3* in granulosa cells have no discernable reproductive phenotype (Li et al. 2008). However, double *Smad2 Smad3* cKO mice using *Amhr2cre* have reduced litter sizes and become infertile after 5 months of age with disrupted follicle development (i.e., fewer antral follicles), luteinized follicles, and reduced ovulation, with severe defects in cumulus cell function. The phenotypes of conditional knockouts for the BMP SMADs (BR-SMAD) have phenotypes that differ dramatically from the other SMAD conditional knockout models. Single conditional mutants *Smad1* or *Smad5*, or *Smad8* KO mice, are viable and fertile. The combinations of double conditional *Smad1 Smad8*, or *Smad5 Smad8*, are also fertile. However, reproductive phenotypes are seen in double conditional *Smad1* and *Smad5*, or triple conditional *Smad1 Smad5 Smad8* mice (Pangas et al. 2008). Both *Smad1 Smad5* dKO or *Smad1 Smad5 Smad8* tKO have initial fertility defects with decreased litters per month and increasing infertility with age. These mice also develop GCTs with full penetrance by 3 months of age. In addition, the majority of *Smad1*, *Smad5* dKO and *Smad1*, *Smad5*, *Smad8* tKO mice show peritoneal implants and lymphatic metastases over time. The

Smad1, Smad5 dKO and Smad1, Smad5, Smad8 tKO models were the first in vivo demonstration that the BMP SMADs may have a critical tumor suppressor function.

The phenotypes of the various SMAD and activin/inhibin knockouts in the ovary suggest a potential interaction between the various BMP and TGF β /activin pathways in controlling granulosa cell growth and differentiation. Of the knockouts generated in the TGF β family, only two TGF β family mouse models develop GCTs: the inhibin α KO and the BR-SMAD cKOs. Part, though not all, of the phenotype of the inhibin α KO has been attributed to activin's tumor promoting activity in part via SMAD3 (Li et al. 2008) (see above). In the BR-SMAD cKO mouse models, an examination of the phosphorylation status of the AR-SMADs demonstrated that SMAD2 and SMAD3 are nuclear and phosphorylated, indicating pathway activation; thus, it has been suggested that part of the phenotype of the BR-SMAD cKO may be due to dysregulated AR-SMAD (i.e., *Smad2* and *Smad3*) pathway regulation (Pangas et al. 2008). Thus, one of the roles of the BR-SMADs may be to antagonize or cross-regulate the AR-SMADs to control cell proliferation. The role of additional signaling pathways in tumorigenesis in the BR-SMAD cKO models is still under investigation.

Disrupting the function of TGF β family ligands and their signaling pathways not only influences somatic cell function, but eventually results in improper oocyte development. As originally proposed by John Eppig (1991), oocytes secrete factors now known to include GDF9 and BMP15, which based on mouse knockout studies are known to control specific somatic cell functions, including optimal expansion of the cumulus oocyte complex prior to ovulation (Elvin et al. 1999a; Pangas and Matzuk 2005; Vanderhyden et al. 2003). Most recently, Eppig et al. (2008, 2007) have published novel results indicating that oocyte-derived factors regulate cumulus cell production of key metabolic substrates presumed essential for oocyte quality and viability (Eppig et al. 2005; Sugiura et al. 2005a, b). Specifically, oocytes do not make their own cholesterol, fatty acids, or glucose. Rather the oocytes, most probably by release of BMP15 and GDF9, control cumulus cell expression of specific genes within the cholesterol biosynthetic pathway and the glycolytic pathway. As noted above, FOXO1 also appears to regulate genes in the cholesterol biosynthetic pathway, and thus there may be a functional link between FOXO1 and the oocyte derived growth factors BMP15/GDF9, though this remains to be determined.

7 New Mediators of Ovulation and Luteinization

Based on recent studies, we know that the LH surge can stimulate PKA, AKT, and RAS signaling cascades, and that each of these appears critical for ovulation (Fan et al. 2008b). Most importantly, the seminal studies of Marco Conti and colleagues have shown that LH rapidly induces in granulosa cells the expression of the EGF-like factors amphiregulin (AREG), betacellulin (BTC), and epiregulin (EREG) (Conti et al. 2005). These factors bind the EGF receptors present on granulosa cells and induce the expression of downstream target genes, *Has2*, *Ptgs2*, and *Tnfaip6*, which in cultured cells are targets of ERK1/2 (Shimada et al. 2006). Disruption of the EGF ligand-receptor signaling pathway in mice compromises ovulation, indicating that activation of this pathway is essential for LH-induced ovulation to occur (Hsieh et al. 2007). Moreover, mice in which ERK1 and ERK2 have been disrupted in granulosa cells exhibit normal follicle growth but fail to ovulate or

luteinize (unpublished observations). Thus, the critical importance of LH induction of the EGF-like factors and activation of the EGF receptor pathway is mediated, in large part by the activation of ERK1/2 in granulosa cells as well as in cumulus cells (Shimada et al. 2006). Specifically, the prostaglandins (PGE₂) and the EGF-like factors produced by granulosa cells then activate specific PGE and EGF receptors present in cumulus cells leading to the expression of specific genes involved in expansion of the cumulus oocyte complex and oocyte maturation (Shimada et al. 2006). The genes involved in expansion include factors essential for making and stabilizing the hyaluronan matrix (*Has2*, *Ptgs2*, *Tnfaip6*, *Vcan*, and *Ptx3*) but also additional genes frequently associated with innate immune responses, including components of the Toll-like receptor pathway, *Cd34*, *Cd52*, *Alcam*, many potent cytokines, such as IL6, as well as transcription factors *Runx1* and *Runx2* (Shimada et al. 2006; Liu et al. 2008b, 2009; Richards et al. 2008).

The impact of cytokines on ovarian function represents a relatively new area of investigation. Recently, IL6 alone has been shown to stimulate expansion of the cumulus oocyte complexes and induce the expression of specific genes involved in this process (Liu et al. 2009). These observations indicate that in clinical situations where levels of IL6 are elevated, such as chronic infections, endometriosis, and possibly PCOS, this and other potent cytokines may disrupt the normal functionality of granulosa and cumulus cells. IL6 acts via specific receptors (IL6ST, also known as gp130) present on cumulus cells as well as the oocyte. Moreover, IL6 can increase the expression of *Stat3* and *Il6st* in cumulus cells and the oocyte present in preovulatory follicles and enhance reproductive outcomes, suggesting that this pathway significantly influences oocyte quality (Liu et al. 2009). Of note, mice null for *gp130/Il6st* exhibit defects in zygotic cell division, suggesting that IL6 and related cytokines regulate oocyte function (Molyneaux et al. 2003). Because the induction of IL6 is regulated not only by AREG (Liu et al. 2009) but also by the progesterone receptor (PGR) (Liu et al. 2009; Kim et al. 2008) and possibly CEBP β (unpublished observations) that are essential for ovulation, it is tempting to speculate that IL6 may mediate some key process downstream of PGR and CEBP β in granulosa cells. Moreover, the expression of SNAP25, an important component controlling neuronal-like secretion of cytokines from granulosa cells, is also regulated by PGR (Shimada et al. 2007). Thus, the importance of locally produced and secreted ovarian cell derived cytokines during ovulation needs to be analyzed further and may be relevant for several ovulation-related processes including rupture, COC transport, and fertilization (Richards et al. 2008). In this regard, it is important to note that cytokines have recently been shown to influence the fertilization process by enhancing sperm motility and capacitation (Shimada et al. 2008).

Because mice null for the nuclear receptor interacting protein *Nrip1* (also known as RIP140) also exhibit impaired ovulation and reduced expression of *Areg*, *Ereg*, and many other ovulation related genes, it is possible that NRIP1 regulates transcription of the *Areg* gene, a critical early event in the ovulation process (Tullet et al. 2005; Nautiyal et al. 2010). Because NRIP1 also impacts metabolic pathways and inflammatory events in other tissues (Nichol et al. 2006), NRIP1 may also regulate additional events critical for ovulation.

Targeted disruption of the transcription factor Steroidogenic Factor 1 (SF1; now known as nuclear receptor subfamily 5, group a, member 1, NR5a1) in mice provided the first major

evidence that this nuclear receptor was essential for pituitary, gonad, and adrenal formation (Luo et al. 1994). Conditional deletion of this gene in granulosa cells has documented further that there is a critical role for SF1 in early follicle formation and development (Pelusi et al. 2008). More recently, a conditional knockout of *Lhr1* (*Nr5a2*; an orphan receptor highly similar to *Nr5a1*) in murine granulosa cells has been reported (Duggavathi et al. 2008). Although *Nr5a2* is also expressed in granulosa cells of small and growing follicles, evidence from the conditional disruption of *Nr5a2* in granulosa cells indicates that it plays a more critical role in events associated with ovulation and luteinization than in the early stages of follicle growth. Impressively, the *Lhr1* null mice exhibit impaired ovulation and luteinization suggesting that this nuclear factor plays a critical role in both of these processes. Thus, in this in vivo context, these two nuclear receptors appear to exhibit distinct, rather than overlapping, functions. One explanation for this may be related to the impact of specific signaling cascades and phosphorylation of either SF1 or LRH1 that alters their ability to bind key regulatory elements in target genes via a switch-type mechanism (Weck and Mayo 2006). Additionally, LRH1 and SF1 exhibit similar functions when overexpressed in cultured rat granulosa cells with the notable exception that SF1, but not LRH1, can override the inhibitor effects of DAX on FSH stimulation of estradiol and progesterone biosynthesis (Saxena et al. 2007). However, an essential role for DAX in the ovary is doubtful based on evidence that targeted disruption of DAX does not alter normal follicle development, but does prevent normal testis development (Yu et al. 1998). Thus, DAX is not a potent determinant of ovarian development. The differences in the functions of SF1 and LRH1 also appear to be related, in part, to genes that are selectively regulated by SF1 (*Amh*, *Inha*) (Pelusi et al. 2008) compared with LRH1 (*Cyp19*, *Cyp11a1*, and *Ptgs2*) (Duggavathi et al. 2008). Recently, mice null for the estrogen-specific sulfotransferase (*Sult1e1*) have been generated and exhibit impaired ovulation and cumulus expansion (Gershon et al. 2007), suggesting that LRH1 or other transcription factors potentially induce expression of this gene in response to the LH surge. Serum and presumably intraovarian levels of estradiol are elevated in these mice disrupting normal feedback mechanisms. In addition, sulfated estradiol is an inactive form unable to bind estradiol receptors thereby potentially altering ovarian cellular functions. However, the mechanisms by which *Sult1e1* disruption impairs ovulation have not been defined. Based on the genes regulated by ERK1/2, LRH1, and NRIP1, it is tempting to speculate that NRIP1 may be an important coregulator and/or activator of LRH1 in the ovary and that ERK1/2 may be required for specific phosphorylation events. Because LRH1 is also important for liver metabolism (Lee and Moore 2008) and embryonic stem cells (Mullen et al. 2007), it will be interesting to determine what specific genes this factor controls in ovarian cells that are distinct from those regulated in liver or embryonic stem cells. One other mutant mouse model in which both ovulation and luteinization are impaired is the *Cebpb* null mouse (Sterneck et al. 1997). Thus, it will be important to determine how ERK1/2, CEBP β , LRH1, and NRIP1 coordinately regulate a select number of genes.

8 New Regulators of Oocyte Maturation and Meiosis

The meiotic arrest of oocytes is controlled by critical levels of cAMP within the oocyte. For many years somatic cells were thought to be the source of cAMP that was delivered to the

oocyte via gap junction because the disruption of gap junctions elicited spontaneous resumption of meiosis (Norris et al. 2008; Gittens and Kidder 2005). However, recent molecular studies have identified and highlighted a critical role for specific G-protein coupled receptors, especially GPR3 and possibly GPR12 in controlling intraoocyte production of cAMP and thereby suppressing meiotic maturation in oocytes of antral follicles (Mehlmann et al. 2004; Hinckley et al. 2005). Specifically, disruption of *Gpr3* in mice led to premature resumption of meiosis and ovarian “aging” (Mehlmann et al. 2004). Human oocytes also express functional *Grp3* but not *Gpr12* (DiLuigi et al. 2008). Collectively, these studies provide the first evidence that oocytes themselves express a receptor that allows these cells to generate their own cAMP. These observations indicated that intra-oocyte cAMP levels were unlikely to be controlled exclusively by transport from somatic cells to the oocyte as previously thought. Furthermore, phosphodiesterase *Pde3a* and adenylyl cyclase 3 (*Adcy3*) are selectively expressed in murine and human oocytes (Vaccari et al. 2008). Disruption of *Pde3a*, which increases cAMP, or *Adcy3*, which reduces cAMP, either prevents or stimulates, respectively, precocious oocyte maturation (Vaccari et al. 2008). Moreover, the phenotype of mice null for both *Gpr3* and *Pde3a* indicates that *Gpr3* is the major source of cAMP that disrupts meiosis in the *Pde3a* null mice (Vaccari et al. 2008) because meiosis is restored in the double mutant mice. These results provide additional evidence for intra-oocyte control of cAMP production and degradation. Although the specific ligands for these orphan G-protein coupled receptors remain to be convincingly characterized, sphingosine 1-phosphate is a likely candidate (Uhlenbrock et al. 2002).

Thus, factors that regulate ligand production or that modify receptor activity provide potential targets for designing molecules that specifically target oocytes and thereby regulate fertility by blocking meiosis or by eliciting premature resumption of meiosis. For example, a molecule that would selectively bind to ZP1, 2, or 3 and deliver a potent signal to the oocyte might be engineered and delivered via novel nanoparticles. In addition, specific PKA anchor proteins (AKAPs) are present in oocytes and appear to be key regulators of the cAMP/PKA pathway that controls meiosis in the mammalian oocyte (Burton and McKnight 2007). Therefore, targeting these molecules might also provide novel ways to control fertility.

Perhaps one of the most dramatic and provocative approaches developed to study the dynamics of follicle and oocyte growth comes from studies of Woodruff and Shea (Pangas et al. 2003; West-Farrell et al. 2008; Xu et al. 2006). These investigators and their colleagues have shown in mice that maintaining a 3-D follicle structure within an inert but supportive extracellular alginate matrix permits in vitro follicle growth and oocyte maturation in response to hormones. Moreover, these in vitro matured mouse follicles could ultimately be stimulated to release oocytes capable of being fertilized in vitro and subsequently give birth to viable, healthy pups. These approaches open new and exciting possibilities for preserving ovarian tissues in woman undergoing radiation treatment and who subsequently wish to conceive. Although tedious and currently untested in nonrodent species, these approaches may provide a way to mature follicles and thereby reduce the genetic changes that are observed in oocytes matured in vitro compared with in vivo. These studies may also provide a method to study differences that may exist between individual follicles or oocytes, and potentially be able to provide a “biomarker” (either somatic or germ

cell) that may be used to determine the capacity of individual eggs when fertilized to develop into viable offspring.

9 Summary

New insights into regulators of early oocyte and follicle formation (*Nr5a1*, *Nobox*, *Sohlh1*, *Lx8*, *Foxo3*, *Foxl2*, *Wnt4*, *Ctnnb1*), follicular growth (*Gdf9*, *Bmp15*, *Foxo1*, *Smads*, *Inha*, *Inhba*, *Inhbb*) as well as ovulation and luteinization (*Cebpb*, *Nr5a2*, *Areg*, *Ereg*, *Btc*, *Nrip1*, *Kras*, *Mapk1/3*, *Il6*) indicate that multiple factors and signal transduction pathways act in a cell specific and context specific manner. To regulate fertility, cumulus oocyte complexes remain an attractive target if one could prevent expansion, alter oocyte/cumulus cell interactions or oocyte maturation by disrupting the actions of specific cytokines or other factors without altering the functions of other major organs. These might provide new avenues for contraceptive research as well as improving fertility in women with endometriosis and PCOS.

Information being derived from new approaches such as the exponential increase in knowledge of microRNAs should provide additional insights into factors and combined sets of factors that regulate genes in a cell and context specific manner (Otsuka et al. 2008; Nagaraja et al. 2008; Hong et al. 2008; Fiedler et al. 2008; Mishima et al. 2008). Because microRNAs control the levels of more than one mRNA, local disruption of these molecules may also provide tools for regulating fertility, cancer, and development. Because the delivery of small molecules by a variety of nanotechnology approaches is being aggressively pursued by many, these approaches may enhance the specificity and cell specific delivery of key regulatory molecules that can impact fertility at critical sites and times.

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