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Ursula-F. Habenicht
R. John Aitken
Editors

Fertility Control

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Preface

The world's population is growing at an unsustainable rate. From a baseline figure of one billion in 1800, global population is predicted to exceed nine billion by 2050 and 87.8% of this growth will be localized in less developed countries. Such uneven population growth will yield a harvest of poverty, malnutrition, disease and environmental degradation that will affect us all. Amongst the complex mixture of political, social, cultural and technological changes needed to address this issue, the development of improved methods of fertility regulation will be critical. The inadequacy of current contraceptive technologies is indicated by recent data suggesting that the contraceptive needs of over 120 million couples go unmet every year. As a direct consequence of this deficit 38% of pregnancies are unplanned and more than 50% end in an abortion, generating a total of 46 million abortions per annum particularly among teenagers. If safe, effective contraceptives were available to every couple experiencing an unmet family planning need, 1.5 million lives would be saved each year (UNFPA 2003).

Progress in contraceptive technology should not only generate more effective methods of regulating fertility, but should also provide a range of methods to meet the changing needs of the world's population. Contraceptive practice was revolutionized in 1960 in the US and 1961 in Europe by the introduction of the oral contraceptive pill by Gregory Pincus, MC Chang and colleagues, based on fundamental hormone research conducted in Germany. While "the pill" continues to represent a highly acceptable and effective method of fertility regulation, we should not lose sight of the fact that this approach has its roots in the endocrinology of the 1920s and was designed to meet the clinical and social needs of the 1960s. During the past 50 years we have seen no radically new forms of family planning designed to meet the contraceptive needs of the twenty-first century. For example, fertility control in the future will have to be linked with the need to prevent the spread of sexually transmitted diseases (STD). Every year at least 340 million new cases of curable STD are notified, one third in young people under 25 years of age (World Health Organization 2001). Recent figures on AIDS indicate that this condition is continuing to spread at a rate of 2.7 million new cases a year, generating an

estimated annual death toll of 2 million (UNAIDS and World Health Organization (2009). Africa has been decimated by the disease and it is now rapidly gaining hold in SE Asia. Chlamydia is also spreading rapidly and is now one of the most commonly diagnosed bacterial sexually transmissible infections (Hocking et al. 2008). The spread of STDs is particularly marked in young women aged 15–25, for whom the risk of infection is approximately six times greater than their male counterparts. For these women, development of dual-purpose methods that simultaneously target pregnancy and STDs are desperately needed. Similarly, the contraceptive strategies we develop for the future should also recognize the increasing desire of men to actively participate in the family planning process. Furthermore, it should be emphasized that whereas in the past approximately 10 years of contraceptive protection was required in a lifetime, nowadays the average couple will require 30 years of contraception to meet their family planning needs, due to earlier onset of sexual activity, later time point for first birth and greater intervals between births. As a consequence we not only have to deal with the differing contraceptive needs demanded by diverse cultural and social environments, but also with the changing needs of individual women over their reproductive lifespan.

Given all the major improvements in healthcare that have been delivered by molecular medicine in the last half-century, it is remarkable that something that touches all of our lives should be so neglected. The major reasons for this state of affairs have been three fold. First, the specification for new contraceptive methods is extremely difficult to achieve. We want the new generation of contraceptive agents to combine absolute efficacy with the complete absence of adverse side effects. Because contraceptives are the only medicinal compounds that we give to perfectly healthy people, the risk-benefit equation is strongly driven the right i.e., all benefit, no risk. Developing pharmaceutical agents that meet such exacting standards will be hard. Secondly, the history of contraceptive development has been beset with the frustration of trying to project radically new methods of fertility control from an extremely narrow science base. It is extremely difficult to interfere with the reproductive system in a controlled, targeted manner, if we do not understand how the system works. Since the pharmaceutical industry is not primarily designed to make fundamental contributions to the science-base, this role has been left to public sector research institutions and, as a result, progress has been painfully slow. This situation has been exacerbated by the third factor, which is the low priority given to basic reproductive research by public sector funding agencies. Infertility is not seen as a life-threatening condition in the same way as cancer, multiple sclerosis or kidney failure, and governmental research priorities tend to reflect this perception, no matter how short-sighted.

Hopefully contraception will not remain a neglected field for much longer. The political climate has recently changed to one that is more sympathetic to reproductive research. In the past decade we have also witnessed the birth of private–public partnerships in order to improve our fundamental understanding of the reproductive process through the creation of coordinated international networks. For example in 1997, the Rockefeller Foundation and Ernst Schering Research Foundation developed one of the first such networks to intensify research on the posttesticular

maturation of spermatozoa, utilizing new approaches in molecular pharmacology [the application of molecular pharmacology for posttesticular activity (AMPPA) network]. Hopefully this will be the predecessor of further targeted networks in the future. With the advent of such initiatives, as well as parallel developments in the fields of pharmacology and drug design, the scene is now set for dramatic improvements in the technologies we shall use to regulate our future fertility.

This volume could not have been produced at a more opportune moment. It brings together contributions from all corners of the globe on all aspects of reproductive biology pertinent to contraceptive development. It contains cutting edge assessments of the molecular mechanisms regulating male and female reproduction and the new opportunities for contraceptive development to emerge as a consequence of this knowledge. It also contains expert evaluations of the potential for product development in the contraceptive field. This book looks forward to a future where men and women will be able to choose from a range of novel, safe, effective, contraceptive methods tailored to their individual needs. Hopefully it will inspire a new generation of young scientists and clinicians to exploit recent gains in our understanding of reproductive mechanisms, to engineer such new approaches to the regulation of human fertility.

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Part I
Female Reproduction

New Insights into Ovarian Function

JoAnne S. Richards and Stephanie A. Pangas

Contents

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.

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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 FRIZZED (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 *RSPO1* 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 *Sf1-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 GSK3β, 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 cells 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 TGFβ 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 βA 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* (phosphatidyllinositol 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 *Sf1-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 Coffer 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 potently 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 TGFβ family in multiple stages of follicle development, though many of these functions are still not understood.

Other members of the TGFβ 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 granulose 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 TGFβ Family in Regulation of Granulosa Cell Growth and Differentiation

The TGFβ 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: βA and βB. Mice null for βA die shortly after birth (Matzuk et al. 1995), but mice deficient for βB have normal size litters but defects in nursing (Vassalli et al. 1994). Ovaries from βB deficient females overproduce the βA subunit (Vassalli et al. 1994), suggesting that any intraovarian reproductive phenotype that is caused by loss of βB 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 TGFβ, 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 TGFβ family in ovarian follicles has also been investigated by deletion of the SMAD transcription factors, which are part of the TGFβ family canonical signaling pathway. SMAD2 and SMAD3 signal for activin, GDF9, and TGFβ, while SMAD1, SMAD5, and SMAD8 signal for the BMPs and AMH. An additional SMAD, SMAD4, is shared by all members of the TGFβ 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/TGFβ 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