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Regulation of the ovarian reserve by members of the transforming growth factor beta family

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Abstract

Genetic or environmental factors that affect the endowment of oocytes, their assembly nto primordial follicles, or their subsequent entry into the growing follicle pool can disrupt reproductive function and may underlie disorders such as primary ovarian insufficiency. Mouse models have been instrumental in identifying genes important in ovarian development, and a number of genes now associated with ovarian dysfunction in women were first identified as causing reproductive defects in knockout mice. The transforming growth factor beta (TGFB) family consists of developmentally important growth factors that include the TGFBs, anti-Müllerian hormone (AMH), activins, bone morphogenetic proteins (BMPs), and growth and differentiation factor 9 (GDF9). The ovarian primordial follicle pool is the source of oocytes in adults. Development of this pool can be grossly divided into three key processes: (1) establishment of oocytes during embryogenesis followed by (2) assembly and (3) activation of the primordial follicle. Disruptions in any of these processes may cause reproductive dysfunction. Most members of the TGFB family show pivotal roles in each of these areas. Understanding the phenotypes of various mouse models for this protein family will be directly relevant to understanding how disruptions in TGFB family signaling result in reproductive diseases in women and will present new areas for development of tailored diagnostics and interventions for infertility.

Keywords

primordial follicle; reproduction; ovary; fertility; activin; BMP

Introduction

One of the characteristics of human reproduction is the cessation of menstrual cycling in women at midlife, leaving an extended period of post-reproductive time. In other long-lived apes, reproductive senescence, or menopause, does not appear to occur (Emery Thompson et al. 2007; Robbins et al. 2006; Wich et al. 2004) and infertility in older female chimpanzees in wild populations appears to be a reflection of somatic aging and a decline in overall health (Emery Thompson et al. 2007). In this regard, alterations to the pool of quiescent ovarian follicles that make up the “ovarian reserve” and which are available for the production of mature oocytes can have detrimental effects on reproductive health and may result in sterility in women. Menopause is thought to be driven by a natural depletion of

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Quote: “In the ovary, various members of [the TGF beta] family control growth and differentiation of the somatic cells and germ cells ”

oocytes in the ovarian reserve (Faddy et al. 1992; Gougeon 1996), and menopausal age is highly heritable (Murabito et al. 2005a; Murabito et al. 2005b; Snieder et al. 1998; van den Berg and Boomsma 2007). Pathologic, early reproductive senescence can also occur; this primary ovarian insufficiency (POI) is defined as amenorrhea for at least 4 months, sex steroid deficiency, and increased follicle-stimulating hormone (FSH) in women under the age of 40 (De Vos et al. 2010; Gosden and Faddy 1998). POI can be caused by genetic, environmental, or iatrogenic factors, such as chemotherapeutic reagents or irradiation during cancer therapies (De Vos et al. 2010). Preventing premature oocyte loss would be beneficial to improving women's quality of life because early reproductive senescence affects not only the ability to have children, but also results in a number of disruptive changes to overall health, including increases in cardiovascular disease, bone loss, and neurological disorders (De Vos et al. 2010; Kodaman 2010; Marino and Misra 2011; Wellons 2011).

Oocytes in primordial follicles are derived from primordial germ cells (PGCs) that are specified early in embryogenesis (reviewed in Edson et al. 2009; Ewen and Koopman 2010). PGCs then undergo a period of proliferation and migration to the developing gonad (reviewed in Molyneaux and Wylie 2004; Richardson and Lehmann). In the ovary, oogonia undergo additional rounds of proliferation before entering meiosis around embryonic day E13.5 in mice or 9-11 weeks of gestation in humans (Kurilo 1981; Le Bouffant et al. 2010; Liu et al. 2010a; McLaren 2000). Oocytes in the developing ovary are found in structures called germ cell cysts, which form both by aggregation and clonal division (Gomperts et al. 1994; Mork et al. 2012; Pepling and Spradling 1998). Primordial follicles form when oocytes in germ cell cysts are separated into individual follicles by invading somatic cells, which eventually form the granulosa cells of the follicle (Figure 1). In mice, primordial follicle assembly begins around embryonic day 17.5 to postnatal day 4 (Pepling and Spradling 2001), with breakdown rates dependent upon strain (Pepling et al. 2010). In humans, assembly begins around 17-19 weeks of gestation (Childs et al. 2010). As primordial follicles form, oocytes become arrested in the dictyate-stage, meiotic prophase I (Paredes et al. 2005), and oocytes will remain arrested until released from meiotic arrest by the mid-cycle surge of gonadotropins at ovulation.

It is estimated that of the approximately 6 million oocytes at mid-gestation in fetal human ovary, only 1-2 million are found at birth, and 300,000 at puberty (Figure 2) (Baker 1963; Block 1952; Faddy 2000). Not all oocytes will become primordial follicles, and most oocytes undergo apoptosis or other forms of cell death (De Felici et al. 2008; Gougeon 1996; Pepling and Spradling 2001; Rodrigues et al. 2009; Tingen et al. 2009b). Recent studies have suggested a rare stem cell-like population exists within the adult ovary that could contribute to folliculogenesis during the reproductive years (White et al. 2012; Zou et al. 2009). This hypothesis is widely debated and not generally accepted, however (Byskov et al. 2011; Kimura et al. 2011; Monget et al. 2011; Vogel 2012; Zhang et al. 2012). Therefore, in the absence of knowledge regarding the physiologic significance of these cells in the adult ovary, this review will focus on understanding the formation and regulation of the ovarian reserve during embryonic and postnatal development.

In mice, breakdown of germ cell cysts and the formation of the primordial follicle pool is nearly complete 2-4 days after birth (Pepling and Spradling 2001) (Figure 1). Ingression of cells from the ovarian surface epithelium, which are thought to give rise to the pre-granulosa cells that surround each primordial follicle in adult mice, also ceases by postnatal day 4 (Mork et al. 2011). Once established, the size of the primordial follicle pool may regulate reproductive lifespan, with a low number of primordial follicles (approximately 1000) found at the time of menopause in women (Gosden and Faddy 1998). The quiescent primordial follicle pool is tapped during follicle activation into the growing pool, and these develop through a well-characterized series of developmental stages, culminating in ovulation

(reviewed in Richards and Pangas 2010) (Figure 1). Primordial follicle activation is thought to be irreversible, with most follicles eventually undergoing atresia (Adhikari and Liu 2009; McGee and Hsueh 2000).

Most genetic causes of POI are unknown, although mutation analysis has identified members of the transforming growth factor beta (TGFB) family as candidate genes (Persani et al. 2011). The TGFB family consists of evolutionarily conserved growth factors that have wide-ranging functions in development and tissue homeostasis. There are 33 TGFB-related proteins in mammals, which are primarily secreted as disulfide linked homoor heterodimers (Derynck et al. 1994). These proteins bind to cell-surface serine/threonine kinase receptor complexes that phosphorylate and activate the intracellular SMAD transcription factors. Phosphorylation of SMADs results in their nuclear accumulation, especially in complexes with other transcriptional co-factors that regulate gene expression (Hill 2009; Massagué 1998). In the ovary, various members of this family control growth and differentiation of the somatic cells and germ cells, as well as being involved in ovulation and fertilization. This review summarizes the role of the TGFB family in aspects of primordial follicle formation, assembly, and activation, with an emphasis on what we have learned from mouse genetic models. Extensive reviews covering the roles of the TGFB family at later stages of follicle development and ovarian function are available (Edson et al. 2009; Knight and Glister 2006; Pangas and Matzuk 2008; Pangas 2011; Rosairo et al. 2008).

BMPs and Gremlin

The largest subgroup of the TGFB family is the bone morphogenetic proteins (BMPs). There are approximately 20 BMPs, subdivided into 7 additional groups based on their structure and function (Chen et al. 2004; Guo and Wu 2012). With respect to development of the germ line, BMPs have one of the earliest functions for members of the TGFB family. BMPs are well-known for their role in primordial germ cell specification during embryonic development, and loss of *Bmp2* or *Bmp4*, or their downstream transcription factors, SMAD1 or SMAD5, leads to reduced or absent PGCs (Table 1) (Chang and Matzuk 2001; de Sousa Lopes et al. 2004; Ewen and Koopman; Hayashi et al. 2002; Lawson et al. 1999; Ying and Zhao 2001; Zhang and Bradley 1996; Zhao and Hogan 1996). Recent studies also show that BMP signaling is required for germ cell survival and migration to the genital ridge (Dudley et al. 2007).

Bmp2, Bmp4, and *Bmp7* are expressed in the developing embryonic ovary (Ross et al. 2007; Yao et al. 2004). The function of BMP2 and BMP4 within the mouse fetal gonad is unknown, although *Bmp7* is necessary for germ cell proliferation in the mouse ovary around embryonic day 10.5-11.5 (Ross et al. 2007). Unlike in the mouse, *BMP7* is not well expressed in the human fetal ovary (Childs et al. 2010), suggesting some differences between mouse and human. Both *BMP4* and *BMP2* are expressed in humans, however: *BMP4* expression is highest during the period of PGC mitosis (8-9 weeks gestation), and decreases with increasing gestation (Childs et al. 2010). *BMP2* shows a reciprocal pattern, with low expression at 8-9 weeks, and increasing expression to later gestation, showing a 7.4-fold increase by 20 weeks (Childs et al. 2010).

There is supporting evidence for a regulatory role for BMPs in modulating germ cell numbers and primordial follicle assembly based on a study of the BMP antagonist, gremlin (*Grem1*). GREM1 is a member of the DAN (differential screening-selected gene, aberrative in neuroblastoma)/cerberus family of proteins that binds to BMP2, BMP4, and BMP7, neutralizing their activity by preventing association with the signaling receptors (Balemans and Van Hul 2002; Gazzerro and Canalis 2006; Wordinger et al. 2008). Mice containing a homozygous null mutation for *Grem1* (*Grem1*$^{-/-}$) die within 48 hours after birth due to

defective kidney and lung formation (Khokha et al. 2003). Kidney development is restored by genetically reducing *Bmp4* levels, indicating that deletion of *Grem1* results in prolonged or over-activated BMP signaling (Michos et al. 2007). While kidney development is affected in *Grem1*$^{-/-}$ mice, the remainder of the urogenital tract appears to develop normally (Khokha et al. 2003; Michos et al. 2004).

Prior to analyzing newborn *Grem1*$^{-/-}$ ovaries, it was predicted that there would be an increased number of oocytes, given the known role of BMPs as positive regulators of germ cell specification and proliferation (Durcova-Hills and Capel 2008; Ross et al. 2007), and that loss of *Grem1* resulted in increased BMP activity. Surprisingly, however, ovaries from newborn *Grem1*$^{-/-}$ mice contain significantly fewer oocytes than their control littermates (Myers et al. 2011). These data might be explained by the known biphasic role of BMP activity on PGC numbers. Low doses (0.5 to 5 ng/mL) of BMP4 increase PGC numbers in mice, while a high dose (500 ng/nL) reduces it (Dudley et al. 2007). Thus in *Grem1*$^{-/-}$ ovaries, increased BMP activity may drive PGC apoptosis. This explanation would be in line with data from human studies, which demonstrate increased germ cell apoptosis in human fetal ovaries treated with a relatively high dose (100 ng/mL) of BMP4 in culture (Childs et al. 2010).

Besides a reduction in the number of oocytes, *Grem1*$^{-/-}$ ovaries also contain defects in assembly of the primordial follicle pool (Myers et al. 2011). At birth, more oocytes in *Grem1*$^{-/-}$ ovaries remain in germ cell cysts than as primordial follicles when compared to their wild-type controls, suggesting a delay in germ cell cyst breakdown. Because of their neonatal lethality, other methods had to be used to further analyze follicle growth, including transplantation of knockout ovaries (Telfer et al. 1990) and the generation of conditional knockout mice (Myers et al. 2011). In transplanted ovaries, newborn *Grem1*$^{-/-}$ or control ovaries were placed under the kidney capsule for three weeks follicle. During this time, follicle development in *Grem1*$^{-/-}$ ovaries proceeded to later stages of follicle development, indicating that the delay in germ cell cyst breakdown at birth is eventually overcome, and preantral- to antral-stage folliculogenesis can occur. Dynamics of follicle growth in *Grem1*$^{-/-}$ ovaries, with respect to numbers and rates, remain to be determined, however. A conditional knockout for *Grem1* was also generated using the *Amhr2cre* recombinase line to delete floxed *Grem1* alleles in somatic cells of developing follicles (Jamin et al. 2002; Jorgez et al. 2004; Myers et al. 2011). This mouse model has normal fertility, and suggests *Grem1* may have its critical function during embryonic ovarian development. Alternatively, it is possible that *Grem1* loss can be functionally compensated for by additional BMP antagonists co-expressed with *Grem1* in follicular granulosa cells (Fenwick et al. 2011; Myers et al. 2011).

A role for the BMPs in primordial follicle activation has been shown in in vitro studies, though not tested in vivo. BMP4 treatment of cultured, 4-day-old rat ovaries increases the proportion of primary follicles and reduces number of primordial follicles (Nilsson and Skinner 2003). Conversely, antibody neutralization of endogenous BMP4 results in smaller ovaries with a progressive loss of oocytes in primordial follicles (Nilsson and Skinner 2003). Culturing 2-day-old mouse ovaries for four days (*i.e.*, at the time of follicle growth from primordial to primary) with exogenous BMP7 also stimulates additional follicle growth (Lee et al. 2004). These data indicate that BMP4 and BMP7 activity may also be important for modulating the growth of the primordial follicle pool. BMP receptors, *Bmpr1a, Bmpr1b*, and *Bmpr2* localize to oocytes and granulosa cells in almost all follicles of the ovary in rats (Shimasaki et al. 1999), suggesting that BMPs can potentially act both on the oocytes and the granulosa cells. While BMP2 is expressed in adult mouse ovary, its effects on early postnatal folliculogenesis have not been determined.

Bmp2, Bmp4, and *Bmp7* are expressed from somatic follicle cells in the postnatal ovary. There are additional BMPs and related growth and differentiation factors (GDFs) expressed only from oocytes — including *Bmp6, Bmp15*, and *Gdf9* during the postnatal period — although their expression pattern and mouse knockout phenotypes do not suggest major roles for these ligands in primordial follicle assembly or activation (Table 1). *Bmp6* is expressed in oocytes of mice in growing follicles and appears to be absent from type 2 (primordial) follicles (Elvin et al. 2000). *Bmp6*$^{-/-}$ ovaries contain normal numbers of primordial follicles at postnatal day 18, with no changes in growing follicles of immature mice (Sugiura et al. 2010). Yet, female *Bmp6*$^{-/-}$ mice have a mild subfertility defect and ovulate fewer oocytes that also have reduced developmental potential (Sugiura et al. 2010). *Bmp15* is not expressed in oocytes until postnatal day 4 in mice, and no change in oocyte endowment or in the dynamics of the primordial follicle pool in *Bmp15*$^{-/-}$ mice have been reported, although *Bmp15*$^{-/-}$ females are subfertile on some genetic backgrounds (Yan et al. 2001). In contrast, *Gdf9*$^{-/-}$ female mice are infertile with a well-described follicular block at the primary follicle stage (Dong et al. 1996). *Gdf9* transcripts can be detected in oocytes of germ cell cysts in E19.5 mouse ovaries (Rajkovic et al. 2004), but no changes in the number of oocytes at birth or in germ cell cyst breakdown in the *Gdf9*$^{-/-}$ mice have been described. In addition, follicle recruitment and initiation of follicle growth appear grossly normal in *Gdf9*$^{-/-}$ ovaries (Dong et al. 1996; Elvin et al. 2000). Interestingly, double mutants for *Bmp15* and *Gdf9* (*Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$) display follicles containing multiple oocytes, which may indicate a defect in the initial formation of primordial follicles (Yan et al. 2001), although the mechanism behind this is unknown.

Activin, Inhibin, and Follistatin

Activins are dimers of two β subunits derived from one of four genes in mammals: *Inhba, Inhbb, Inhbc*, or *Inhbe*. Activin A (βA:βA homodimers) and activin B (βB:βB homodimers) are the most commonly expressed isoforms, and both have important developmental and physiologic roles (Chang et al. 2002; Matzuk et al. 1995a; Vassalli et al. 1994; Wiater and Vale 2008). Activin signaling can be inhibited by expression of the extracellular binding protein, follistatin (FST), which shows high affinity for activin and prevents activin from binding to its signaling receptor (Thompson et al. 2005). Activin production or signaling also can be prevented by expression of inhibin, a heterodimer containing the same β subunits as activin, but with dimerization to dissimilar α subunit – a product of the inhibin α (*Inha*) gene. Inhibin prevents activin function by acting as a competitive binding protein to the activin receptors, preventing their signaling (Zhu et al. 2012).

Inhibin, activin, and follistatin all function within the ovary at various developmental stages, though there are gaps in our understanding of their definitive role in primordial follicles. Both activin βA null mice (*Inhba*$^{-/-}$) and follistatin null (*Fst*$^{-/-}$) mice die perinatally, with craniofacial defects, amongst other gross deficits (Matzuk et al. 1995a; Matzuk et al. 1995b). Activin βB null (*Inhbb*$^{-/-}$) mice are viable and produce live offspring, but have lactation problems resulting in the death of their pups (Vassalli et al. 1994). To date, the dynamics of the primordial follicle pool have not been analyzed in either *Inhba*$^{-/-}$ or *Inhbb*$^{-/-}$ lines. Studies on the expression of *Inhba* and *Inhbb* in the embryonic mouse ovary suggest that only the *Inhbb* isoform is normally expressed at E12.5 (Yao et al. 2006). Yet, the *Inhbb*$^{-/-}$ mouse is able to produce pups, suggesting that some compensation by *Inhba* may occur. Overlap and compensation between ligands of the TGFB family is common, and partial redundancy between activin isoforms has been demonstrated in mice that have the *Inhbb* gene “knocked-in” to the *Inhba* locus (termed the *Inhba*^{BK} allele) (Brown et al. 2000). Expression of *Inhbb* from the *Inhba* locus rescues the embryonic lethality and craniofacial defects of *Inhba*$^{-/-}$ mice (Brown et al. 2000), although female mice homozygous for the *Inhba*^{BK} allele have unexplained fertility defects, including smaller ovaries with fewer

preantral follicles. These data, plus results from double conditional knockouts for *Inha* and *Inhbb* in granulosa cells that show a dose-dependent infertility phenotype with loss of the β-subunits genes, suggests that *Inhbb* acts as a hypomorphic *Inhba* allele (Pangas et al. 2007).

Follistatin is a high-affinity antagonist of activin (Schneyer et al. 1994; Thompson et al. 2005). *Fst* knockout mice ($Fst^{-/-}$) mice have normal numbers of PGCs through E15.5 (Yao et al. 2004), but have lost all germ cells by birth due to apoptosis that begins around E16.6 (Yao et al. 2004). This appears to be due to activin-driven apoptosis of germ cells (Liu et al. 2010b), and accordingly, deletion of the activin β subunit (*Inhbb*) from $Fst^{-/-}$ restores normal ovarian development (Yao et al. 2006). Thus, changes in follistatin-activin signaling in the developing embryonic ovary could have clinically important implications in determining the size of the primordial follicle pool. There appears to be some differences between mouse and human, however, with respect to follistatin and the activin system in ovary development. The role of follistatin in the human fetal ovary is not certain because *FST* is not expressed at the time of oogonia proliferation and primordial follicle formation (Martins da Silva et al. 2004). Also, unlike in mice, both *INHBA* and *INHBB* isoforms appear to be expressed in the human ovary from 14-19 weeks of gestation, with increasing expression of activin βA at the time of germ cell cyst breakdown (17-19 weeks of gestation) (Martins da Silva et al. 2004). Thus, there may be species-specific effects of the activin-follistatin system with respect to primordial follicle assembly. It is also possible that activin may play the same role in both mouse and human, but its activity may be regulated by other mechanisms.

Follistatin may be found as three isoforms, (FST288, FST303, and FST315), which are generated by alternative splicing and post-translational processing. These isoforms have similar binding affinity for activin, but differ in their expression pattern and cell-surface binding activity (Sidis et al. 2006). Of the three isoforms of follistatin, FST288, which can bind cell-surface proteoglycans, has the strongest neutralizing activity for activin (Sidis et al. 2006). Mice generated to only express FST288 survive to adulthood, but are subfertile (Kimura et al. 2010). FST288-only mice have declining numbers of primordial follicles with age and an early cessation of reproduction at eight to nine months (Kimura et al. 2010). Surprisingly, FST288-only female mice start with greater numbers of primordial follicles at postnatal day 8.5, although these numbers return to wild-type levels by sexual maturity (Kimura et al. 2010). Consistent with a role for the follistatin-activin system in regulating oocyte numbers in the developing ovary, neonatal ovaries from FST288-only mice had more germ cells with reduced levels of apoptosis and an extended period of germ cell cyst breakdown (Kimura et al. 2011). Interpretation of these studies is complicated, however, because FST288 mice also express lower levels of follistatin mRNA and protein (Kimura et al. 2011). Data from this study have led to the suggestion that loss of one of the other follistatin isoforms or reduced levels of FST288 in the fetal gonad may lead to increased activin activity and more germ cells at birth (Kimura et al. 2011), a statement that appears to be in conflict with embryonic studies on the $Fst^{-/-}$ mouse, which show that embryonic activin expression drives germ cell apoptosis (Yao et al. 2006; Yao et al. 2004). This conflict could be resolved by an analysis of the oocyte endowment in ovaries of newborn $Inhba^{-/-}$ $Inhbb^{-/-}$ double knockout mice, but this has not yet been reported.

Mice null for *Inha* ($Inha^{-/-}$) are viable at birth, but these mice are infertile, develop sex-cord stromal tumors, and die by 3-4 months of age (Matzuk et al. 1992). Postnatal follicle development is severely disrupted in the $Inha^{-/-}$ ovary. By postnatal day 12, there are reduced numbers of primordial follicles and concomitant increases in primary and secondary follicles along with a precocious appearance of antral follicles in $Inha^{-/-}$ ovaries. This suggests premature activation of the primordial follicle pool and accelerated preantral follicle development in $Inha^{-/-}$ mice. In addition to quantitative changes to the stages of

follicles growing within the *Inha*$^{-/-}$ ovary, there are qualitative changes in the association of oocytes with their surrounding somatic granulosa cells. Specifically, proliferation of the granulosa cells surrounding the oocyte in *Inha*$^{-/-}$ primordial follicles appears to be increased (Myers et al. 2009). In wild-type ovaries, primordial follicles measure less than 20 μm and the oocyte occupies the majority of the mass of the follicle. In *Inha*$^{-/-}$ ovaries, oocytes of that size are often found in follicles over 50 μm in diameter, demonstrating a remarkable increase in somatic cells that make up the primordial follicle. Expression of several key growth factors, including Kit ligant (*Kitl*) and anti-Mullerian hormone (*Amh*) (see sections below), are also altered in *Inha*$^{-/-}$ ovaries and *Inha*$^{-/-}$ mice overexpress the activin β subunits, which eventually results in activin-driven, cancer cachexia-like death (Coerver et al. 1996). The interplay of these different growth factors on primordial follicle activation and regulation of granulosa cell and oocyte growth in *Inha*$^{-/-}$ mice, specifically as they contribute to the disordered follicle growth, still needs to be determined.

Anti-Müllerian hormone

Anti-Müllerian hormone (AMH), also called Müllerian inhibiting substance (MIS), is a member of the TGFB family that was named for its function in development of the male reproductive tract, where it induces regression of the Müllerian duct in male embryos (Behringer et al. 1994; Josso et al. 2006). In the adult female, *Amh* expression is restricted to the ovary and is expressed in granulosa cells of developing follicles. Granulosa cell expression of *Amh* begins as pre-granulosa cells differentiate to the cuboidal stage at the transition from primordial to primary follicle (Figure 1) (Sadeu et al. 2008). *Amh* continues to be highly expressed in granulosa cells of growing follicles until the early antral stage (Durlinger et al. 2002b), when its expression is suppressed in FSH-sensitive mural granulosa cells of preovulatory follicles, but remains expressed in cumulus cells (Baarends et al. 1995; Diaz et al. 2007). The latter localization data support a hypothesis that *Amh* expression is at least partly controlled by factors produced by the oocyte (Salmon et al. 2004).

Experiments in mice null for *Amh* (*Amh*$^{-/-}$) initially did not suggest any impact of AMH loss on female reproductive function because *Amh*$^{-/-}$ female mice had normal estrous cycles and litter sizes (Behringer et al. 1994). A detailed analysis of follicle growth dynamics, however, showed that during the reproductive life of *Amh*$^{-/-}$ mice, the number of primordial follicles decreased more rapidly as compared to their normal litter mates (Durlinger et al. 1999). This was due to an increase in the number of growing follicles (Durlinger et al. 2002a). While loss of *Amh* in mice causes premature primordial follicle activation, this effect takes several months to establish, as even at 13 months of age, *Amh*$^{-/-}$ mice retain a few primordial follicles, albeit at very low numbers (Durlinger et al. 1999). These data suggest that AMH acts as a modifier of the rate of primordial follicle activation (Durlinger et al. 2002a). Multiple growth factors appear to serve this function in the ovary, either as positive or negative modulators (see section above, *BMP and Gremlin*). In human cortical biopsies that contain primordial follicles, addition of AMH at high levels results in the maintenance of primordial follicles throughout a 7-day culture period (Carlsson et al. 2006) — a result that also supports a regulatory role of AMH on primordial follicle activation.

How AMH secretion by neighboring growing follicles modulates activation of the primordial follicle pool is not entirely clear. The type-II binding receptors for AMH (AMHR2), is expressed in the rodent ovary as early as E17.5 in mice, in granulosa cells of developing secondary and small antral follicles, but not in primordial or primary follicles at a detectable level (Arango et al. 2008; Baarends et al. 1995; Jorgez et al. 2004). These data suggest the effects of AMH on primordial follicle activation are indirect. Recent studies using recombinant AMH on cultured newborn rat ovaries in culture have also shown that

AMH delays primordial follicle assembly (Nilsson et al. 2011). One unexplored facet to these data would be a finding from a recent study suggesting that precursors to pre-granulosa cells, which surround oocytes during primordial follicle formation, arise from the ovarian surface epithelium shortly after birth in mice (Mork et al. 2011). *Amhr2* expression is detectable in 2-day old mouse ovaries, a time when there are no follicles larger than primordial, but *Amhr2* has not been localized to a particular cell type (Durlinger et al. 2002a). Given that AMH has been shown to inhibit in vitro invasion and migration of epithelial ovarian cancer cell lines that express its receptor AMHR2 (Chang et al. 2011) and that AMHR2 localizes to the surface epithelium in human ovaries (Yuan et al. 2006), it is interesting to speculate that exogenous AMH could have inhibited the migration of cells from the ovarian surface epithelium in the study by Nilsson and colleagues (Nilsson et al. 2011). These results also imply that AMH activity/availability should be tightly restricted during primordial follicle assembly to prevent disruption of the breakdown process.

While the mouse knockout for *Amh* suggests a regulatory effect on overall female follicular development in mice, the application of serum AMH levels as a clinically relevant marker is growing, though controversial (Loh and Maheshwari; Nelson et al. 2012). Serum AMH levels have been proposed to be a marker for monitoring the ovarian reserve, and has potential applications for measuring ovarian function in a number of ovarian diseases, such as POI, polycystic ovary syndrome (PCOS), granulosa cell tumor recurrence, as well as a monitor of ovarian function following chemotherapy or as a predictor of success in in vitro fertilization (reviewed in La Marca and Volpe 2006; Ledger; van Houten et al.). Because AMH is expressed from growing follicles and not the primordial follicle pool, it is an indirect measure of ovarian reserve.

Increasing evidence suggests that measuring the growing follicle pool can accurately reflect the state of the quiescent pool, however, because the two are interdependent (Peters 1979). Whether or not there is a more direct measure of the ovarian reserve is unknown, as primordial follicles are generally believed to be metabolically quiescent, though recent studies suggest this follicle class may be engaged in a higher level of metabolic activity than previously thought (John et al. 2008). Identifying a factor secreted directly from primordial follicles that can be measured in sufficient quantity in body fluids seems unlikely, but possible. A spatial analysis of post-natal mouse ovaries suggested that follicles are less likely to start growing when there are one or more primordial follicles nearby, and predicts that primordial follicles inhibit each other via a diffusible factor; this factor may be a known inhibitory molecule or a negative regulator of a stimulatory factor (Da Silva-Buttkus et al. 2009). The presence of such a factor and how its expression is regulated could shed light on why some primordial follicles remain dormant while others are activated.

TGFB

There are three mammalian isoforms of TGFB: TGFB1, TGFB2, and TGFB3. Knockout mice for *Tgfb1* (*Tgfb1*$^{-/-}$) display a high degree of embryonic or perinatal lethality, but in some cases, homozygous null *Tgfb1* mice live to 3-4 weeks of age and die due to inflammatory disorders (Dickson et al. 1995; Kulkarni et al. 1993; Shull et al. 1992). *Tgbf2* and *Tgfb3* knockout mice have perinatal lethal phenotypes (Kaartinen et al. 1995; Proetzel et al. 1995; Sanford et al. 1997). There is little information regarding a role for TGFB isoforms in oocyte endowment or primordial follicle assembly and activation. Newborn ovaries from all three knockout mice (*Tgfb1*$^{-/-}$, *Tgfb2*$^{-/-}$ or *Tgfb3*$^{-/-}$) have been analyzed (Memon et al. 2008). None of the isoforms are highly expressed in mouse ovaries from E13.5 to E16.5, although *Tgfb3* expression doubles during this time (Memon et al. 2008). While no differences were detected in postnatal day 0 ovaries for *Tgfb1*$^{-/-}$ or *Tgfb3*$^{-/-}$ mice, *Tgfb2*$^{-/-}$ ovaries, but not testes, had an increased number of germ cells, suggesting

that *Tgfb2* may have an embryonic function in regulating germ cell numbers in the female, potentially by mediating apoptosis (Memon et al. 2008).

Integration of TGFB family signaling with KITL-KIT function

Part of the function of the TGFB family in controlling follicle development may, in part, involve their regulation of kit ligand (*Kitl*), a growth factor necessary for embryonic and adult germ cell development. *Kitl* is expressed from ovarian somatic cells and is important for signaling to the kit receptor (KIT), found on germ cells and oocytes (Otsuka and Shimasaki 2002). *Kit-Kitl* mutations are known for causing infertility and reproductive defects in mice at multiple stages of oogenesis and folliculogenesis, including defects in PGC migration, survival, and proliferation, as well as generating defects in follicle development (reviewed in Edson et al. 2009; Hutt et al. 2006). Activin, GDF9, and BMP7 all suppress *Kitl* expression (Coutts et al. 2008; Elvin et al. 1999; Joyce et al. 2000; Lee et al. 2004; Pangas et al. 2007), while BMP15 upregulates it (Otsuka and Shimasaki 2002). There are two alternative spice forms of kit ligand: a soluble form (KITL-1) and a more potent membrane-bound form (KITL-2) (Miyazawa et al. 1995). Activin selectively suppress the membrane-bound form of KITL in human ovaries (Childs and Anderson 2009), while GDF9 suppresses both isoforms (Joyce et al. 2000). These data suggest that signaling by activin and GDF9 will not have the same effect with respect to downstream KITL function.

One of the signaling cascades that KITL-KIT activates is the phosphoinositide-3-kinase (PI3K) signaling pathway (Adhikari and Liu 2009). Activation of PI3K signaling leads to phosphorylation and activation of the protein kinase AKT, a main effector of this pathway. Among other activities, AKT inactivates the forkhead box O (FOXO) transcription factors, leading to their nuclear exclusion and degradation. Deletion of *Foxo3* in mice results in global activation of the primordial follicle pool so that by postnatal day 14, $Foxo3^{-/-}$ ovaries are devoid of quiescent primordial follicles (*i.e.*, those without an enlarged oocyte) and are sterile by 15 weeks of age (Castrillon et al. 2003). This activity of primordial follicle growth suppression is not restricted to the postnatal period; deletion of *Foxo3* in oocytes in adult primordial follicles also causes global activation of the primordial follicle pool (John et al. 2008). *Foxo3* has also been over-expressed in mouse oocytes, and this results in retarded oocyte growth, follicle development, and anovulation leading to infertility (Liu et al. 2007). These transgenic mice also have decreased expression of BMP15, suggesting that FOXO3 negatively regulates *Bmp15*, thereby inhibiting the development of granulosa cells (Liu et al. 2007). Likely other factors are involved because $Bmp15^{-/-}$ mice do not show the same phenotype (Liu et al. 2007; Yan et al. 2001). Conditional deletion of *Pten* (phosphatase and tensin homolog deleted on chromosome 10), an inhibitor of PI3K signaling, in oocytes of primordial follicles also causes premature activation of the primordial follicle pool, enhanced AKT signaling, and suppression of *Foxo3* (Reddy et al. 2008). Yet, deletion of *Pten* in oocytes from the primary stage onward has no effect of fertility or follicle development, and thus while the PTEN/PI3K-AKT-FOXO3 pathway in oocytes is essential for controlling primordial follicle activation, is likely not required at later stages of follicle development (Jagarlamudi et al. 2009).

While KITL can simulate the intra-oocyte PI3K pathway, the regulatory network that controls the PI3K pathway has not been fully delineated, nor does it appear to be fully dependent on KITL signaling (Adhikari and Liu 2009; John et al. 2008). Mice with a mutation in the kit receptor (Y719F), which inhibit KIT's activation of the PI3K pathway, have normal follicle activation (John et al. 2009; Kissel et al. 2000). Other pathways also activate the PI3K signaling cascade — including platelet derived growth factor (PDGF), keratinocyte growth factor (KGF), nerve growth factor (NGF), and glial-derived growth

factor (GDNF) (reviewed in Adhikari and Liu 2009) — all of which have been shown to be expressed in the ovary and have stimulatory effects on the transition from primordial to primary follicles (Dole et al. 2008; Kezele et al. 2005; Nilsson et al. 2006) or are required for primordial follicle formation and growth (Dissen et al. 2001).

Questions for future research and conclusions

Much remains to be understood about the development, growth, and regulation of the primordial follicle pool. While it is clear that members of the TGFB have been implicated in the formation and regulation of the primordial follicle pool, our knowledge about their function is incomplete, as is the application of this knowledge to reproductive technologies and in other clinical capacities. For example, if menopause age is mainly determined by the size of the ovarian reserve, then manipulating this resource could have the potential to extend reproductive health, and hence overall health of women. Such manipulations would require an extensive knowledge about the signal transduction pathways that regulate oocyte proliferation, primordial follicle assembly, as well as survival. While decreases in the ovarian reserve clearly reduce reproductive lifespan in mice and women, experiments in mice currently do not support the idea that simply increasing the initial pool of primordial follicles will lead to a longer reproductive period. These studies instead suggest that the ovary is able to limit the size of the ovarian reserve, even if the number of primordial follicles is artificially increased (Flaws et al. 2001; Tingen et al. 2009a). How this set point is reached is currently unknown.

Modifying activation of the primordial follicles from the quiescent pool may also have potential clinical uses. Long-term inhibition of primordial follicle growth is likely not feasible and would be disruptive to overall ovarian function and health. As suggested by over-activation studies of *Foxo3* in mice, inhibiting growth of primordial follicles would lead to oocyte loss and infertility (Liu et al. 2007). On the other hand, increasing primordial follicle activation may be useful for in vitro for assisted reproductive technologies. For example, pathway inhibitors to PTEN have been used to activate primordial follicles in human cortical sections, and following xenotransplantation, follicles had developed to the preovulatory stage (Li et al. 2010). Therefore, a scenario of coaxing primordial follicles out of dormancy may be a key breakthrough for generating sufficient numbers of eggs from cryopreserved ovaries obtained from cancer patients or in women with some forms of infertility (Adhikari and Liu 2009). But manipulation of oocytes, in vitro or in vivo, should always be approached with caution because of potentially increased congenital malformations or disease risk in their offspring (Davies et al. 2012).

Several issues need to be overcome to address some of these questions using mouse models. Primordial follicle assembly and activation is difficult to study using many of the existing knockout mouse models for the TGFB family because follicle assembly occurs just before and following birth, and many of the knockout mice for the ligands, their receptors, or downstream transcription factors have embryonic or perinatal lethal phenotypes (Table 1). Consequently, not all have been analyzed during the embryonic period or at the neonatal period prior to their death, either with respect to oocyte endowment or follicle activation (Table 1). In addition, while there are a large number of oocyte-specific cre recombinase lines that span a wide developmental period and follicle stages (Hammond and Matin 2009; Pangas and Matzuk 2008; Sun et al. 2008), current conditional knockouts for follicular granulosa cells rely upon *cre-loxP* recombination using *cre* expression from the *Amhr2* or *Cyp19* promoters, which are more appropriate for deletion in growing preantral and mural stage follicles (Boerboom et al. 2005; Fan et al. 2009; Jorgez et al. 2004; Pangas et al. 2006). Together, the lethality of the phenotype and the availablility of cre recombinase models for somatic cells leave much of the lifespan between primordial follicle assembly

through activation unaccounted for using in vivo models. For understanding the development of the somatic cells that surround the primordial follicle, a new generation of cre recombinase lines will have to be developed in order to tune the temporal deletion of floxed alleles. Unfortunately, unlike oocytes, somatic cells of the ovary have fewer “unique” genes to use for cell-type specific cre recombinase expression, which increases the chances for additional defects due to loss outside the ovary.

While some differences may exist between the development and regulation of the ovarian reserve in mice and humans, many of the gene mutations now associated with POI in women were first discovered by analysis of mouse knockouts, such as *Nobox, Figla*, and *Gdf9*, (Bouilly et al. 2011; Dong et al. 1996; Qin et al. 2009; Rajkovic et al. 2004; Soyal et al. 2000; Zhao et al. 2008; Zhao et al. 2007). These data underscore the conservation of signaling pathways that control many aspects of follicle development between species. Thus, mouse models will continue to be important in identifying pathways that regulate various aspects of primordial follicle assembly and activation.

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Abbreviations

AMH[R]	anti-Müllerian hormone [receptor]
BMP[R]	bone morphogenetic protein [receptor]
FSH	follicle-stimulating hormone
FST	follistatin
FOXO3	forkhead box 3
GDF	growth and differentiation factor
GREM	gremlin
INH	inhibin/activin isoforms
KIT/L	kit receptor/kit ligand
PGC	primordial germ cell
PI3K	phosphoinositide 3-kinase
POI	primary ovarian insufficiency
PTEN	phosphatase and tensin homolog
TGFB	transforming growth factor beta

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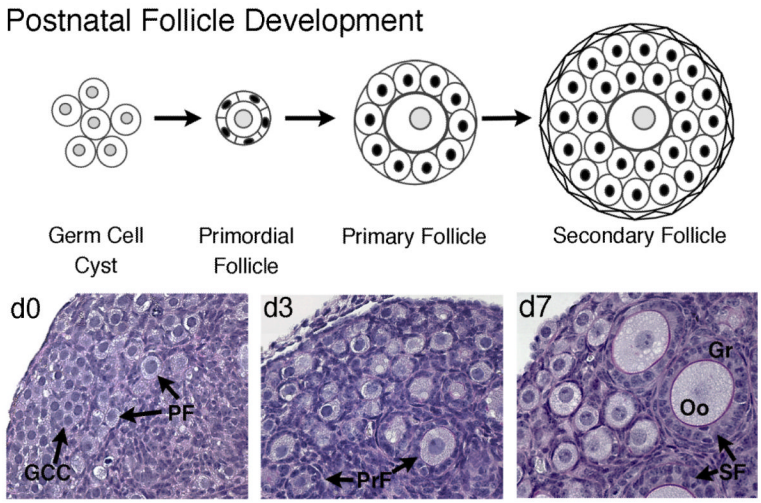

Figure 1.

Formation of primordial follicles and early activation to the growing follicle stage in mice. Germ cell cysts (GCC) are found in mouse ovaries during late gestation, and breakdown to form primordial follicles (PF) shortly before and after birth. Primordial follicles contain a single oocyte arrested in prophase-I of meiosis, and are surrounded by a flattened epithelium of pre-granulosa cells. Primordial follicles activate into the growth phase to become primary follicles (PrF), characterized by the transition of somatic cells into granulosa cells (Gr) and an increase in size of the oocyte, though it still remains in meiotic arrest. Primary follicles (PrF) are easily visible in the postnatal day 3 (d3) ovary (middle lower panel). Secondary follicles develop from primary follicles by postnatal day 7 (d7) (left right panel), and are characterized by increased proliferation of the granulosa cells, development of the third cell layer, the thecal cells, and increased growth of the oocyte (Oo). Secondary follicles develop a fluid-filled antrum under the influence of pituitary gonadotropins, developing to the final stages as preovulatory follicles, and are either stimulated to ovulate or undergo atresia (not shown).

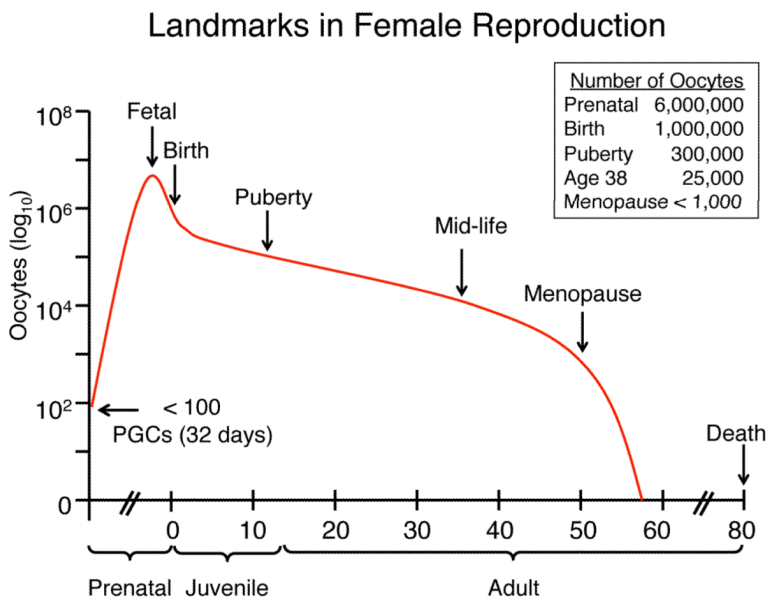

Figure 2.

Changes in the ovarian reserve in the human ovary. Less than 100 primordial germ cells are specified during early embryogenesis, and increase to over 7 million at five months of gestation. Primordial follicle formation in the human ovary begins at approximately 18-19 weeks of gestation and continues to the time of birth (Fulton et al. 2005). The number of oocytes decreases to 1-2 million at birth, and continues to decrease over the reproductive lifespan. Menopause occurs in women around age 51, at which time approximately 1000 follicles remain (Faddy 2000; Gosden and Faddy 1998). Figure is drawn from numbers presented in (Faddy 2000; Gosden and Faddy 1998).

Table 1

Phenotypes of homozygous null (−/−) mouse knockouts for ligands and antagonists of the TGFB family with respect to oocyte endowment, primordial follicle assembly, and primordial pool activation.

Knockout line (−/−)	Reproductive Phenotype	Oocyte Endowment	Primordial Pool Assembly	Primordial Pool Activation	Reference
Ligand					
Amh	Fertile	No change (PND21)	N/D	Increased	(Durlinger et al. 2002a)
*Bmp2**	N/A	PGC reduced	N/A	N/A	(Ying and Zhao 2001)
*Bmp4**	N/A	PGC absent	N/A	N/A	(Lawson et al. 1999)
Bmp6	Defects in ovulation	No change (PND18)	N/D	No change	(Sugiura et al. 2010)
*Bmp7***	N/A	Decreased (E11.5)	N/D	N/D	(Ross et al. 2007)
Bmp15	Subfertile	None noted	N/D	N/D	(Yan et al. 2001)
Gdf9	Infertile	None noted	N/D	N/D	(Dong et al. 1996)
Inha	Infertile/granulosa cell tumors	N/D	None noted	Defective/ Accelerated	(Myers et al. 2009)
*Inhba***	N/A	N/D	N/D	N/A	(Matzuk et al. 1995a)
Inhbb	Fertile but lactation defects	N/D	N/D	N/D	(Vassalli et al. 1994)
*Tgbf1**	N/A	No change	N/D	N/A	(Memon et al. 2008)
*Tgfb2***	N/A	Increased	Accelerated	N/D	(Memon et al. 2008)
*Tgfb3***	N/A	No change	N/D	N/A	(Memon et al. 2008)
Antagonists					
*Fst***	N/A	Germ cell loss (E15.5)	N/A	N/A	(Yao et al. 2004)
*Grem1***	N/A	Decreased (NB)	Delayed	N/A	(Myers et al. 2011)

N/D, not determined; N/A not applicable due to oocyte loss or lethality; (E) denotes embryonic day; NB, newborn; PND, postnatal day. Single asterisk indicates embryonic lethality; double asterisk indicates perinatal/neonatal lethality.

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