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Ontogeny of the ovary in polycystic ovary syndrome

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Abstract

Activation of primordial follicles into the growing pool, selection of the dominant follicle, and its eventual ovulation require complex endocrine and metabolic interactions as well as intraovarian paracrine signals to coordinate granulosa cell proliferation, theca cell differentiation, and oocyte maturation. Early preantral follicle development relies mostly upon mesenchymal-epithelial cell interactions, intraovarian paracrine signals, and oocyte-secreted factors, whereas development of the antral follicle depends on circulating gonadotropins as well as locally derived regulators. In women with polycystic ovary syndrome (PCOS), ovarian hyperandrogenism, hyperinsulinemia from insulin resistance, and altered intrafollicular paracrine signaling perturb the activation, survival, growth, and selection of follicles, causing accumulation of small antral follicles within the periphery of the ovary, giving it a polycystic morphology. Altered adipocyte-ovarian interactions further compound these adverse events on follicle development and also can harm the oocyte, particularly in the presence of increased adiposity. Finally, endocrine antecedents of PCOS occur in female infants born to mothers with PCOS, which suggests that interactions between genes and the maternal-fetal hormonal environment may program ovarian function after birth.

Keywords

Androgens; antimullerian hormone; growth differentiation factor-9; insulin; polycystic ovaries

Polycystic ovary syndrome (PCOS) is the most common cause of infertility in women (1), so it is essential to understand what endocrinologic, molecular, metabolic, and/or genetic factors contribute to this disease and how they alter ovarian follicle development (2–4). According to the Rotterdam criteria, PCOS is characterized by two of the following three features: [1] clinical or biochemical hyperandrogenism, [2] oligoanovulation, and [3] polycystic ovaries (PCO), excluding other endocrinopathies (5). Patients with PCOS also often present with elevated serum levels of luteinizing hormone (LH) and insulin. Although primordial follicles in the ovaries of PCOS patients leave the resting pool, most arrest at the small antral stage preceding dominant follicle selection, giving rise to many small follicles

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forming a ring around the ovary as a characteristic of PCO. Abnormal follicle growth in PCOS accompanies elevated levels of LH, insulin, and androgens; altered expression of transforming growth factor-β (TGF-β) family members; and comparatively low levels of follicle-stimulating hormone (FSH) (4, 6). Studies have indicated that the expression and impact of these factors may vary in PCOS patients by phenotype and may be further influenced by the degree of adiposity (7–10).

This review integrates what is currently known about normal follicular development with specific events that are altered in the follicles of PCOS patients. Mechanisms by which LH hypersecretion, hyperandrogenism, hyperinsulinemia, and TGF-β-related events alter ovarian follicle development are discussed, along with the potential roles of the insulin-like growth factor-I (IGF-I)/AKT/Forehead BoxO (FOXO) pathway and WNT signaling in these events. Due to space limitations, not all aspects of follicle development are covered in depth.

PREANTRAL FOLLICLE DEVELOPMENT

Overview

Primordial follicles are recruited into a cohort of growing follicles, from which one antral follicle is selected to become dominant while the others undergo atresia. Each primordial follicle contains an oocyte arrested at the diplotene stage of prophase one, surrounded by squamous granulose cells. With follicle growth, the oocyte begins to synthesize messenger ribonucleic acid (mRNA), while squamous granulosa cells enlarge into a complete single layer of cuboidal granulosa cells, forming the primary follicle (11, 12).

The Primordial to Primary Transition

Primordial follicles can remain in the quiescent “resting” stage for many years. The precise signals that initiate the transition of a primordial follicle to a growing primary follicle are incompletely understood but are independent of gonadotropins (13). Rather, factors derived from oocyte and granulosa cells activate primordial follicles or inhibit them (12, 14). In the oocyte, the PI3K pathway is critically involved in maintaining oocyte quiescence. Disruption of *Pten*, *Foxo3*, or other PI3K pathway genes in mice leads to oocyte activation, global transition of primordial follicles into the growing pool, and premature ovarian failure (15–18). Conversely, granulosa cell antimüllerian hormone (AMH) production, beginning in primary follicles, acts to reduce the number of primordial follicles leaving the resting pool (19–22). Although granulosa cell AMH produced in response to oocyte-derived factors (23, 24) inhibits primordial follicle growth, disruption of *Amh* gene expression does not cause a reciprocal activation of all primordial follicles, implying that other factors, including granulosa cell-derived kit ligand (KL) and its receptor c-kit on oocytes (11, 12, 25), also contribute to the initiation of primordial follicle development and oocyte growth (25–27). Whether this initial transition process is altered in the ovaries of PCOS patients remains unclear.

Formation of Primary Follicles

Once follicles leave the primordial stage to enter the growing pool, specific changes in the oocyte, granulosa cell, and theca cell functions occur, including [1] transition of granulosa

cells from a flattened fibroblastic-like morphology to a cuboidal shape, [2] appearance of the zona pellucida, and eventually [3] formation of the theca cell layer external to granulosa cells and resting upon the basal lamina (Fig. 1).

Formation of the theca cell layer in primary follicles critically depends on oocyte-derived growth differentiation factor 9 (GDF9) (28, 29) and the granulosa-derived factor KL (11, 12). In addition, GDF9 enhances androgen production in cultured small follicles by regulating theca cell androgen production either directly or indirectly (25, 30–32). Theca cell-derived androgens, in turn, serve essential regulatory roles by increasing the expression of FSH receptors (*Fshr*) in vivo and in vitro (33, 34). In primates, testosterone administration to adult female rhesus monkeys increases the number of growing preantral and small antral follicles (35, 36); up-regulates mRNA expression of FSH receptors, IGF-I receptors, and IGF-I in proliferating granulosa cells (35, 37, 38), and enhances IGF-I and IGF-I receptor mRNA expression in primordial follicle oocytes (37). These androgen actions likely occur via the androgen receptor (AR), which is present in human and mouse primary follicles (39, 40) and if disrupted in mice reduces *Fshr* and *Kitl* expression, impairs folliculogenesis, and induces premature ovarian failure (41–43). Moreover, targeted loss of AR signaling exclusively in murine granulosa cells of preantral and antral follicles also reduces fecundity, induces follicular atresia, and impairs oocyte fertilization as well as preimplantation embryogenesis (44, 45).

Receptors for LH (LHCGR) are present in theca (but not granulosa) cells of small secondary follicles (but not primordial follicles), allowing LH-stimulated androgen production in follicles at this early stage (46). The factors that induce LH receptors are unknown but could be GDF9 or other oocyte- or granulosa cell–derived factors (IGF-I or IGF-II?), retinoic acid signaling (47), or other yet to be identified factors.

Insulin also acts through its own receptors on theca cells, stroma, granulosa, and oocytes to promote the primordial to primary follicle transition (48, 49). This is important for many women with PCOS who have hyperinsulinemia from insulin resistance beyond that predicted by body mass index (BMI) alone, with 50% to 70% of such women demonstrating insulin resistance (50). Hyperinsulinemia in PCOS results from abnormal postreceptor signal transduction, which reduces insulin-mediated glucose uptake (9) without affecting steroidogenesis (51, 52). Thus, insulin excess stimulates theca cell CYP17a activity (53), amplifies LH- and IGF-I-stimulated androgen production (54, 55), elevates serum free testosterone levels through decreased hepatic sex hormone-binding globulin (SHBG) production, and enhances serum IGF-I bioactivity through suppressed IGF-binding protein (IGFBP) production, thereby perpetuating ovarian hyperandrogenism (52). High insulin levels could theoretically act through IGF-I receptors to exert some of these effects; insulin stimulation of human granulosa cell steroidogenesis, however, is mostly mediated through its own receptor because this action is inhibited by blocking with antibody to the insulin receptor, but not to the IGF-I-receptor (56). Insulin (at high levels) may also enhance GDF9-mediated increases in androgen production in primary and small follicles (30).

Given this background, hyperandrogenism, hyperinsulinemia, and/or LH hypersecretion in PCOS patients would be expected to stimulate early follicle development, increase *Fshr*

expression prematurely or to an exaggerated level, and render granulosa cells prematurely more responsive to FSH, perhaps through enhanced FSH-stimulated cyclic adenosine 3′ :5′ monophosphate (cAMP) production (57, 58). Conversely, the ability of AMH to decrease the expression of *Fshr* and the response of granulosa cells to FSH (59) would be expected to blunt the effects of FSH, at least temporarily, in early growing follicles but not necessarily in PCOS follicles (as will be discussed).

In support of this, in vitro studies of PCOS theca cells show intrinsically increased androgen biosynthesis and augmented expression of several steroidogenic enzymes (60, 61), presumably from the enhancing effects of the retinoic acid pathway (47) and dysregulation of theca cell mitogen activated protein kinase signaling (62). However, androgen production has not been established for normal or PCOS primordial or primary follicles, and reports using in situ hybridization indicate that the ovaries of anovulatory women with PCOS have increased primary follicle growth with reduced oocyte GDF-9 mRNA (63, 64). Lower levels of GDF9 in PCOS follicles at this stage might be expected to reduce theca cell organization or androgen production, but this has not yet been investigated.

Insulin may compensate for reduced GDF9. Furthermore, histologic examination of human ovaries from women with PCOS has found an enhanced number of growing primary follicles and a reciprocally decreased proportion of primordial follicles, independent of ovulatory status or atresia (65, 66). This may be related to the lower level of AMH (but not necessarily AMHR2 levels) that is observed in primordial and transitional follicles in anovulatory PCOS ovaries (39, 67). Primary PCOS follicles also show increased granulosa cell proliferation and enhanced oocyte growth (63), with decreased atresia of preantral PCOS follicles grown in vitro, possibly from enhanced survival (68). However, the roles of GDF9, AMH, and AMHRII during the primordial to primary transition in normal and PCOS follicles remain to be clearly defined with a larger number of samples, especially for GDF9 and AMHR2 expression, and functional data. Studies in nonhuman primates and other species will be especially important in this regard.

The Primary to Secondary Follicle Transition

Secondary follicles develop over several months, acquire additional steroid receptors, and become physiologically coupled by gap junctions (Fig. 2 and Fig. 3) (12, 69). As these secondary follicles grow, they become more dependent on gonadotropins, likely because of the spatial changes within the follicle and receptor levels/activity. As granulosa proliferate and an antrum begins to form, the granulosa cells and the theca cells become spatially distanced from the oocyte and its secreted factors. Thus, at the secondary stage, LH may become more important than GDF9 in promoting theca androgen biosynthesis and affecting granulosa cell responsiveness to FSH relative to AMH.

Little is known about secondary follicle development in humans due to the scarcity of this follicle stage in archived ovarian tissue (65, 68). Nevertheless, IGF-II mediates FSH-induced growth of cultured human preantral follicles (70). In PCOS, therefore, when LH and/or androgens are elevated, an increased response of granulosa cells to FSH may lead to FSH-stimulated expression of aromatase (*Cyp19*) and other genes along with additional developmental changes (46, 71) that promote FSH action despite the repressor effects of

AMH, particularly in the presence of insulin excess. This may allow FSH and AMH to functionally coexist.

The IGFI/II/FOXO Pathway

Insulin-like growth factor-I or IGF-II (in humans) is expressed in the granulosa cells of small growing follicles and can enhance FSH-mediated granulosa cell differentiation. This is related to activation of the PI3K and ERK1/2 pathways and to regulation of Forehead BoxO transcription factors. Both FOXO1 and to a lesser extent FOXO3 are highly expressed in granulosa cells of mouse, rat, nonhuman primates, and humans (72–75). These transcription factors have been evolutionarily conserved from *Caenorhabditis elegans* to man, are downstream of AKT in the PI3K pathway, and impact reproductive success (75–77). Therefore, FOXO1/3 are targets of both IGF-I/IGF-II and presumably the insulin pathways in granulosa cells.

Studies have shown that *Foxo1/3* mutant mice are completely infertile and exhibit impaired follicular development as well as impaired apoptosis (77). Detailed molecular analyses of the mutant ovaries in these mice document that FOXO1/3 interact with activin to control genes that promote follicle growth and interact with bone morphogenetic protein-2 (BMP2) to direct apoptosis. What controls this switch remains to be determined but is obviously critical for determining the fate of follicles, of which 99% undergo apoptosis.

The roles of FOXO1/3, IGF-I, and IGF-II in preantral PCOS follicles have not been fully explored, although increased circulating IGF-I bioavailability in women with PCOS could have a permissive effect (78). Because IGF-I receptors are present on mouse oocytes, this signaling pathway may impact oocyte quality as well. In vitro studies using synthetic IGF-I analog with low IGFBP affinity show that an inappropriate increase in IGF bioavailability is detrimental to bovine oocytes of preantral follicles (79).

The WNT/Frizzled Pathway

The WNT/Frizzled pathway is essential for specification of the mouse and human embryonic ovary and is crucial for the transition of secondary follicles to the antral stage (80, 81). Its actions, due in part to activation of β-catenin (CTNNB1), include enhancement in gene expressions of *Fshr* and aromatase (*Cyp19a1*) (80, 82, 83). The actions of WNT4 are tightly linked to those of FOXL2, a transcription factor that also is essential for embryonic specification of the ovary and suppresses steroidogenic gene expression while promoting activin expression (73, 84). Microarray data show that WNT signaling is repressed in PCOS theca cells (85), with several genes of the WNT signaling pathway dysregulated in ovaries of both PCOS patients and androgen-treated female-to-male transsexuals, implicating an androgen influence on this pathway (86). However, the expression levels and functions of WNT4/5A in granulosa cells of normal and PCOS patients remain to be determined.

ANTRAL FOLLICLE DEVELOPMENT

Overview

Growth of follicles from the primordial to preovulatory stage in women takes approximately 6 months, with the final 2 weeks of antral follicular development depending on changing circulating gonadotropin levels (87). Antral follicle development is characterized by slower oocyte growth (maximum diameter 140 μm), extracellular fluid formation, granulosa cell differentiation into mural and cumulus phenotypes, and selection of a dominant follicle (11, 12). Early antral follicles in humans are responsive to FSH (12, 69). In follicles 6–8 mm in size, granulosa cells begin to express CYP19A1 (aromatase) (88), allowing theca-derived androgens to undergo aromatization to estrogens by FSH-stimulated granulosa cells. The LH-stimulated thecal cell androstenedione production via CYP17A1 is enhanced by granulosa cell-derived paracrine factors (89). These paracrine factors include inhibins, IGF-I, and IGF-II as well as retinoic acid, which stimulate thecal cell androgen production; conversely, follistatin binds to activin to inhibit its androgen-suppressing effect (90).

Progressive growth of the antral follicle coincides with gradual acquisition of oocyte developmental competence, defined as the ability of the oocyte to complete meiosis and undergo fertilization, embryogenesis, and term development (6). Oocytes from healthy large antral follicles, exposed to an appropriately timed progression of changes in the intrafollicular microenvironment, are more likely to fertilize and undergo successful embryogenesis than similarly matured oocytes from small antral follicles (6).

Hyperandrogenism

Elevated androgens appear to increase pituitary LH pulsatility and secretion, leading to enhanced theca cell stimulation through reduced hypothalamic sensitivity to steroid feedback, which can be established in utero or postnatally (91–95). Increased androgen biosynthesis appears to enhance the expression of FSH receptor, leading to an increased estradiol (E_2) responsiveness of PCOS granulosa cells to FSH in vitro (96, 97). Despite this phenomenon, follicular arrest in PCOS patients occurs at the stage when granulosa cells normally begin to express aromatase and secrete E_2 (6, 88). Androgen excess together with premature differentiation of granulosa cells overexpressing LH receptor preempts FSH action on E_2 production in favor of progesterone synthesis (56, 98–101), perhaps in combination with a local inhibitor of FSH action (96). Elevated expression of LH receptor mRNA and genes associated with differentiation, such as STAR and *RUNX2*, have also been reported in the cumulus cells of lean PCOS patients but not of obese PCOS patients (102), indicating that altered cumulus cell functions likely impact oocyte quality and maturation in this context.

Androstenedione biosynthesis and the steroidogenic enzyme CYP17A1 are elevated in PCOS theca (60, 61, 96). As a result, serum androstenedione levels positively correlate with antral follicle number in normal women and those with PCOS (103, 104), while antiandrogen therapy to PCOS patients improves PCO morphology (105). Small PCOS follicles also have elevated 5α-reductase activity, which elevates 5a–reduced androgen levels to concentrations capable of inhibiting, rather than increasing, granulosa cell

aromatase activity in vitro (58, 106, 107). Consistent with this finding, dihydrotestosterone (DHT) impairs gonadotropin-stimulated E_2 secretion in female rhesus monkeys (108) and can inhibit gran-ulosa cell proliferation in rodents in certain contexts (109). Clearly the effects of DHT are dose and concentration dependent, and the beneficial effects of androgens can support follicle development (110) in part by regulating the FSH receptor (33, 34).

High androgens in small antral PCOS follicles may also decrease rates of in vitro maturation, fertilization, and embryo development compared with immature oocytes from normal women (111) by interfering with E_2-mediated effects that promote oocyte cytoplasmic maturation (112–114).

Hyperinsulinemia

Insulin can enhance granulosa cell responsiveness to LH (56, 98), implying that premature follicle luteinization may occur in hyperinsulinemic PCOS patients (99). As evidence, cultured granulosa cells from small PCOS follicles exhibit premature responsiveness to LH due to early expression of LH receptors (3, 99, 100), leading to enhanced progesterone production (101). Such premature follicle luteinization facilitated by insulin excess may also affect human oocyte development as well because insulin receptors are present in oocytes (48). As an example, insulin together with FSH up-regulates LH receptor expression in cultured mouse cumulus-oocyte complexes and reduces blastocyst development (115). One caveat of these studies, however, is the extremely high levels of insulin that were used.

Hyperinsulinemic PCOS patients undergoing gonadotropin therapy develop a larger number of follicles between 12 and 16 mm in diameter (116). Of relevance, mRNA expressions of insulin receptor (*INSR*) and specific fatty acid binding proteins (FABP) are elevated in cumulus cells of obese PCOS compared to lean PCOS patients (102) and the INSR also may be elevated in oocytes of PCOS versus non-PCOS patients (205). These distinctly different patterns of insulin-related gene expression suggest that the cumulus cells and oocytes of obese PCOS patients have different responses to insulin than those of lean PCOS patients. Because insulin can bind directly to oocytes, a direct effect of insulin excess may alter oocyte quality (117).

Increased Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor A (VEGF-A) and its related protein members (VEGF-B, VEGF-C, and VEGF-D) of the platelet-derived growth factor family are potent cytokines that promote angiogenesis and vascular permeability (118). Vascular endothelial growth factor gene expression occurs in normal and PCOS ovaries (119) and increases after human chorionic gonadotropin (hCG) administration in human luteinized granulosa cells (120, 121), elevating VEGF protein levels in blood and follicles as well as in peritoneal fluids of women at risk of or with ovarian hyperstimulation syndrome (OHSS) (121–123). Ovarian VEGF synthesis and vascular blood flow as well as serum VEGF levels are increased in PCOS patients (124–126). Consequently, enhanced VEGF-mediated vascular permeability and fluid extravasation can occur in PCOS patients undergoing controlled ovarian stimulation, increasing their risk of developing the complications of OHSS, including

ascites, intravascular fluid depletion, hemoconcentration, hypercoagulation, hypotension, pleural effusion, hematologic and liver dysfunction, renal failure, and respiratory distress (127). This increased risk of OHSS also is linked with hyperinsulinemia because in vitro VEGF-A production by luteinized granulosa cells of PCOS patients compared with normal women is preferentially enhanced by synergistic interactions between LH/hCG and insulin (128). Whether other factors, including angiopoietins, further alter the ovarian vascular network in PCOS, as they do in the DHEA-treated rodent model of PCOS (129), remains to be determined.

Increased AMH Production

Antimüullerian hormone is normally produced by the granulosa cells of growing follicles (67, 130), so low AMH levels occur in primordial and primary follicles, increase to maximal levels in large preantral and small antral stages, and then decline in granulosa cells but not cumulus cells during final follicular maturation (67, 131–133). As a marker of growing follicles in normal women undergoing in vitro fertilization (IVF), serum AMH levels positively correlate with the number of antral follicles, the serum androgen concentration, and the oocytes retrieved, and negatively correlate with amount of recombinant human FSH administered (132, 134). Intrafollicular AMH levels also negatively correlate with FSH levels in the follicles of normal women undergoing IVF (135).

Women with PCOS have elevated circulating and intrafollicular AMH levels related to an increase in the number of follicles as well as from hypersecretion by granulosa cells themselves (59, 136–139). With AMH production in anovulatory PCOS significantly higher on the basis of the granulosa cell per se, raised serum AMH levels in PCOS patients represent both enhanced production by granulosa cells and an increased number of follicles. Consequently, serum AMH levels are elevated in normoandrogenic women with PCO undergoing ovarian stimulation for IVF, and are further increased in hyperandrogenic women with PCO, independent of antral follicle number (133). Some investigators have suggested that serum AMH levels (>35 pmol/L, or >5 ng/mL) may serve as a surrogate marker of PCOS, although insufficient data exist regarding the ability of circulating AMH determinations to discriminate PCOS phenotypes by patient age (140).

An inverse relationship between serum AMH and E_2 levels in PCOS agrees with the ability of AMH to decrease FSH receptor mRNA expression and impair FSH-induced aromatase activity in vitro (59, 138, 139, 141). However, other data show that mRNA levels for *Amh*, *Fshr*, and *Ar* are all higher in small and large follicles obtained from hormone-stimulated PCOS patients compared with those from control patients (142). These results indicate that in PCOS antral follicles the inhibitory effects of AMH are reduced/altered. The question is why? The possible explanations are multiple and need to be resolved. For example, follicle development may differ among women, with lean PCOS patients exhibiting follicles at a more differentiated stage and obese patients having follicles at a less differentiated stage (see Fig. 2). Alternatively, androgens that enhance FSH-receptor expression (33) when combined with the mitogenic actions of growth factors (i.e., LH, epidermal growth factor, TGF-β, IGF-I, or insulin) on PCOS granulosa cells (6, 12, 48) may overcome AMH inhibition of FSH-dependent aromatase activity. In light of these considerations, serum

AMH and LH levels are positively linked in severe PCOS (143, 144), with AMH secretion by cultured PCOS granulosa cells enhanced by LH (136), perhaps from premature differentiation due to early expression of LH receptors (3, 99, 100). Collectively, serum AMH concentrations in PCOS are [1] positively predicted by LH and testosterone levels as well as antral follicle number, [2] variably related to *Fshr* expression or action, [3] negatively predicted by BMI (144), and [4] variably reduced by weight loss (145, 146) or metformin (134, 147), suggesting complex interactions between LH, androgen, and insulin with ovarian function in obese women with PCOS.

Inhibins, Activins, and Follistatin

Granulosa cell-derived inhibins and activins belong to the TGF-β superfamily. Follistatin binds activin with high affinity to inhibit its action (148). In addition, FSH increases activins and inhibin, which in turn regulate granulosa cell, theca cell, and pituitary *Fshb* cell functions (29, 84).

Inhibin A occurs in follicles as small as 6.5 mm and increases in parallel with α-subunit and βA-subunit mRNA expression during antral follicle growth (149, 150). Inhibin A suppresses FSH synthesis by inhibiting *Fshb* gene transcription (151) and enhances LH-stimulated androgen biosynthesis (152). In contrast, intrafollicular inhibin B levels do not necessarily increase, nor does its βB-subunit mRNA expression vary, by follicle size (149, 150). Activin promotes follicle growth by stimulating granulosa cell proliferation while delaying luteinization, increasing granulosa cell FSH receptor expression and E_2 synthesis (i.e., enhanced FSH-induced *Cyp19*, *Ccnd2*, and *Inha* and other genes) and decreasing androgen production (148, 153). Activin actions are balanced by bone morphogenetic proteins (BMPs) within the ovary, such as BMP6 and BMP15 (oocyte), BMP4 (theca), and BMP2 (apoptotic granulosa cells) (84, 154, 155). Collectively, activins promote follicular development by enhancing granulosa cell responsiveness to FSH and by suppressing androgen synthesis, while inhibins produced by the dominant follicle stimulate theca cell androgen production (89, 148–150).

Inhibin α-subunit and βA-subunit mRNA levels are reduced in granulosa cells of small PCOS follicles (156). Inhibin A and B concentrations also are decreased in some, but not all, small PCOS follicles, despite normal amounts of activin and follistatin unbound to activin (150, 157). Although exogenous FSH stimulates the growth of PCOS follicles, intrafollicular inhibin A levels in PCOS patients receiving gonadotropin-releasing hormone (GnRH) analog/gonadotropin therapy for IVF remain reduced (158). On the other hand, serum inhibin B levels arising from the multiple small follicles are elevated in PCOS patients (159) and are suppressed by exogenous hCG and endogenous insulin (160), linking defective inhibin biosynthesis with follicular arrest, likely by reducing FSH levels (151).

High follistatin and low activin A levels also have been reported in the circulation of some PCOS patients (161, 162). Follistatin levels in small human antral follicles, however, are 10-fold greater than those of activin A levels, which brings into question the role of activin A bioactivity in these follicles (163). Thus, the overall impact of the activin and inhibin pathways on PCOS remains unclear.

Adipocyte-Ovarian Interactions

Adiposity-dependent insulin resistance, although not necessarily intrinsic to PCOS, has become inextricably linked with the syndrome (164). Greater total and abdominal obesity as well as insulin resistance, menstrual irregularity, and hyperandrogenism occur in women with classic PCOS, while weight loss in obese women with PCOS lowers circulating LH, androgen, and insulin levels, thereby improving hirsutism and menstrual and ovulatory dysfunction as well as dyslipidemia (4, 165–169).

In addition to obesity, insulin resistance in PCOS is greater than that predicted by BMI, with 40% to 50% of women with PCOS being nonobese (170). Nevertheless, adipose function is important because adipose tissue secretes many factors that not only regulate adipocytes, macrophages, and pluripotential cells but also likely alter ovarian function in PCOS (171, 172). These factors may act indirectly or directly on granulosa cells (i.e., IL6, leptin, adiponectin) or cumulus cells and oocytes (leptin, adiponectin) (173). Adiponectin has many functions (174), including its ability to promote oocyte maturation and blastocyst formation (175), and is reduced in the circulation of women with PCOS (176), together with tumor necrosis factor-α-induced dysregulation of adiponectin secretion by PCOS adipocytes in vitro (177). In addition, leptin impairs FSH-stimulated steroidogenesis in human granulosa cells, thereby reducing ovarian responsiveness to FSH (178–181). Obesity also lowers LH pulse amplitude and alters LH pharmacokinetic structure (182, 183), contributing to reduced serum LH levels in obese women with PCOS (184, 185), who may be more susceptible to the modulatory effects of insulin excess than LH.

THE DIFFERENTIATED PREOVULATORY FOLLICLE

Overview

The hallmarks of normal preovulatory follicles are acquisition of LH receptors on mural granulosa cells, elevated expression of aromatase (*Cyp19a1*) with increased E_2 production, and markedly enhanced responsiveness of granulosa cells to FSH and LH via increased cAMP production (Fig. 4) (186–189). Only preovulatory follicles ovulate in response to the LH surge, and only preovulatory follicles express high levels of LH receptor in granulosa cells, a response mediated by the coordinate actions of FSH and E_2 or FSH and androgens (187).

With the midcycle LH surge, the preovulatory follicle shifts steroidogenesis from androgen and estrogen to progesterone production (87). Critical events induced in granulosa cells by the LH surge depend on induction of epidermal growth factor-like factors (amphiregulin, epiregulin, and β-cellulin) (190, 191), which activate the epidermal growth factor receptor, leading to activation of RAS and the MEK/MAPK1/3 (ERK1/2) pathway. This pathway is essential for initiation of meiosis, cumulus cell oocyte complex expansion, and follicle rupture (190–192). As a result, bidirectional cumulus-cell–oocyte signaling through cGMP (193–196) and gap junctions (197, 198) is reduced to permit the oocyte to undergo germinal vesicle breakdown and produce a haploid metaphase II oocyte (i.e., nuclear maturation) capable of cytoplasmic maturation, fertilization, and initial embryonic development (199–202).

Oocyte Developmental Competence

Terminally differentiated follicles of classic PCOS patients undergoing ovarian stimulation for IVF remain hyperandrogenic (203, 204) and contain normal appearing metaphase II oocytes with distinctly abnormal gene expression profiles (205) involving signal transduction, cell metabolism, DNA transcription, and RNA processing. These genes often contain promoter sequences with putative binding sites for androgen receptor, peroxisome proliferating receptor-γ, and/or peroxisome proliferating receptor γ-retinoid × receptor, linking hyperandrogenism with insulin resistance in the developmental fate of the oocyte. With androgen and insulin levels in the follicles of IVF patients determined by PCOS and BMI, respectively (203, 206), obese PCOS patients experience a high miscarriage rate after transfer of normal appearing embryos into a surrogate uterus (207).

Impaired oocyte developmental competence, however, is not a universal finding in PCOS, and it is related to many factors (207–213). For example, impaired oocyte competence has been linked with elevated follicle fluid levels of tumor necrosis factor-α (214) and interleukins (215), whereas other adipokines have a positive action on cumulus-oocyte functions (173, 175). This constellation of findings may involve ovarian cell lipotoxicity, as shown in mice fed a high-fat diet, whereby poor oocyte quality is associated with increased lipid content and reduced mitochondrial membrane potential, cumulus-oocyte complexes with increased gene expression of endoplasmic reticulum stress markers, increased granulosa and cumulus cell apoptosis, and abnormal embryogenesis (168, 216–220). The degree to which ovarian cell lipotoxicity contributes to impaired oocyte developmental competence in women with PCOS remains to be determined.

Ovarian Aging

Anovulatory women with PCOS can resume ovulation as serum AMH and androgen levels diminish with age (221–225). Despite an age-related decline in AMH, serum AMH levels remain twofold to threefold higher in women with PCOS compared with normal women in the fourth decade of life, predicting an estimated delay in menopause of about 2 years (134, 226, 227). Whether delayed ovarian aging in PCOS is biologically relevant is unclear, although PCOS patients undergoing ovarian stimulation for IVF between the ages of 22 and 41 years do not exhibit the reduced number of oocytes retrieved and live-birth rates typical of IVF patients of similar age with tubal factor infertility (228). Whether prolonged survival of PCOS follicles in vivo, despite increased primordial follicle recruitment, accounts for a larger follicle pool throughout reproductive life remains to be established (68).

ENDOCRINE ANTECEDENTS TO PCOS

Some evidence indicates that PCOS has been evolutionarily conserved and may have a genetic basis (4). In addition, infant girls born to mothers with PCOS exhibit overproduction of AMH, which persists into prepubertal life (229) and is variably improved, along with exaggerated E_2 responsiveness to leuprolide administration when their mothers received metformin during pregnancy, particularly beginning at conception (230, 231). Increased ovarian volume and hyperinsulinemia also occur in the prepubertal daughters of mothers with PCOS, accompanied by hyperandrogenism later in puberty (232, 233).

These data also suggest that epigenetic changes in fetal life may impact the developmental origins of PCOS, assuming a critical time interval of fetal susceptibility beginning in midgestation when developmental programming occurs (91, 234). In humans, monkeys, and sheep, species characterized by ovarian follicular differentiation by birth, experimentally induced prenatal testosterone excess programs permanent PCOS-like phenotypes, including multifollicular ovaries (92, 234–237). This agrees with an increased prevalence of PCOS in women with classic congenital adrenal hyperplasia and congenital adrenal virilizing tumors (2, 238, 239). Such prenatal testosterone treatment in rhesus monkeys and sheep induces maternal glucose intolerance, causing transient hyperinsulinemia and decreased activin A availability in their respective female fetuses, which could affect ovarian steroidogenesis, follicular proliferation, and germ cell survival in utero (240–243). Because maternal androgen in normal pregnancy does not usually program PCOS in offspring due to placental aromatization (244), a plausible hypothesis for transgenerational programing of ovarian function is that metabolic disorders of pregnancy, including PCOS, induce androgen overproduction by the midgestational human fetal ovary, which reprograms ovarian function after birth in susceptible female offspring (4, 91).

FUTURE DIRECTIONS

Although PCOS is the leading cause of anovulatory infertility in women (1), the endocrine and molecular events that control this disease remain to be fully understood. With obesity as one of the fastest-growing medical problems worldwide,itiscrucial to understand how PCOS and obesity interact to disrupt the intrafollicular environment and oocyte development. Lean and obese PCOS patients likely represent different ends in a continuum of reproductive dysfunction that requires these two types of PCOS patients to be analyzed separately.

In lean PCOS patients, LH hypersecretion and ovarian hyperandrogenism appear to be the primary drivers of altered folliculogenesis, with insulin enhancing gonadotropin actions on ovarian steroidogenesis and granulosa cell differentiation (see Fig. 2). In obese PCOS patients, metabolic factors such as glucose-insulin homeostasis, adipogenic dysfunction, and possibly lipotoxicity may be more important than LH as primary drivers of altered folliculogenesis (see Fig. 3). Whereas loss of weight can restore ovulation and fertility in many obese PCOS patients, this is not true of the lean PCOS patients, in whom ovulation induction is necessary.

In lean and obese PCOS patients alike, we need to identify androgen target genes in all cell types of the human ovary. Is the FSH receptor gene the primary androgen target or are there more? Does testosterone, acting as an androgen or through aromatization, promote follicle survival, as it does for mouse and rhesus preantral follicles in vitro (40, 245, 246)? We also need to know the critical insulin target genes in these same ovarian cell types, using in vitro models whereby physiologic and pathophysiologic rather than pharmacologic amounts of insulin are used. Although high insulin levels might act through IGF-I receptors in follicular cells to exert some effects, too many in vitro studies have used 5–10 mg/mL of insulin, which are levels that far exceed the physiologic limit and therefore preclude meaningful interpretations.

In lean PCOS patients, we need to understand what controls the persistently elevated expression of intrafollicular AMH and whether it contributes to ovarian dysfunction, follicle survival, and impaired oocyte quality (247). Serum levels of LH and AMH are positively correlated in PCOS patients (144), agreeing with the ability of LH to stimulate AMH production by cultured PCOS granulosa cells (136). That such PCOS granulosa cells secrete AMH in response to LH implies the presence of LH receptors in these prematurely differentiated cells and further suggests that AMH overproduction may be under the tripartite control of LH, androgen, and insulin. However, AMH expression can also be regulated by BMPs (human), activin (mouse), FSH, and the FoxoBoxO transcription factors (77, 248), implicating SMADs and FOXOs as well as cAMP in regulating transcription of the AMH promoter (249). The AMH promoter contains orphan nuclear receptor (SF1 or LRH1) response elements and putative GATA and AP1 binding sites (249). Transcription factors, especially LRH1 and GATA4, are highly expressed in granulosa cells of growing follicles (75, 250, 251) and impact follicle development (252, 253), potentially interacting with FOXO1 or SMADs to regulate AMH expression. Increased phosphorylation of LRH1 is likely, given the high levels of LH and insulin that can activate the mitogen-activated protein kinase (MAPK) pathway (192). Estradiol has also been implicated in controlling the responsiveness of the AMH promoter to FSH (254). Do potential regulatory loops between activin, AMH, and FSH (or LH) exist in PCOS granulosa cells, and, if so, does it reflect higher levels of FSH receptor (induced by androgens), predict AMH or E_2 responsiveness to FSH in vitro (96, 136), or interact with other inflammatory, adipogenic, or other metabolic factors?

Equally important are the regulatory mechanisms governing bidirectional cumulus cell-oocyte signaling. From an “oocentric” point of view, the ability of oocyte-derived factors such as BMP15 and GDF9 to regulate cumulus cell proliferation, differentiation, and steroidogenesis emphasizes the role of the oocyte in determining its own developmental fate and protecting itself against its own microenvironment (197, 198, 255). Oocyte-derived factors also appear to increase AMH in primary follicles, joined by granulosa- or theca-derived factors during secondary follicle growth, and by cumulus cells of growing and preovulatory follicles. Furthermore, studies have indicated that midrange follicular fluid levels of AMH are optimal for maximal oocyte quality and IVF success (256), suggesting that oocyte-mediated, paracrine control of follicle growth may account for the heterogeneity in follicular development among the cohort of PCOS follicles undergoing gonadotropin stimulation. Conversely, a granulosa cell-cumulus cell connection is required for generating cGMP that passes through gap junctions to suppress the resumption of meiosis in oocytes, at least in antral follicles (190, 193, 195).

Finally, because experimental constraints exist on studying human oocytes, animal models and in vitro follicle cultures must continue to pioneer the earliest aspects of ovarian and oocyte physiology (20, 40, 92, 197, 198, 234, 257–261). Such models need to explore how developmentally relevant endocrine/paracrine factors and genes interact to promote optimal epigenetic and genetic expression in the oocyte for successful fertilization and preimplantation embryogenesis. They also need to define critical times during fetal development when the maternal endocrine status might permanently alter the ovarian physiology of the fetus and modify ovarian function after birth. With such information, new

clinical strategies targeting long-term correction of follicle development in PCOS could improve fertility, optimize follicular responsiveness to ovulation induction, and enhance pregnancy outcomes by IVF, while decreasing the risk of multiple gestation and its adverse consequences on maternal-fetal health.

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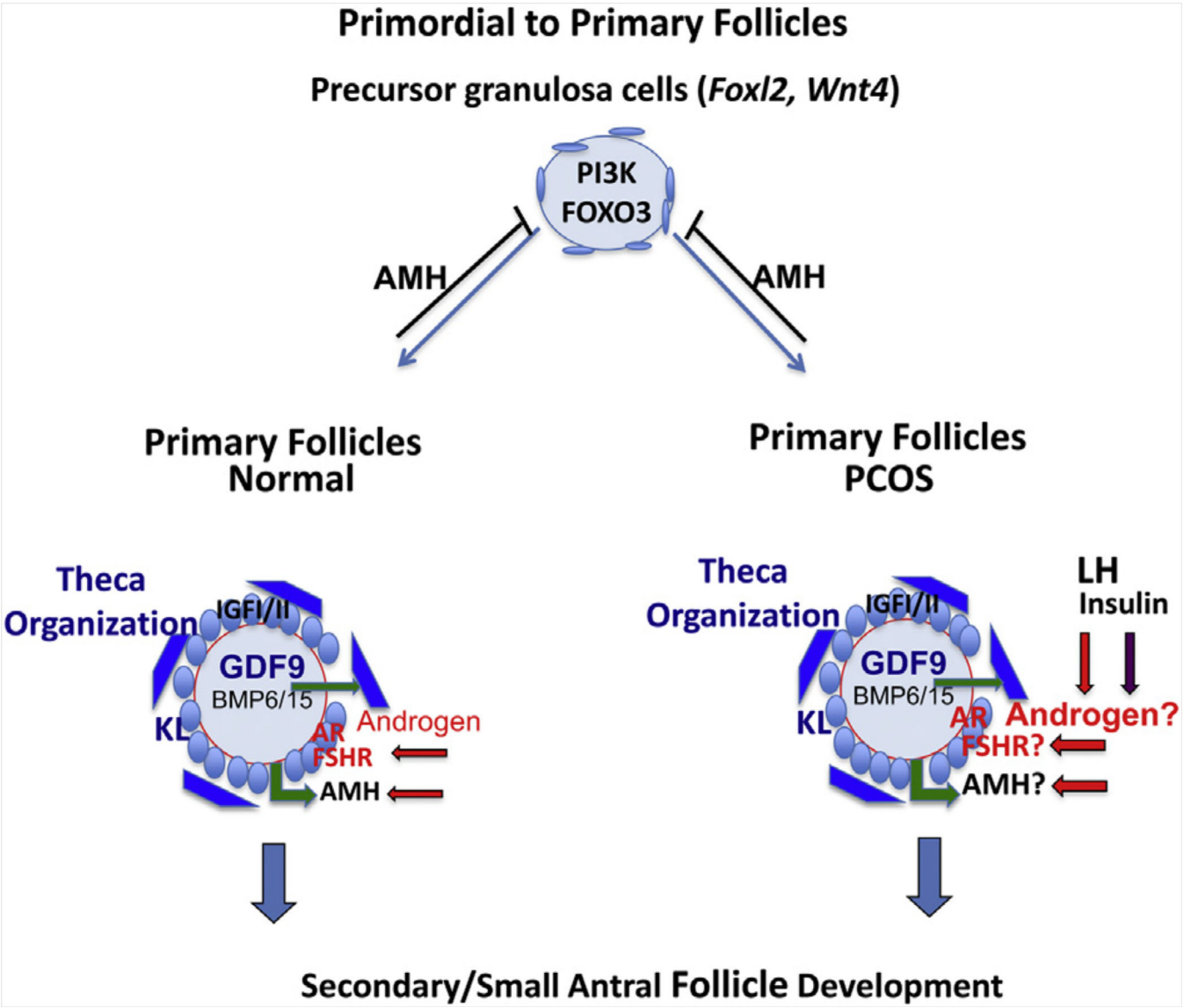

FIGURE 1.

Transition of primordial to primary follicles. Embryonic specification of the female gonad and formation of primordial follicles depends on WNT4 and FOXL2. Quiescent primordial follicles leave the resting pool by mechanisms that involve changes in both oocyte (activation of the PI3K pathway) and somatic cell (levels of AMH) functions. Primary follicle formation is independent of gonadotropins but is associated with changes in granulosa cell function and morphology leading to expression of IGF-I/-II, FSHR, AMH, AR, and KL. Changes in oocyte functions lead to the production of the zona pellucida and expression of GDF9. KL and GDF9 coordinate to regulate formation of the theca cell layer. GDF9 also appears to enhance theca cell androgen production, which in turn can increase the expression of FSHR. In PCOS, high levels of insulin and LH may act on cognate receptors in theca cells, once organized, to further increase androgen production. Levels of FSHR (and AMH?) are elevated; however, some data indicate that AMH is lower (262). *Large gray circle*: oocyte; *red line*: zona pellucida; *small blue circles*: granulosa cells; *dark*

blue boxes: theca cells. Oocyte factor and GDF9 signaling is denoted by *green arrows*; LH and androgen signaling by *red arrows* and letters; and insulin signaling by *purple arrows. Blue arrows* indicate that these primary follicles go on to develop into secondary follicles.

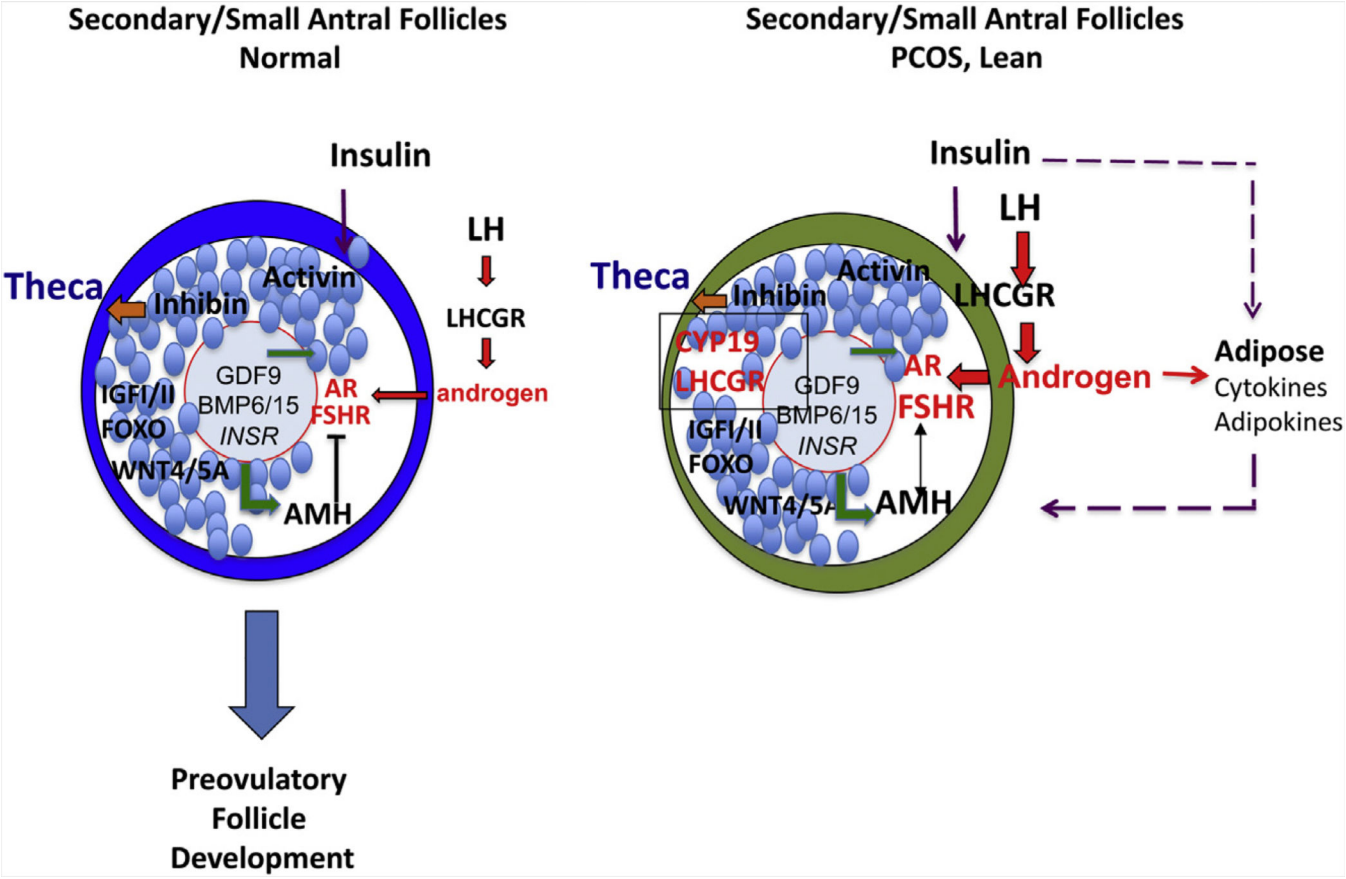

FIGURE 2.

Transition from primary to secondary/small antral follicles. Granulosa cells proliferate and follicles grow in response to multiple factors including activin, IGF-I, WNT4/5a, and FSH. The transcription factor FOXO1 impacts both follicle growth and follicle atresia (see the text for details). Consequently, as the distance between the oocyte and the theca layer is increased, theca cell production of androgens becomes more dependent on LH and possibly inhibin-α. Levels of AMH increase and negatively regulate FSH action in granulosa cells to prevent premature maturation. In lean PCOS patients, elevated LH and insulin levels increase theca cell androgen production, which enhances FSHR expression and premature differentiation of granulosa cells via induction of aromatase (CYP19a1), LH receptor (LHCGR), and other genes including *RUNX2* (102). WNT4/5a may contribute to the enhanced actions of FSH to induce CYP19a1. Enhanced FSH actions override those of elevated AMH. *Large gray circle*: oocyte; *red line*: zona pellucida; *small blue circles*: granulosa cells; *large blue circle*: normal theca cells; *green circle*: PCOS theca. Oocyte factor and GDF9 signaling is denoted by *green arrows*; inhibin by the *orange arrow*; LH and androgen signaling by *red arrows* and letters; and insulin signaling by *purple arrows* and letters. *Large blue arrow* indicates continued normal follicular growth.

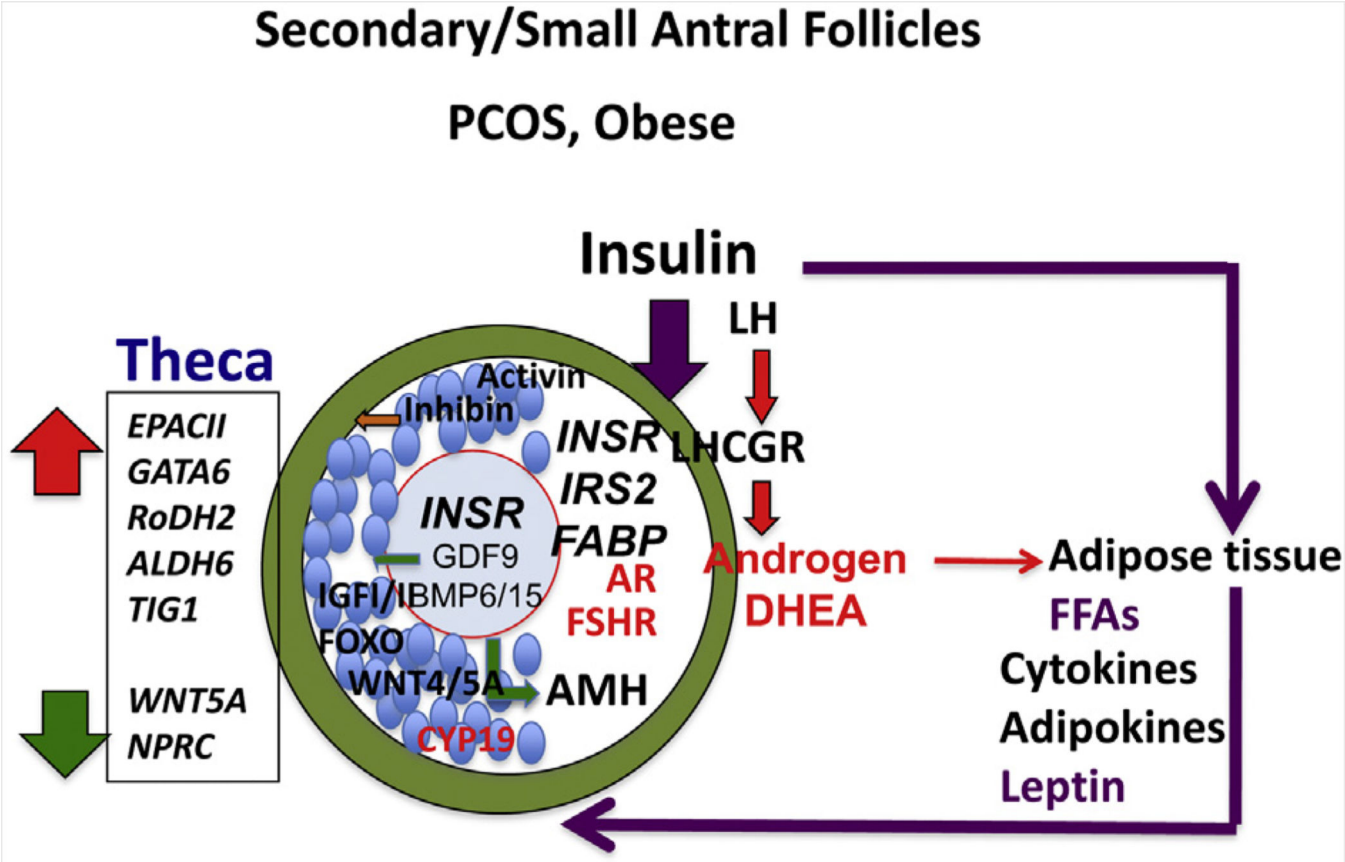

FIGURE 3.

Transition from primary to secondary/small antral follicles. In obese PCOS patients, hyperinsulinemia from adiposity-dependent insulin resistance and altered adipogenesis impair follicular development by mechanisms that appear to differ from those in lean PCOS patients. Insulin may have a greater influence than LH on theca, granulosa, and likely oocyte functions. Adipokines also likely alter each cell type within the follicle. Note that insulin receptor (*INSR*) expression may be elevated in oocytes of PCOS patients compared to non-PCOS patients (205). Cumulus cells of obese PCOS patients also express higher levels of INSR and specific fatty acid binding proteins (FABPs) (102). Theca cells produce elevated levels of androgens, especially DHEA, in response to LH and elevated expression of *CYP17A1* and *CYP11A1*, which are targets of GATA6 and retinoic acid (RA) (47). The PCOS theca cells exhibit increased expression of enzymes controlling RA production and enhanced responses to RA, including steroidogenesis and the target gene *TIG1*. These responses may be related to cAMP signaling through exchange protein activated by cAMP II (EPAC II). Conversely, PCOS theca cells exhibit reduced expression of WNT signaling and cGMP signaling pathways, but the functional significance of this remains to be determined. Oocyte factor and GDF9 signaling is denoted by *green arrows*; inhibin by the *orange arrow*; LH and androgen signaling by *red arrows* and letters; and insulin signaling by *purple arrows* and letters.

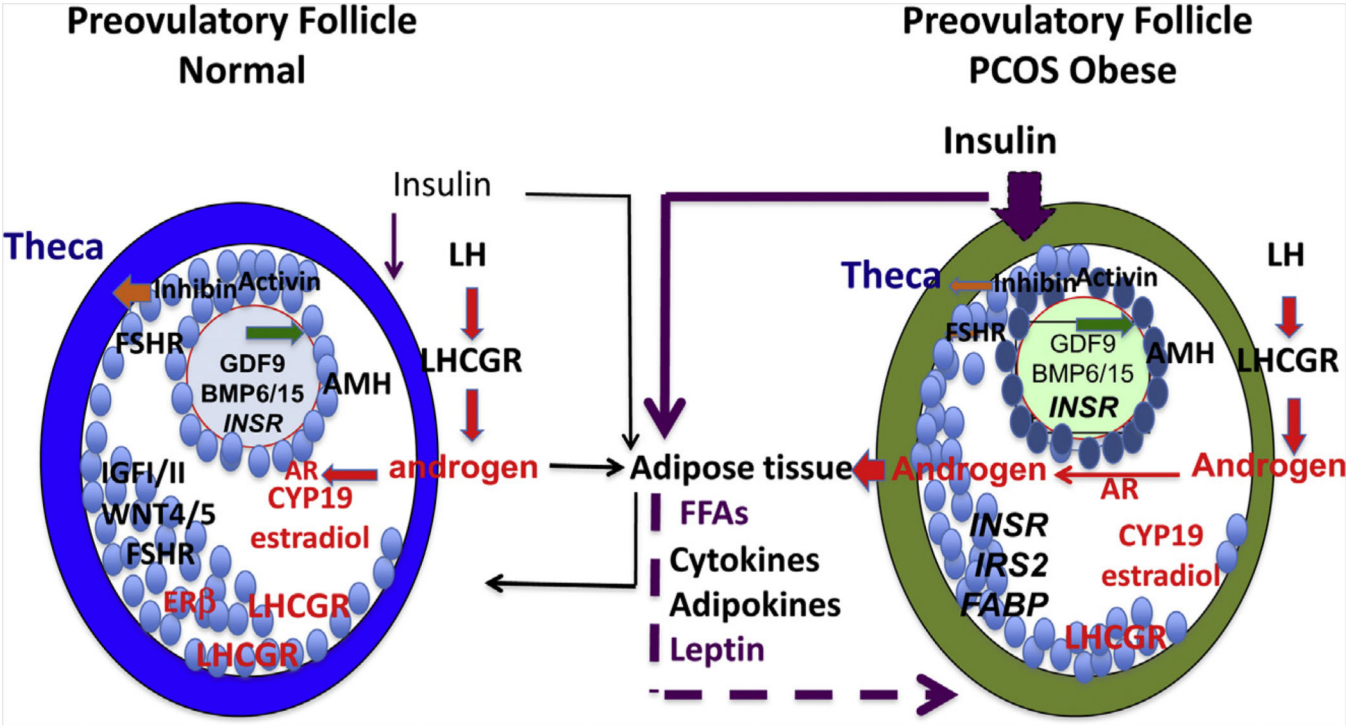

FIGURE 4.

Preovulatory follicles. The hallmarks of preovulatory follicles are the induction of LH receptors (LHCGR) and elevated expression of aromatase (CYP19A1). In obese individuals with PCOS, preovulatory follicles that are selected and ovulate (either in response to weight reduction, exogenous hormone, and/or metformin) express high AMH and remain hyperandrogenic. Thus, despite lowered insulin action or improved adipogenic function to facilitate the growth of follicles that can be ovulated, theca androgen production is central to the PCOS condition. However, direct effects of insulin and/or androgens may harm oocytes at this late stage or earlier stages of follicular development. Oocyte factor and GDF9 signaling is denoted by *green arrows*; LH and androgen signaling by *red arrows* and letters; and insulin signaling by *purple arrows*.