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Review Series

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The ovary: basic biology and clinical implications

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Introduction

The ovary is a highly organized composite of germ cells (oocytes or eggs) and somatic cells (granulosa cells, thecal cells, and stromal cells) whose interactions dictate formation of oocyte-containing follicles, development of both oocytes and somatic cells as follicles, ovulation, and formation of the corpus luteum (the endocrine structure that forms from the ovarian follicle after ovulation and is required for establishing and maintaining pregnancy) (Figure 1). Many events in the adult ovary are controlled by two hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), secreted from the anterior pituitary gland under the control of pulses of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Figure 1). Low-frequency GnRH pulses stimulate a slight increase in FSH levels early in a woman's menstrual cycle, enhancing follicle growth, while high-frequency GnRH pulses lead to a sharp rise in levels of LH just before mid-cycle (an event known as the "LH surge"), triggering ovulation and formation of the corpus luteum (Figure 1). The ovary also has a key role in these processes, ensuring the timely release of fertilizable oocytes and the maintenance of luteal cell function, a necessity for pregnancy, by directing feedback mechanisms to the hypothalamus and pituitary. For example, estrogen produced by the cells of the developing follicle both inhibits GnRH production in the hypothalamus and elicits elevated GnRH pulses, which trigger the mid-cycle LH surge that initiates ovulation. Thus, fertility depends on highly orchestrated endocrine events involving multiple organ systems.

Disruption of this finely controlled network can lead to many clinical syndromes including premature ovarian failure (POF), polycystic ovarian syndrome (PCOS), ovarian hyperstimulation syndrome, ovulation defects, poor oocyte quality, and cancer. The goal of this Review is to cover some of the advances in our understanding of the basic biology of the mammalian ovary that may shed light on specific ovarian dysfunctions that lead to infertility. We primarily focus on recent developments using mouse genetic models to study follicle growth and ovarian function because of the inherent difficulty of obtaining human ovarian tissue at defined stages of follicular growth. We emphasize the roles of bone morphogenetic proteins (BMPs), activins, and SMADs, WNT signaling, and recently uncovered aspects of the FSH and LH signaling cascades. We focus on these areas because specific mutant mouse models have unveiled how these signaling cascades regulate

critical events controlling follicular development, ovulation, and luteinization and because we believe that they have relevance to understanding ovarian dysfunctions in women.

Factors that control oocyte development, primordial follicle formation, and early follicle growth

Gonadal development. During embryogenesis, the mammalian gonad first develops adjacent to the urogenital ridges and is given the term the "bipotential" or "indifferent" gonad because it develops identically at this time in both male and female embryos. Differentiation into a testis (male) or ovary (female) does not occur until after the primordial germ cells (PGCs) have migrated from the yolk sac and colonized the indifferent gonad. In mice, colonization occurs approximately 9.5–11.5 dpc. Once the PGCs colonize the indifferent gonad, in female mice, they undergo a period of proliferation, followed by differentiation into oocytes that enter meiosis (at approximately E13.5), and in male mice, they differentiate into prospermatogonia that become quiescent until after birth. Development of germ cells into either male or female states depends on their interactions with the somatic cells of the gonad (see following paragraph) (Figure 2). Multiple growth factors modulate PGC mitosis, including members of the TGF- β family: activin, BMPs, and TGF- β 1 (reviewed in ref. 1). In mice, BMPs have an important role in PGC proliferation, and BMP2 and BMP4 increase the number of PGCs in culture (2, 3). *Bmp7*-null mice have reduced numbers of germ cells after 11.5 dpc, likely as a result of a proliferation defect between 10.5 and 11.5 dpc (4). In humans, activin increases female germ cell proliferation before the germ cells enter meiosis (5), while in mice, activin and TGF- β inhibit PGC proliferation (6), indicating the possibility of species-specific regulation. In mouse ovaries, the regulation of activin or BMP function appears to be critical for germ cell survival, as mice lacking follistatin (which is encoded by the gene *Fst*), an extracellular antagonist of activin, have normal numbers of PGCs and germ cells at E11.5–15.5 but lose 90% of germ cells at E16.5, with an almost complete loss by birth (7).

Several factors determine whether the gonad will be an ovary or a testis, including members of the WNT/FZD/ β -catenin (which is encoded by the gene *Ctnnb1*) signaling cascade. Ovaries of *Wnt4*^{-/-} mice are abnormal and exhibit structures similar to testicular tubules (8), indicating that WNT4 is a determinant of the female gonad. Mutations in the human R-spondin homolog (*RSPO1*) gene, which encodes a WNT pathway adapter molecule, indicate that this molecule is also a primary female sex-determining factor (9). Specifically, XX individuals with a mutant *RSPO1* gene

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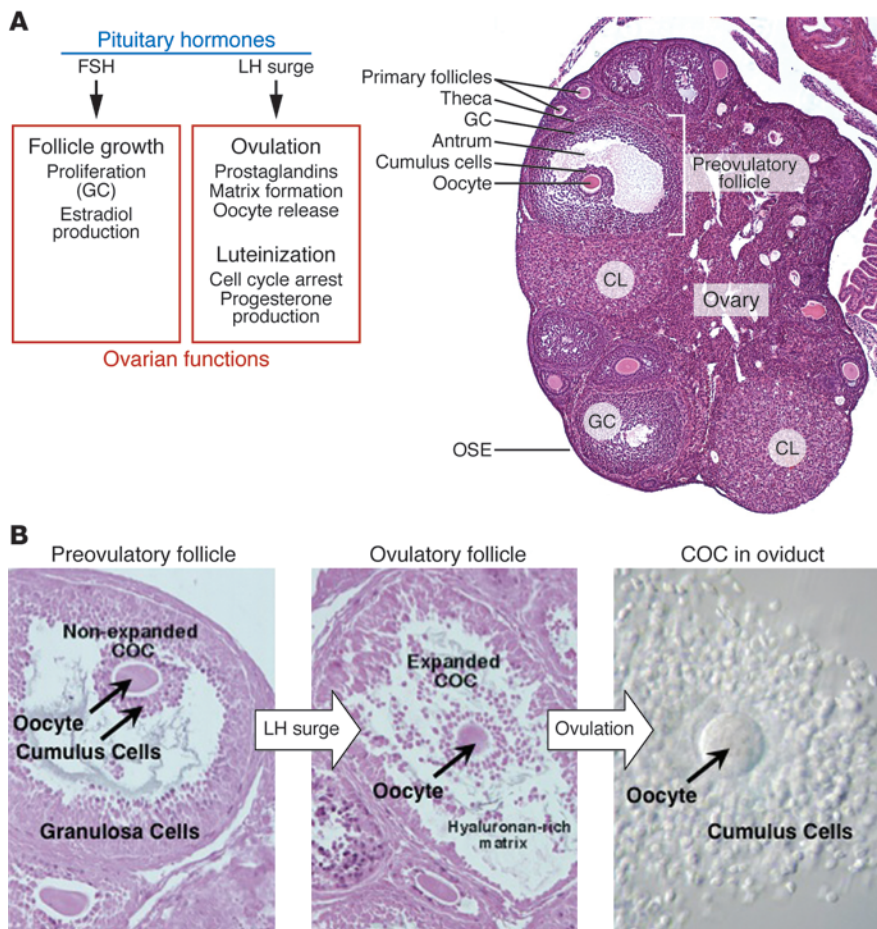


Figure 1

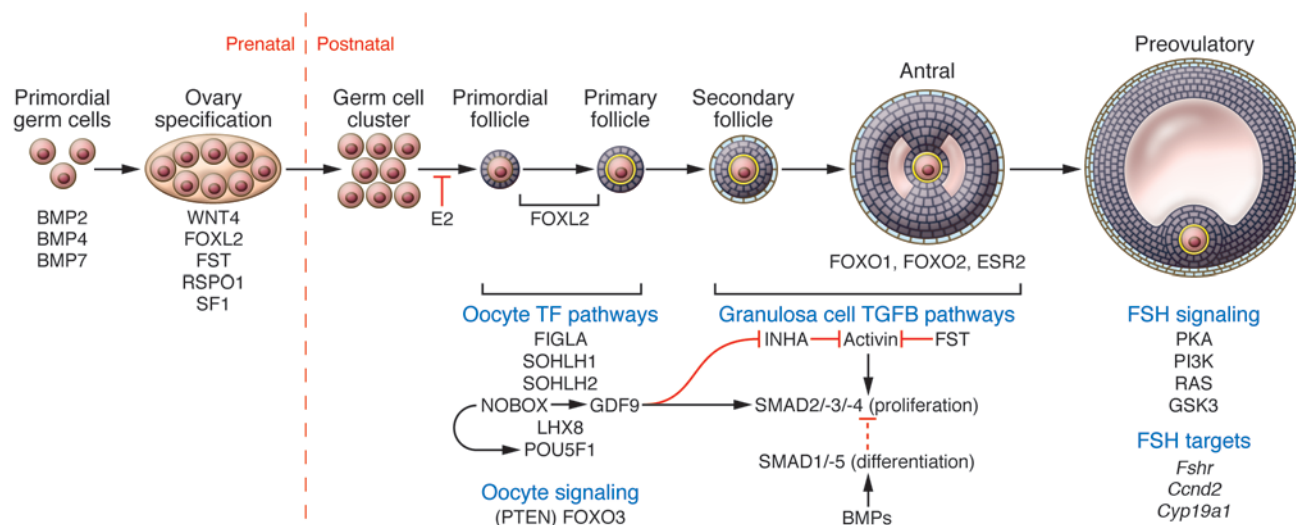
Summary of hormonal control of the ovary during follicle growth, ovulation, and luteinization. (A) Left: The pituitary gonadotropins FSH and LH are part of the hypothalamic-pituitary-gonadal axis that coordinately regulates the menstrual (in humans) or estrous (in nonhuman mammals) cycle by extensive feedback loops. FSH controls follicular granulosa cell (GC) growth and estradiol production, while LH controls ovulation and follicular luteinization. Right: A cross section of a mouse ovary is shown, demonstrating the main cell types and follicle stages. Ovarian follicles are composed of a single oocyte surrounded by somatic cells (granulosa cells) and thecal cells. Follicles grow from primordial (not shown) to primary and secondary stages independent of the pituitary gonadotropins. FSH stimulates growth to the preovulatory follicle stage, characterized by granulosa cells that directly surround the oocyte (cumulus cells) and those that make up the bulk of the wall of the follicle. Following the LH surge, the follicle erupts through the ovarian surface (OSE), and the remaining cells of the follicle terminally differentiate to form a corpus luteum. Original magnification, $\times 5$. (B) Ovulation in the mouse. Left: The preovulatory follicle contains an oocyte surrounded by cumulus cells that are separated from the mural granulosa cells by a fluid-filled cavity. Middle: Following the LH surge, the COC undergoes a process called cumulus or COC expansion, in which the cumulus cells make and become embedded in a hyaluronan-rich matrix. Right: The cumulus cells accompany the oocyte into the oviduct following release of the entire COC from the ovarian follicle. Original magnification, $\times 40$.

exhibit testicular-like gonads, lack Müllerian structures (oviduct and uterus), and present masculinized external genitalia as a consequence of testicular androgen biosynthesis. Moreover, female *Rspo1*^{-/-} mice also demonstrate gonadal sex-reversal and oocyte loss (9–10). Remarkably, mice lacking both WNT4 and the transcription factor forkhead box L2 (FOXL2) exhibit complete and functional sex reversal of the ovary to a testis in the XX genotype (11). These intriguing recent results document unequivocally that there are organizers of ovary versus testis development and that

Rspo1, *Wnt4*, and *Foxl2* are all important for ovary development, at least in mice. Furthermore, XY male mice engineered to express stable β -catenin, a downstream target of WNT signaling, exhibit partial male-to-female sex reversal with ovarian structures totally lacking germ cells or seminiferous tubule demise and germ cell loss (12). Thus, β -catenin also appears to be one key mediator of WNT signaling that is essential for normal ovary development, at least in the embryonic mouse gonad.

PGCs divide to form syncytia (nests or cysts) of oocytes that break down to form primordial follicles, which represent the quiescent follicle reserve in the ovary. Each primordial follicle contains an immature oocyte arrested early in meiosis, surrounded by a flattened epithelium that will eventually become the granulosa cells. Germ cell syncytia breakdown occurs prenatally in humans and shortly after birth in mice (Figure 2). It is associated with massive germ cell loss, such that oocyte numbers are reduced from approximately 6 million in the fetal human ovary to 1 million at birth (13–15). Inappropriate germ cell syncytia breakdown can lead to the generation of polyovular follicles (i.e., follicles that contain more than one oocyte). Polyovular follicles are present in some mouse strains (16, 17), in mice with certain TGF- β family member mutations (e.g., mice overexpressing the α subunit of inhibin, a TGF- β family member that inhibits FSH secretion [ref. 18], mice lacking activin in granulosa cells [ref. 19], and mice lacking oocyte-expressed *Bmp15* [ref. 20]), and in mice with altered Notch signaling (21). Polyovular follicles are also seen in human ovaries at birth, but rarely in adults (22). Follicles with more than one egg can be identified in women undergoing in vitro fertilization (IVF) procedures (23), but the clinical significance of polyovular follicles on fertility has not been established in humans; however, polyovular follicles are not a primary cause of infertility in mice (24, 25). This might be because individual oocytes develop at different rates within the same follicle (23), and while multiple oocytes might be developing

inside the follicle, only one may be at the appropriate maturation stage to be fertilized. Polyovular follicle formation can be induced by exposure of neonatal mice to estrogen (26–30). In this context, estrogen reduces activin expression, which suggests a direct role for activin in normal primordial follicle formation (19, 26). This effect of estrogen underscores the need for further research on the effect of endocrine disruptors — environmental and dietary compounds that act like hormones — in reproductive diseases. Along these lines, increasing evidence from rodent studies indicates that

**Figure 2**

Signaling pathways controlling ovarian follicle growth in the mouse. PGCs are specified by the BMP pathway, then proliferate and migrate to the indifferent gonad. The BMPs are major determinants of PGC specification and proliferation in the mouse. During the postnatal period, clusters (or nests) of germ cells break down to form primordial follicles, which upon activation become primary follicles. Estradiol (E2) inhibits the breakdown of germ cell clusters to primordial follicles. A number of mice lacking oocyte transcription factors (TFs) (NOBOX, SOHLH1, SOHLH2, and LHX8) show loss of follicles at the primordial follicle-to-primary follicle transition or before primordial follicle formation (FIGLA). *Foxo3* is also expressed in oocytes, and deletion of *Foxo3* (or the PI3K inhibitor *Pten*) in oocytes results in premature activation of the primordial follicle pool and oocyte loss. *Foxl2* is expressed in somatic granulosa cells, and deletion results in arrest and subsequent death of follicles before the primary follicle stage. FOXL2 also likely functions at late stages in folliculogenesis. NOBOX regulates other TFs (e.g., *Pou5f1*) and also *Gdf9*, the product of which is secreted by the oocyte to regulate granulosa cell function, including suppression of *Inha*. GDF9 and activin signal through SMAD2/3. Regulation of activin by its inhibitors, such as inhibin or follistatin, is critical after the secondary follicle stage and involves many follicle stages. In vitro experiments implicate FOXO1 and ESR2 as important regulators in granulosa cells of growing follicles. FSH is a key regulator of the antral and preovulatory stage through multiple signaling cascades, and many of the FSH targets at the preovulatory stage are coregulated by activin (*Fshr*, *Cyp19a1*) and/or β -catenin (*Cyp19a1*). See text for discussion and references for each indicated gene pathway.

neonatal exposure to genistein, the phytoestrogen found in soy, disrupts normal reproduction (30–36). The effects of phytoestrogens and other estrogen-like compounds on the reproductive tract, fertility, and reproductive cancers are not well understood.

Transcription factors and early follicle growth. A number of transcription factors whose expression, at least in adult tissues, appears to be restricted to germ cells or oocytes, are necessary for early folliculogenesis (Figure 2) (37). Factor in the germline α (FIGLA) was the first of these transcription factors to be identified (38), and *Figla*^{-/-} mice are sterile because primordial follicles fail to form (39). *Figla* encodes a basic helix-loop-helix (bHLH) transcription factor that regulates expression of the zona pellucida genes *Zp1*, *Zp2*, and *Zp3*, which encode the egg coat (38). Other germline-expressed, bHLH transcription factor-encoding genes are known, including spermatogenesis and oogenesis bHLH transcription factors 1 and 2 (*Sohlh1* and *Sohlh2*). Mice null for either gene have a similar female phenotype: postnatal oocyte loss leading to female sterility (40, 41). Both *Sohlh1*^{-/-} and *Sohlh2*^{-/-} mice have deficiencies in ovarian expression of other transcription factor-encoding genes: lim homeobox protein 8 (*Lhx8*), POU-type homeodomain-containing DNA-binding protein (*Pou5f1*; also known as *Oct4*), newborn ovary homeobox (*Nobox*), and *Figla*. Deletion of *Nobox* also causes female sterility and postnatal oocyte loss, and the gene product has been shown to directly regulate expression of growth differentiation factor 9 (*Gdf9*) and *Pou5f1* (42, 43). It is unclear how these transcriptional networks intersect to control oocyte development; however, if a woman were to have impaired expression of any one of these

transcription factors, evidence from mice indicates that she might develop POF. Indeed, while these transcription factors were first identified and functionally studied in mice, recent studies have discovered mutations in both *NOBOX* and *FIGLA* in women with POF (44, 45), highlighting the importance of mouse models for identifying candidate disease-causing genes in humans.

Disruption of the *FOXL2*-encoding gene leads to abnormal follicle development and POF in mice (46) and humans (47). As discussed in the previous section, *Foxl2* is expressed in the early stages of gonadal development and has been shown to direct ovarian and oppose testis development. In early postnatal *Foxl2*-null ovaries, the expression of some genes increases, e.g., nuclear receptor subfamily 0, group B, member 1 (*Nr0b1*; also known as *Dax1*) and *Wnt4*, while the expression of others decreases, e.g., nuclear receptor subfamily 5, group A, member 2 (*Nr5a2*; also known as *Lrh1*), aromatase (*Cyp19a1*), *Fst*, and *Apoa1*. Additional genes regulated in the ovary by FOXL2 at later stages of follicle development include inhibin β B subunit (the product of which forms either inhibin B when in complex with inhibin α subunit or activin B when it homodimerizes), *Nr5a2*, sterol regulatory element-binding transcription factor 1 (*Srebf1*), PPAR γ coactivator 1 α (*Ppargc1a*), cholesterol side chain cleavage (*Cyp11a1*), and steroidogenic acute regulatory protein (*Star*) (48–50). These results indicate that in addition to its role in embryonic ovarian development, FOXL2 likely affects specific basic metabolic aspects required for the proliferation and differentiation of somatic cells in the postnatal ovary. A missense mutation (402C→G) in human *FOXL2* has been linked to adult

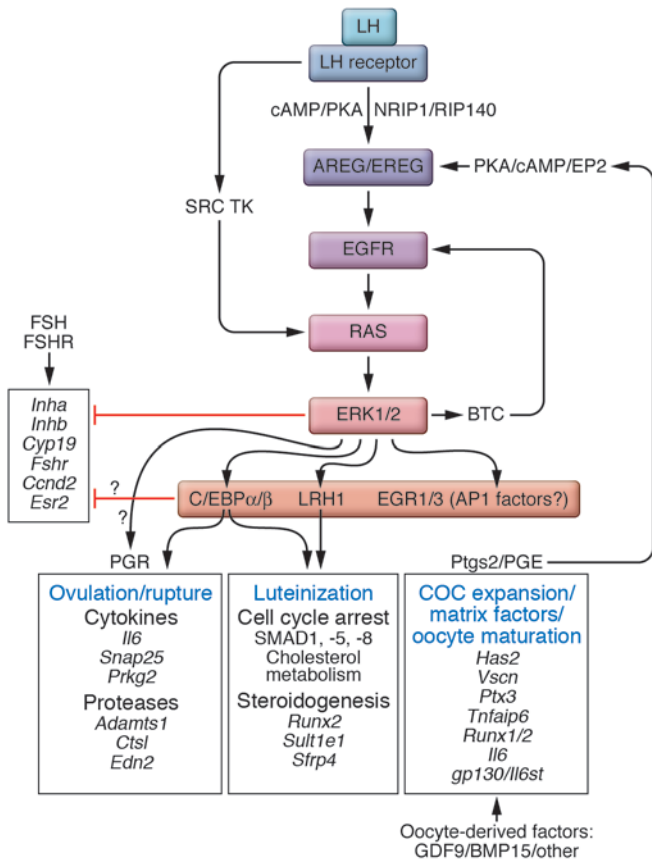


Figure 3

LH-mediated pathways to ovulation and luteinization. LH induces ovulation, COC expansion, oocyte maturation, and luteinization in pre-ovulatory follicles. These events are mediated by LH activation of the PKA pathway and NRIP1, which induce the expression of the EGF-like factors (AREG, EREG). AREG and/or EREG in turn activate the EGFR signaling cascade, including RAS and ERK1/2. This hypothesis is based on the observations that when *Erk1* and *Erk2* are disrupted in granulosa cells, global changes occur in gene expression patterns that control ovulation, COC expansion, resumption of meiosis, and luteinization. Moreover, activation of ERK1/2 is essential to turn off the FSH-regulated gene expression program that controls genes essential for preovulatory follicle growth and differentiation. C/EBP α and C/EBP β , NR5A2 (also known as LRH1), and possibly PGR appear to be among the key transcription factors that are activated by ERK1/2 phosphorylation and affect ovulation and luteinization. Other transcription factors that are targets of ERK1/2 include members of the AP1 family and EGR1/3, whose specific functions in ovulation and luteinization remain to be defined. Additionally, oocyte-derived factors such as GDF9 and BMP15 affect cumulus cell and granulosa cell functions in a gradient-dependent manner. The functions of specific genes are discussed in the text. TK, tyrosine kinase.

In contrast to FOXO3, which is expressed in oocytes, FOXO1 is expressed preferentially and at elevated levels in granulosa cells of growing follicles in mice and humans (Figure 2). Thus far, loss-of-function analysis of the role of this transcription factor in the ovary has been precluded because *Foxo1*-null mice are embryonic lethal (57). Although conditional alleles of *Foxo1*, *Foxo3*, and *Foxo4* have been generated, they have not yet been disrupted in a cell-specific manner in the ovary (58, 59). However, expression of FOXO1 mutants in granulosa cells indicates that FOXO1 may affect specific genes controlling granulosa cell proliferation (60), differentiation (60), or metabolism (61). Expression of a constitutively active nuclear form of FOXO1 in granulosa cells both suppresses expression of cyclin D2 (*Ccnd2*), *Cyp19a1*, FSH receptor (*Fshr*), and LH/choriogonadotropin receptor (*Lhcgr*) and acts as a potent negative regulator of essentially all genes in the cholesterol biosynthetic pathway, including *Srebf1* and *Srebf2* (60, 61). Thus, FOXO1 appears to be a critical factor in granulosa cells. Because FOXO1 is a downstream target of FSH and IGF1 and is presumed to be critical for metabolic homeostasis, and possibly apoptosis, modulation of FSH, IGF1, or insulin signaling might lead to clinical problems and may underlie some cases of infertility associated with diabetes.

New regulatory mechanisms that control follicle growth and differentiation

FSH signaling pathways. Although many of the early stages of follicle growth occur independently of pituitary gonadotropins (i.e., FSH and LH), FSH is required for granulosa cell differentiation and these cells rely on FSH to facilitate follicular growth (Figure 2 and Figure 3). Although FSH activates adenyl cyclase, leading to production of cAMP and activation of PKA, FSH also activates other signaling cascades independently of cAMP: for example, pathways involving PI3K (likely via an SRC tyrosine kinase) (62), RAS (62), or glycogen synthase kinase 3 β (GSK3 β) (63). Activation of PI3K leads to phosphorylation and activation of AKT, which in turn phosphorylates and thereby inactivates FOXO1 (63). Although, as mentioned above, the effects of disrupting *Foxo1* expression in granulosa cells have not yet been analyzed in vivo, disruption of *Pten* expression in granulosa cells, which leads to increased activa-

forms of ovarian granulosa cell tumors (GCTs), the most common type of malignant ovarian sex cord–stromal tumor (51). This mutation is unlikely to disrupt FOXL2 DNA binding, but rather alters protein-protein interactions (51). While this study found that the 402C→G mutation rarely occurs in juvenile GCTs (51), juvenile GCTs show reduced expression of *FOXL2* (52), indicating that FOXL2 may regulate multiple effects in granulosa cells that are context and stage specific.

Other members of the forkhead box transcription factor family also affect ovarian function. These include members of the FOXO family that are evolutionarily conserved and act downstream of insulin and IGF1, as well as FSH, to direct cell survival, apoptosis, and proliferation. Disruption of the *Foxo3* gene in mice leads to inappropriate oocyte activation, premature entry of primordial follicles into the growing pool, and POF (53) (Figure 2). Upon exhaustion of the primordial follicle pool, ovaries become devoid of growing follicles and the mice become infertile. Conversely, forced overexpression of *Foxo3* in oocytes reduces the number of growing follicles (54). As 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, oocyte-specific disruption of the *Pten* gene generates an ovarian phenotype identical to that of *Foxo3*^{-/-} mice: premature oocyte activation and release of primordial follicles into the growing pool (55). Specific targets of FOXO3 that restrict oocyte activation and proliferation of surrounding somatic cells remain to be defined (56). Although not yet observed in humans, mutation in the *FOXO3* gene might be the underlying cause of unexplained POF or streak ovaries (i.e., remnant gonadal tissue devoid of oocytes).



tion of the PI3K pathway and therefore increased phosphorylation and degradation of FOXO1, results in enhanced proliferation of granulosa cells, ovulation, and formation of corpora lutea that persist for unusually prolonged periods of time (64). 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 are likely 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 (64). Consistent with this, disruption of *Pten* in somatic cells of the mouse ovary causes distinct effects from those observed when it is disrupted in oocytes, as described above (*Transcription factors and early follicle growth*) (53–55). Furthermore, although natural mutations or disruption of *Pten* in other tissues lead to tumor formation, disruption of *Pten* only in granulosa cells does not lead to GCTs (64), perhaps because other factors affect the PI3K pathway in these cells. Because FOXO1 expression is high in human granulosa cells, this pathway is likely to be regulated in a manner similar to that in the mouse, but until we have *Foxo1*^{-/-} mice, the precise functions in vivo will remain unclear.

During the later stages of follicular growth (Figure 2), activins and estradiol, the predominant estrogen in humans, enhance the actions of FSH (65, 66) (Figure 2). In mice, activins activate the transcription factors SMAD2/3 and SMAD4, which coordinately regulate several FSH-induced genes including those encoding the cell cycle regulator CCND2 and the steroidogenic enzyme aromatase (67), which converts theca cell-derived androgens to estradiol. Estradiol, acting primarily via estrogen receptor beta (ERS2), has recently been shown to suppress expression of phosphodiesterase 1C (*Pde1c*), thereby increasing intracellular levels of cAMP induced by FSH (66). Because activins facilitate proliferation of granulosa cells and enhance FSH actions, in part by increasing estradiol, these interactions may help explain why, when activin actions are unopposed by inhibins (as occurs in mice lacking inhibin α subunit, which is encoded by *Inha*), activins promote GCT growth. The WNT signaling target β -catenin has also recently been implicated in enhancing *Cyp19a1* expression (68), indicating that FSH-induced phosphorylation of GSK3 β may be physiologically relevant in activating this arm of the WNT signaling cascade (69). Although disruption of the *Ctnnb1* gene in mice does not lead to overt changes in follicular development (70), proper activation of β -catenin in the adult ovary appears to be essential for normal tissue maintenance because overexpression of a constitutively active form of β -catenin leads to abnormal follicle development and, eventually, to development of GCTs (71). Moreover, the tumor phenotype induced by overexpression of the constitutively active form of β -catenin is enhanced when the tumor suppressor *Pten* is also concomitantly disrupted in granulosa cells, and these mice die by 6 weeks of age (72).

FSH and LH have recently been shown to activate RAS, in part via an SRC tyrosine kinase-mediated process, leading to the phosphorylation and activation of downstream kinases, in particular MEK1 and ERK1/2 (62) (Figures 2 and 3). Strikingly, KRAS is expressed at high levels in the granulosa cells of both small follicles and antral follicles (follicles at the late stages of follicular development that are characterized by the presence of a fluid-filled cavity known as the antrum adjacent to the oocyte; Figures 1 and 2), but its role remains to be determined (73). Expression in granulosa cells of a constitutively active form of KRAS that is frequently associated with various cancers, including ovarian surface epithe-

lial (OSE) cell cancer (KRAS^{G12D}), does not stimulate proliferation or tumor formation (73). Rather, KRAS^{G12D}-expressing granulosa cells cease dividing, do not undergo apoptosis, and fail to differentiate; that is, they are cell-cycle arrested. As a consequence, small abnormal follicle-like structures devoid of oocytes persist and accumulate in the ovaries of the KRAS^{G12D} mutant mice. Even when *Pten* is disrupted in *Kras*^{G12D} mutant mice, GCTs do not form (74), which indicates that granulosa cells are extremely resistant to the oncogenic insults of mutant *Kras* and loss of *Pten*. By contrast, if the *Kras* and *Pten* mutations are engineered in OSE cells, aggressive tumors appear within 6 weeks of age (74). These results indicate that the elevated levels of FSH or LH observed in patients with POF or PCOS may lead to increased activation of the RAS/ERK1/2 pathway as well as the PKA pathway, causing an imbalance in granulosa cell functions. These pathways need to be analyzed in more detail in clinical samples.

TGF- β family members. The TGF- β family of growth factors has wide-ranging roles in female reproduction (75). Although various family members are expressed by the major ovarian cell types (i.e., oocytes, granulosa cells, and thecal cells) (Figures 1 and 2), many of their effects center on the control of granulosa cell growth and differentiation, both of which affect folliculogenesis and oocyte development. Disruption in the ovarian function of the TGF- β family has resulted in mouse models that mimic human diseases such as POF. For instance, follistatin is an extracellular binding protein that binds to members of the TGF- β family and acts as a ligand antagonist. *Fst* conditional knockout female mice have been generated using *Ambr2-cre*, which expresses cre recombinase in adult female ovaries, predominantly in granulosa cells (76, 77). These mice demonstrate some aspects of POF, with few remaining follicles found by eight months of age (76). In addition, *Fst* conditional knockout mice show increased levels of gonadotropins, with decreased serum testosterone, mimicking the hormonal profile observed in women with POF. However, mutations associated with follistatin in human cases of POF have not been reported. The mechanism behind the premature loss of fertility in *Fst* conditional knockout mice is unknown, but because follistatin is a strong inhibitor of activin, part of the phenotype potentially results from increased activin activity.

Lack of inhibin α also results in increased activin because inhibins and activins are related proteins that share common β subunits: inhibin A is a heterodimer of the inhibin α and β A subunits, and inhibin B is a heterodimer of the inhibin α and β B subunits, while activin A and B are homodimers of inhibin β A subunit and inhibin β B subunit, respectively. Inhibins are also functional antagonists of activins (78–80). *Inha*^{-/-} mice develop sex cord-stromal tumors as adults and die of cancer cachexia-like syndrome (81, 82). In addition, *Inha*^{-/-} female mice are infertile prior to gross tumor development, as determined by their inability to ovulate and the lack of late-stage follicle development (83). Inhibin does not appear to be essential for embryonic oocyte development, germ cell syncytia breakdown, or primordial follicle formation because the ovaries of newborn *Inha*^{-/-} mice have normal numbers of oocytes and develop equivalent numbers of primordial follicles as the ovaries of wild-type mice in the three days after birth (84). However, marked changes in the ovaries of *Inha*^{-/-} and wild-type mice occur during the period of secondary follicle to early antral follicle development (Figure 2), which occurs after 6 days of age. At this point, ovaries in *Inha*^{-/-} mice are much larger and contain more advanced follicle types than those in wild-type mice. In addi-



tion, granulosa cell growth is uncoupled from oocyte growth, as evidenced by overly large follicles containing inappropriately small oocytes. The lack of oocyte growth is likely related to decreased expression of *Kitl*, as the gene encoding the receptor for the *Kitl* gene product is expressed in oocytes and is critical for oocyte growth and development (85). Both GDF9 and activin are known to decrease expression of *Kitl* (19, 86), and both *Gdf9* and the genes encoding inhibin β A and β B subunits are overexpressed in *Inha*^{-/-} ovaries (84). Additionally, there is loss of oocyte-expressed *Bmp15* and granulosa cell-expressed anti-Müllerian hormone (*Amb*), two growth factors that also modulate follicle growth. Thus, deletion of *Inha* results in multiple changes in the local hormonal milieu and causes infertility.

Mice lacking inhibin β A subunit (and thus lacking activin A and inhibin A) die shortly after birth (87), while mice deficient for inhibin β B subunit (and thus lacking activin B and inhibin B) have normal size litters but defects in nursing (88). Ovaries from inhibin β B subunit-deficient females overproduce the inhibin β A subunit (88), suggesting a possible compensatory gain in activin A activity. However, activin A and activin B are not fully redundant. For example, generating a mouse that expresses inhibin β B subunit in place of inhibin β A subunit rescues the perinatal lethality and craniofacial defects of inhibin β A subunit deficiency but is insufficient for normal folliculogenesis and female reproduction to occur (89). Moreover, stepwise removal of activin subunits by conditional deletion in granulosa cells culminates in female sterility only when all activin subunits are absent (19). In the activin-deficient ovary (conditional deletion of inhibin β A subunit in granulosa cells with global deletion of inhibin β B subunit), there are multiple defects in folliculogenesis (19). The most obvious is the progressive accumulation of corpora lutea, which is accompanied by increases in serum FSH and progesterone. As noted above, in granulosa cells, activin appears to play a predominant role as a growth promoter, and in support of this hypothesis, no ovarian tumors develop in activin-deficient mice. The growth-promoting role of activin in GCTs has been established by demonstrating that GCT growth in *Inha*^{-/-} mice is slowed by deletion of the gene encoding the activin type II receptor; deletion of *Smad3*, which encodes a transcription factor that acts downstream of activin; or injection into mice of a chimeric activin binding receptor–murine Fc protein (81, 82, 90–93).

SMAD transcription factors are key intracellular mediators of the canonical TGF- β family signaling pathway: SMAD2 and SMAD3 (AR-SMADS) signal downstream of activin, GDF9, and TGF- β , while SMAD1, SMAD5, and SMAD8 (BR-SMADS) signal downstream of the BMPs and AMH. An additional SMAD, SMAD4, participates in signaling triggered by all members of the TGF- β family. Conditional mutations of all SMADs have been generated in granulosa cells either as single or multiple mutations (94–96). Conditional deletion of *Smad4* results in age-dependent infertility, and because SMAD4 is involved in mediating signals triggered by all members of the TGF- β family, the phenotype affects multiple stages of folliculogenesis. Ovaries in *Smad4* conditional knockout mice show defects in steroidogenesis, ovulation, and the function of cumulus cells (which are a subpopulation of granulosa cells whose main functions in developing follicles are to support oocyte development), and the mice eventually develop POF (95). Unlike in activin-deficient mice, ovaries in *Smad4* conditional knockout mice exhibit increased preantral follicle death, a decrease in the number of antral follicles, and no accumulation of corpora lutea.

Similar to the activin-deficient ovary, small follicles luteinized prematurely, and even though *SMAD4* is a known tumor suppressor gene, no tumors developed in *Smad4* conditional knockout mice.

A similar phenotype to *Smad4* conditional knockout female mice is seen in female mice with granulosa cell conditional knockout of *Smad2* and *Smad3* (94). SMAD2 and SMAD3 have redundant functions in granulosa cells because mice with single conditional knockout of either *Smad2* or *Smad3* in granulosa cells have no reproductive phenotype (94). However, mice lacking both proteins have reduced litter sizes and become infertile after five months of age with disrupted follicle development (i.e., increased preantral follicle atresia and fewer antral follicles), luteinized follicles, reduced ovulation, and severe defects in cumulus cell function. The phenotypes of mice with conditional knockout of the BR-SMADs differ dramatically from those of other SMAD conditional knockouts. Single granulosa cell conditional knockout of either *Smad1* or *Smad5* and global *Smad8* knockout generates mice that are viable and fertile, as are double granulosa cell conditional knockout *Smad1/Smad8* and *Smad5/Smad8* mice (96). However, double granulosa cell conditional knockout *Smad1/Smad5* mice and triple granulosa cell conditional knockout *Smad1/Smad5/Smad8* female mice demonstrate infertility with age and develop GCTs with full penetrance by three months of age. The BR-SMAD phenotype is similar to the juvenile form of human GCTs (97). In addition, the majority of double granulosa cell conditional knockout *Smad1/Smad5* and triple granulosa cell conditional knockout *Smad1/Smad5/Smad8* mice show peritoneal implants and lymphatic metastases over time. These mice were the first in vivo demonstration that the BR-SMADs may have a critical tumor suppressor function in ovarian granulosa cells (96).

The various phenotypes of the SMAD and activin/inhibin knockouts in the ovary suggest potential interactions between the BMP and TGF- β /activin signaling pathways, in particular the observation that *Inha*^{-/-} and BR-SMAD conditional granulosa cell knockout mice develop GCTs (Figure 2). Part of the phenotype of *Inha*^{-/-} mice can be attributed to the tumor-promoting activity of activin via SMAD3 (94). In the BR-SMAD conditional granulosa cell knockout mice, 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 conditional granulosa cell knockout mice may be due to dysregulation of the AR-SMADs (i.e., SMAD2 and SMAD3) (96) that promotes granulosa cell proliferation. The role of additional signaling pathways in tumorigenesis in the BR-SMAD conditional granulosa cell knockout mice is still under investigation. In addition, as members of the TGF- β family signal through SMAD-independent pathways – including ERK1/2, JNK, PI3K/mTOR, and p38 MAPK pathways (98–101) – it is possible that tumor development results from activation of one or more of these noncanonical pathways by the BMPs when the BR-SMADs are not present.

New insights into factors controlling ovulation and luteinization

The LH surge terminates preovulatory follicle growth and initiates the processes of ovulation, oocyte meiosis, expansion of the cumulus cell oocyte complex (COC) (during which cumulus cells make a hyaluronan-rich matrix that surrounds the oocyte prior to ovulation), and luteinization (Figure 3) (102, 103). As a consequence, the FSH program of gene expression is turned off while genes controlling matrix formation and luteinization are turned on (74). Recent



studies show that the LH surge stimulates not only PKA but also PI3K/AKT and RAS signaling cascades, and that each of these is critical for ovulation (73). LH rapidly induces in granulosa cells the expression of the EGF-like factors amphiregulin (AREG), betacellulin (BTC), and epiregulin (EREG) (104) in a PKA-dependent manner. These factors bind their cognate receptors present on granulosa cells and cumulus cells, activate RAS, and induce expression of downstream target genes, including hyaluronan synthase 2 (*Has2*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), and TNF- α -induced protein 6 (*Tnfaip6*), each of which is a target of ERK1/2 in cultured cells (103). 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 (105). Moreover, mice in which ERK1 and ERK2 have been disrupted in granulosa cells exhibit normal follicle growth, but in response to LH, the COCs fail to expand, oocytes fail to re-enter meiosis, and follicles fail to either ovulate or luteinize (106). Genes expressed in preovulatory follicles fail to be suppressed, and genes known to regulate COC expansion, ovulation, and luteinization fail to be induced or activated. Thus, ERK1/2 controls a master switch that mediates the global reprogramming of granulosa cells downstream of EGF-like-factor activation of the EGF receptor pathway. Strikingly, ERK1/2 are also essential in the female mouse pituitary to initiate the LH surge (107). Thus, both the pituitary and the ovary exhibit ERK1/2 dependence during this critical process of ovulation and luteinization. The extent to which the activation of the ERK1/2 kinases is altered in infertile women has not been thoroughly examined.

Several transcriptional regulators are known to affect ovulation and appear to help mediate the effects initiated by ERK1/2 (Figure 3). Mice lacking nuclear receptor-interacting protein 1 (*Nrip1*; also known as RIP140) exhibit impaired ovulation and reduced expression of *Areg*, *Ereg*, and other ovulation-related genes (108). Therefore, NRIP1 may regulate transcription of the *Areg* gene, a critical early event in the ovulation process. Targeted disruption of *Nr5a2* in granulosa cells blocks ovulation and luteinization with little effect on the early stages of follicular development (109), indicating that NR5A2 (also known as LRH1) may be an ERK1/2 target in preovulatory follicles. By contrast, NR5A1 (also known as SF1) is essential for pituitary, gonad, and adrenal formation (110). Conditional deletion of the *Nr5a1* gene in granulosa cells shows that NR5A1 is essential for proper early follicle formation and development but does not appear to compensate for NR5A2 in granulosa cells of ovulating follicles (111). In this *in vivo* context, these two nuclear receptors appear to exhibit distinct, rather than overlapping, functions (112). The differences in the functions of NR5A1 and NR5A2 appear to be related to genes that are selectively regulated by NR5A1 (e.g., *Amb* and *Inba*) (111) compared with NR5A2 (e.g., *Cyp19*, *Sult1e1*, *Cyp11a1*, *Nos3*, and *Ptgs2*) (109). Targeted disruption of the NR5A2-regulated gene estrogen-specific sulfo-transferase (*Sult1e1*) leads to impaired ovulation and cumulus expansion (113), suggesting that NR5A2 potentially induces expression of this gene in response to the LH surge. Because endogenous estradiol levels are high in *Sult1e1*^{-/-} mice, this may contribute to the anovulatory phenotype, but the exact molecular mechanisms affected by high estradiol at this time have not yet been defined. One other mutant mouse in which both ovulation and luteinization are impaired is the CCAAT/enhancer-binding protein β (C/EBP β)-deficient mouse (114). Importantly, C/EBP β is a known phosphorylation target of ERK1/2 in granulosa cells

(106). Based on the genes regulated by ERK1/2, NRIP1, NR5A1, and C/EBP β , it is tempting to speculate that NRIP1 may be an important co-regulator and/or activator of NR5A1 and/or C/EBP β in the ovary and that ERK1/2 may be required for specific phosphorylation events. Thus, it will be important to determine how ERK1/2, C/EBP β , NR5A1, and NRIP1 coordinately regulate a select number of genes that control ovulation versus luteinization. Clearly, ovulation defects in women could be attributed to altered functions in one or all of these factors.

The expression of genes frequently associated with innate immune responses, many potent cytokines, such as IL-6, and runt-related transcription factor 1 (RUNX1) and RUNX2 are also impaired in mice lacking ERK1/2 (103, 115–117) (Figure 3). Recently, IL-6 alone has been shown to stimulate COC expansion and induce the expression of specific genes encoding proteins involved in this process (115). These observations indicate that in clinical situations where levels of IL-6 are elevated, such as chronic infections, endometriosis, and possibly PCOS, this and other cytokines may disrupt normal granulosa cell and cumulus cell functions. Moreover, IL-6 and leukemia-inhibitory factor (LIF) increase the expression of *Stat3* and IL-6 signal transducer (*Il6st*) in cumulus cells and the oocyte present in preovulatory follicles and enhance reproductive outcomes, suggesting that this pathway impacts oocyte quality (115). Mice lacking *Il6st* exhibit defects in zygotic cell division, suggesting that IL-6 and related cytokines affect oocyte functions (118). Whereas inclusion of IL-6 or LIF might improve the quality of oocytes obtained in IVF procedures in women, abnormally elevated levels of cytokines, as occurs in endometriosis and chronic infections, might impair oocyte quality in women. Because the induction of IL-6 is regulated not only by AREG (115) but also by progesterone receptor (PGR) (115, 119) and C/EBP β , both of which are essential for ovulation, it is tempting to speculate that IL-6 may mediate some key process downstream of PGR and C/EBP β in granulosa cells or cumulus cells. Moreover, the expression of synaptosomal-associated protein 25 (SNAP25), an important component controlling neuronal-like secretion of cytokines from granulosa cells, is also regulated by PGR (120). Thus, the importance of locally produced and secreted ovarian cell-derived cytokines during ovulation needs to be analyzed further, and these cytokines may affect several ovulation-related processes including follicle rupture, COC transport, and fertilization (117). In this regard it is important to note that cytokines have recently been shown to affect the fertilization process by enhancing sperm motility and capacitation.

Conclusions

New insights into regulators of early oocyte development, follicle formation, follicular growth, ovulation, and luteinization indicate that multiple factors and signal transduction pathways act in cell- and context-specific manners to regulate fertility. Analyzing these genes might provide new targets for contraceptive research as well as improve fertility in women with endometriosis and PCOS. The ability to manipulate the size of the primordial follicle pool opens up the possibility of extending ovarian function, not only for reproductive purposes, but also to diminish the effects of diseases associated with menopause, such as osteoporosis, heart disease, and some cancers. Recent advances in follicle culture (121–123) may also provide a way to preserve an ovarian reserve for women undergoing chemotherapy. Information being derived from other new approaches, such as the exponential increase in knowledge of



microRNAs, should provide additional insights into the factors, and combined sets of factors, that regulate genes in a cell- and context-specific manner (124–128). Finally, advances in ovarian cancer research will continue to be made with mouse models that recapitulate tumors in women, as well as defining criteria for tumor progression (129).

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