[Frontiers in Bioscience S3, 276-285, January 1, 2011]

WNT signaling and the regulation of ovarian steroidogenesis

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1. ABSTRACT

One of the major functions of the ovary is the biosynthesis of steroid hormones, which are essential for the development of secondary sexual characteristics at puberty, for subsequent ovarian function, and for the establishment and maintenance of pregnancy. Increases in our understanding of the molecular mechanisms governing the control of ovarian steroidogenesis have greatly improved our understanding of the female reproductive cycle, as well as the pathogenesis of reproductive disorders such as polycystic ovarian syndrome and premature ovarian failure. The pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) are the main endocrine regulators of ovarian steroidogenesis, and act by directly or indirectly modulating the activity of a multitude of intracellular signaling pathways. The WNT/CTNNB1 pathway, which is now believed to be a significant contributor to the regulation of ovarian steroidogenesis, could be one of the pathways modulated by gonadotropin signaling. This review will focus on the emerging role of WNT/CTNNB1 signaling in the regulation of steroidogenesis, with emphasis on potential mechanisms of interaction with FSH/LH signaling in ovarian granulosa and luteal cells.

2. INTRODUCTION

2.1. Steroidogenesis in the growing follicle

Estrogens regulate processes as diverse as folliculogenesis, sexual behavior, bone remodeling, lipid metabolism and carcinogenesis (1). In mammals, circulating estrogens are mainly produced by the granulosa cells of developing ovarian follicles (2). However, another cell type is needed for estrogen production by the ovary, namely the theca cells, which are separated from the granulosa cell compartment by a basement membrane. The classic "two-cell, two-gonadotropin" model describes the cooperation between theca and granulosa cells to ensure the production of estradiol (the most bioactive ovarian estrogenic steroid) during the follicular phase (Figure 1A) (3). The theca cells possess all of the cellular and enzymatic components required for *de novo* androgen production from circulating cholesterol. These androgens subsequently diffuse through the basement membrane and serve as substrates for the granulosa cells, which express the enzymes required to convert them to estradiol.

Theca cell steroidogenesis is mainly under the control of LH, which activates the cAMP signaling pathway via a G protein-coupled receptor (4). LH/cAMP

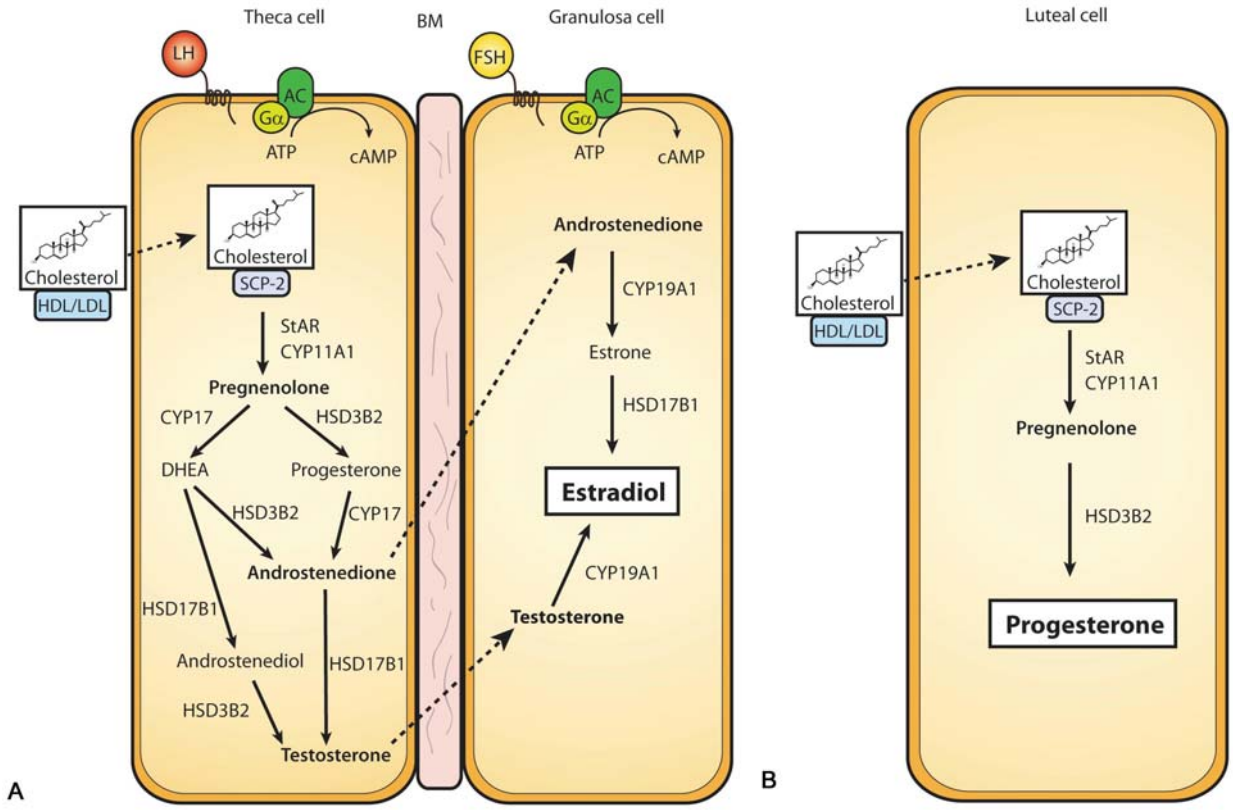

Figure 1. Enzymatic control of steroidogenesis in the growing follicle and in the corpus luteum.A) In the growing follicle, LH-stimulated theca cells convert cholesterol into androstenedione and testosterone which can diffuse through the basement membrane. These androgens are then further transformed into estradiol by FSH-stimulated granulosa cells. B) In the corpus luteum, luteal cells transform cholesterol into pregnolone and then progesterone. Dashed arrows represent molecule translocations and plain arrows indicate an enzymatic conversion. Abbreviations: AC, adenylyl cyclase; ATP, adenosine-5'-triphosphate; BM, basement membrane; cAMP, cyclic adenosine monophosphate; CYP11A1, P450 cholesterol side-chain cleavage; CYP17, cytochrome P450 17β-hydroxylase/C17–20 lyase; CYP19A1, cytochrome P450 aromatase; DHEA, dehydroepiandrosterone; Gα, alpha subunit of G protein; FSH, follicle-stimulating hormone; HDL, high-density lipoprotein; HSD17B1, 17β-hydroxysteroid dehydrogenase; HSD3B2, 3β-hydroxysteroid dehydrogenase; LDL, low-density lipoprotein; LH, luteinizing hormone; SCP-2, sterol carrier protein 2; StAR, steroidogenic acute regulatory protein.

induces the expression of the key steroidogenic enzymes cytochrome P450 cholesterol side-chain cleavage (CYP11A1), cytochrome P450 17β-hydroxylase/C17–20 lyase (CYP17), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (HSD3B2) and steroidogenic acute regulatory protein (StAR) (5). To permit androgen production, theca cells must first acquire cholesterol from circulating high- and low-density lipoproteins (6). Because of its hydrophobic nature, cholesterol must bind to carrier proteins such as SCP-2 inside the cell to reach the mitochondria (7). Once cholesterol reaches the outer mitochondrial membrane, it is translocated to the inner membrane by StAR, which possibly cooperates with peripheral-type benzodiazepine receptor (PBR) and hormone sensitive lipase (8-10). CYP11A1, which is located in the inner leaflet of the mitochondria, performs the first step of steroidogenesis by converting cholesterol to pregnenolone. This steroid is then converted mainly to androstenedione in most species via the successive actions of CYP17 and HSD3B2, along with lesser quantities of other androgens as illustrated in Figure 1A. The growing follicle can also produce modest quantities of progesterone via conversion of pregnenolone by HSD3B2.

Whereas theca cell steroidogenesis is under the control of LH, granulosa cell estrogen production is regulated by FSH. As for LH signaling, FSH binding leads to the activation of the cAMP pathway, which in turn stimulates the expression of cytochrome P450 aromatase (CYP19A1) and 17β-hydroxysteroid dehydrogenase (HSD17B1) (11). CYP19A1 catalyzes the irreversible transformation of thecal androstenedione into estrone, and HSD17B1 converts estrone to the more active estradiol (12).

2.2. Steroidogenesis in the corpus luteum

The secretion of progesterone is essential for ovulation, and subsequently for embryonic implantation and the maintenance of pregnancy. The chain of events leading to progesterone secretion begins with the

preovulatory LH surge. The latter stimulates the granulosa and theca cells to undergo a terminal differentiation process called luteinization which is characterized by growth arrest, tissue remodeling, angiogenesis and changes in gene expression that will lead to the formation and function of the corpus luteum (CL) (13). Luteal cells express a specific set of steroidogenic enzymes that is distinct from those of their granulosa and theca cell progenitors (Figure 1B). CYP11A1, as in theca cells of growing follicles, serves to convert cholesterol to pregnenolone. However, in luteal cells, downregulation of CYP17 allows pregnenolone to be converted mainly to progesterone by 3β-HSD instead of being converted to DHEA (14). In the event of pregnancy, the integrity and function of the CL is maintained by luteotrophic or antiluteolytic signals, or otherwise it regresses.

2.3. WNT/CTNNB1 signaling

The WNTs are a large family of secreted glycoproteins involved in processes including embryogenesis and cancer development (15, 16). WNTs bind to the Frizzled (FZD) family of seven transmembrane-spanning cell surface receptors, and to the lipoprotein-related receptor proteins LRP5 and LRP6 which serve as co-receptors. The resulting biological signal can be transduced via at least three different pathways, referred to as the canonical (WNT/CTNNB1), planar cell polarity (c-jun kinase) and WNT/Ca^{2+} pathways (17-19). Signaling via the canonical pathway results in the inactivation of a multiprotein complex that includes AXIN, glycogen synthase kinase 3β (GSK3β) and adenomatosis polyposis coli (APC). The latter complex normally sequesters and phosphorylates the multifunctional signaling effector CTNNB1 (β-catenin), resulting in its degradation by the proteasome (20). Hypophosphorylated CTNNB1 therefore accumulates in response to a canonical WNT signal, and can then translocate to the nucleus where it can bind to transcription factors such as T-cell factor (TCF) and lymphoid enhancer factor (LEF) to activate target genes.

3. WNT SIGNALING IN THE OVARY

The first evidence of the importance of WNT signaling in the ovary came in a report by Vainio et al, who observed major ovarian developmental defects in *Wnt4*-null mice (21). Notably, these mice had a near-complete depletion of their oocyte reserve at birth. Subsequent studies indicated that *Wnt4* may play a role in the migration and sorting of adrenal and gonadal cells during early development and in sex-specific vasculogenesis (22, 23). Unfortunately, *Wnt4*-null mice die shortly after birth due to congenital kidney defects, precluding analyses of *Wnt4* function in the postnatal ovary. More recently, two independent studies demonstrated that the targeted deletion of the WNT signaling effector *Rspo1* results in an ovarian phenotype very similar to that observed in *Wnt4*-null mice (24, 25). *Rspo1*-null animals survive until adulthood, and females have morphologically abnormal ovaries and are mostly sterile (24). It remains unclear however if the latter phenotype is the result of *bona fide* follicular development defects caused by altered WNT signaling in the adult ovary, or if it is the indirect result of the severe ovarian and reproductive tract developmental defects that occur in these mice during embryogenesis.

In the postnatal ovary, several descriptive studies have reported the regulated expression of various WNTs and WNT signaling pathway components in the granulosa and/or cumulus cells of the developing follicle, as well as in the corpus luteum (26-34). A handful of functional studies have now shown that this pathway is of fundamental importance for the normal functioning of the ovary, and can affect a wide range of physiological processes. Female *Fzd4*-null mice are sterile due to abnormal development and function of the corpus luteum, including altered morphology, gene expression and vasculogenesis (35). WNT2 has been shown to positively regulate granulosa cell proliferation *in vitro* via the WNT/CTNNB1 pathway (36), although *Wnt2*-null mice appear fertile (37). Accordingly, the constitutive activation of the WNT/CTNNB1 pathway in granulosa cells via the expression of a dominant-stable mutant of CTNNB1 was shown to cause granulosa cell tumor development in a transgenic mouse model (38). Finally, a recent study defined the roles of *Wnt4* in the postnatal ovary, using a conditional targeting strategy to circumvent the perinatal lethality phenotype of *Wnt4*-null mice. Knockdown of *Wnt4* expression in granulosa cells resulted in infertility linked to decreased numbers of antral follicles. Furthermore, WNT4 was shown to regulate a number of genes involved in late follicle development and ovulation/luteinization *in vitro* and *in vivo*, including *Adamts1*, *Ptgs2*, *Vegfa* and *Cdkn1a* (39). Together, these studies indicate the involvement of WNT signaling in several phases of follicular and luteal development.

3.1. Ovarian WNT signals that regulate steroidogenesis

One of the key mechanisms by which WNT signaling may regulate ovarian function is through the regulation of steroidogenesis. The earliest evidence for this came from the *Fzd4*-null mouse model, in which decreased expression of the steroidogenesis-related genes *Lhcgr* (LH receptor) and *Cyp11a1* was observed in the CL (35) (Figure 2B), although it was unclear if this indicated a direct regulation of these genes by the WNT pathway, or an indirect consequence of defective CL formation and/or premature regression. The first direct evidence of the influence of WNT signaling on ovarian steroidogenesis came in a study by Parakh et al, which demonstrated that CTNNB1 is required for FSH-induced *Cyp19a1* expression through a functional interaction with the transcriptional factor NR5A1 (Steroidogenic Factor-1) (40) (Figure 2A). This finding was confirmed in a subsequent study using CTNNB1 gene knockdown experiments in primary granulosa cell cultures (41). Another recent report showed that LH causes the phosphorylation and inactivation of GSK3β in bovine luteal cells, and that this effect is cAMP-dependant (42). Inactivation of GSK3β resulted in the stabilization of CTNNB1, its increased association with the StAR promoter, and resultant increases in StAR expression and progesterone synthesis (Figure 2B).

While these data indicate that the canonical WNT signaling pathway may contribute to regulating ovarian steroidogenesis, the only actual WNT protein so far reported to modulate steroidogenesis is WNT4. Indeed, granulosa cells that overexpress WNT4 were shown to

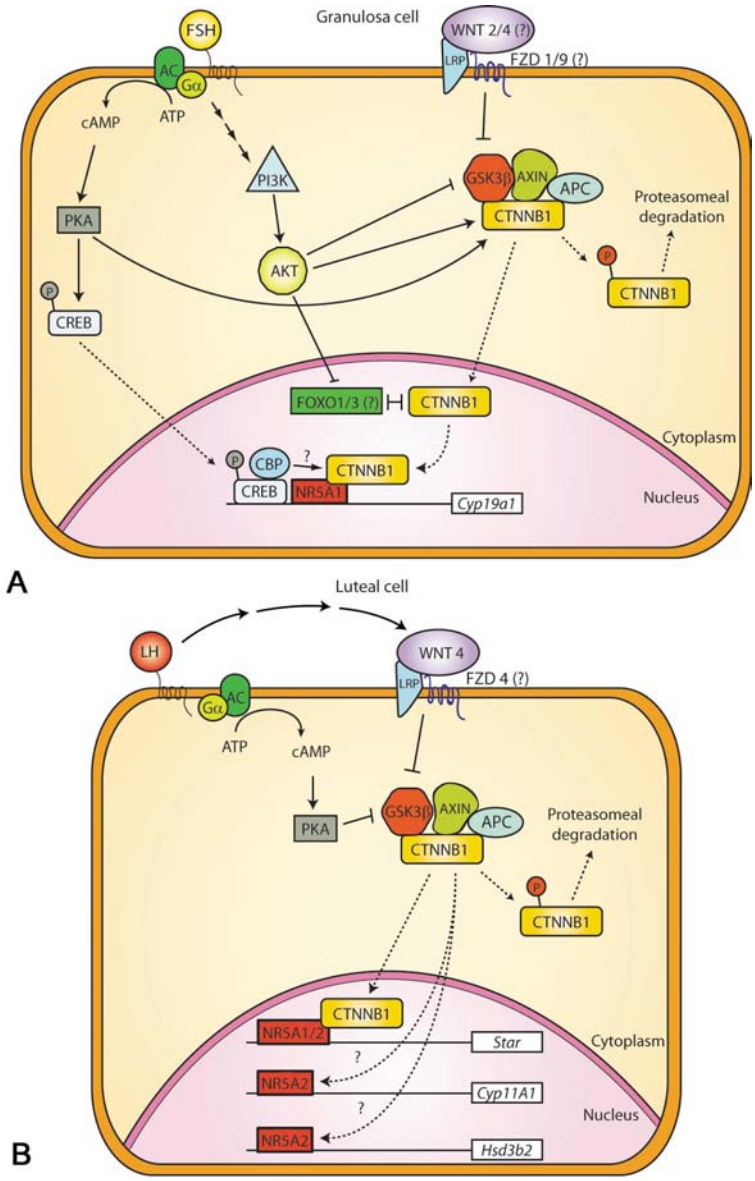

Figure 2. Potential mechanisms of interaction between gonadotropin and WNT/CTNNB1 signaling. A) In granulosa cells. B) In luteal cells. Dashed arrows represent subcellular translocations, plain arrowheads indicate positive regulation, blunted arrowheads indicate negative regulation. Abbreviations: AC, adenylyl cyclase; APC, adenomatosis polyposis coli; ATP, adenosine-5'-triphosphate; cAMP, cyclic adenosine monophosphate; CPB, CREB binding protein; CREB, cAMP-response element-binding protein; *Cyp11a1*, P450 cholesterol side-chain cleavage; *Cyp19a1*, cytochrome P450 aromatase; Gα, alpha subunit of G protein; GSK3β, Glycogen synthase kinase 3 beta; FOXO1/3, Forkhead box O1/O3, FSH, follicle-stimulating hormone; *Hsd3b2*, 3β-hydroxysteroid dehydrogenase; Fzd, frizzled receptor; LH, luteinizing hormone; LRP, lipoprotein receptor-related proteins; NR5A1/2, nuclear receptor 5A1/2; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; *Star*, steroidogenic acute regulatory protein.

overexpress *StAR*, *Cyp11a1* and *Cyp19a1*, whereas these same genes were downregulated in eCG/hCG-treated immature *Wnt4* conditional-null mice relative to controls, resulting in reduced serum progesterone levels (39). This same study also suggested that WNT4 signals via CTNNB1 due to substantial overlap between the downstream target genes of each protein (as determined by microarray analysis), although this remains to be rigorously demonstrated.

4. OVARIAN WNT SIGNALING MECHANISMS; INTERACTIONS WITH GONADOTROPIN SIGNALING?

As detailed above, recent studies are starting to demonstrate that gonadotropin and WNT signaling could act in concert to modulate ovarian steroidogenesis. Indeed, many signaling components implicated in steroidogenic gene regulation belong to common pathways (Figure 2).

In this section, we will integrate current knowledge of gonadotropin and WNT signaling cascades to identify potential mechanisms of synergy and crosstalk between these pathways.

4.1. PKA/CREB

Agonist binding to the G-coupled FSH receptor triggers the activation of multiple signaling pathways, including the cAMP/PKA cascade. The latter leads to the phosphorylation and activation of the cAMP-response element-binding protein (CREB). Studies have shown that the expression of *Cyp19a1* is directed by the ovary-specific promoter II, which contains a non-canonical cAMP response element-like sequence (CLS) that promotes *Cyp19a1* transcription upon CREB binding (43, 44). It has also been shown that *Cyp19a1* transcription requires a functional interaction between CREB and NR5A1, which in turn associates with CTNNB1 on the *Cyp19a1* promoter (40, 45). The acetyltransferase CREB binding protein (CBP), which is expressed in the ovary (46), can bind CREB and enhance the transactivation activity of CTNNB1 via the acetylation of a single lysine residue (47, 48), although this has not yet been shown to occur in granulosa cells or in the context of *Cyp19a1* regulation. In addition, it has also been reported that PKA is able to phosphorylate CTNNB1, and thereby increasing its transcriptional activity and promoting its binding to transcriptional co-activators (49) (Figure 2A). The cAMP/PKA pathway may therefore increase the ability of CTNNB1 to enhance *Cyp19a1* transcriptional activity via post-transcriptional modifications occurring both outside and inside the cell nucleus.

As mentioned above, LH also activates the cAMP/PKA pathway in luteal cells, causing GSK3β phosphorylation and the stabilization of CTNNB1 (42). Available evidence suggests that this phosphorylation is mediated directly by PKA (42). Whether or not any of the numerous other intracellular pathways that are activated by LH may modulate WNT/CTNNB1 signaling has not yet been determined. Likewise, whether or not PKA also acts downstream of FSH in granulosa cells to enhance WNT/CTNNB1 signaling in the same manner as LH also remains to be determined. Importantly, LH induces a rapid and marked increase in WNT4 expression in luteinizing granulosa cells, providing a mechanism of direct activation of the CTNNB1 pathway (39) (Figure 2B).

4.2. PI3/AKT

As cAMP response elements have been identified in only a small subset of FSH-regulated genes, FSH has long been thought to signal via CREB-independant mechanisms. It has been shown that FSH can activate the transcription of ovarian genes such as CYP19A1 in a cAMP/PKA-independent fashion, via activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (50-52). This pathway acts via the activation of a number of downstream effectors, including the transcription factors Forkhead box O1 (FOXO1) and FOXO3 (53). In the unphosphorylated state, these transcription factors bind to DNA to promote or repress genes involved cell cycling, metabolism or survival (54). Following activation of the PI3K/AKT pathway, AKT-mediated phosphorylation of three specific amino acids results in the nuclear export and degradation of FOXO proteins, thereby inhibiting their activity (54) (Figure 2). One means by which FSH/AKT/FOXO may regulate WNT/CTNNB1 signaling may be by sequestration of the nuclear pool of CTNNB1 by FOXO. Indeed, in oxidative stress signaling, a physical association between FOXO and CTNNB1 is thought to antagonize CTNNB1/TCF transcription by diverting the limited pool of CTNNB1 from TCF- to FOXO-mediated transcription (55, 56). In granulosa cells FSH signaling via PI3K/AKT could therefore decrease nuclear FOXO protein levels, thereby releasing CTNNB1 to interact with transcription factors including NR5A1 (Figure 2A). In agreement with this model, a recent study using adenoviral vectors to express FOXO1 gain-of-function mutants showed that constitutively active (i.e., nuclear) forms of FOXO1, with or without a functional DNA binding domain, were able to block FSH-induced *Cyp19a1* expression (57). Since FOXO interaction with DNA was not required, these results support the notion that the coupling/sequestering of CTNNB1 by FOXO1 could be responsible for the inhibition of *Cyp19a1* transcription. Further studies will thus be required to determine if FOXO proteins and CTNNB1 interact in granulosa cells, and the effect that this may have on FSH-regulated ovarian gene regulation and steroidogenesis.

A second level of crosstalk between the PI3/AKT and the WNT/CTNNB1 pathways could occur via the inhibition of GSK3β, one of the kinases responsible for CTNNB1 phosphorylation and subsequent degradation (Figure 2A). AKT phosphorylates GSK3β on Ser-9, inactivating it and thereby leading to an increase in the levels of hypophosphorylated CTNNB1 (58-60). Furthermore, a recent study showed that AKT could also phosphorylate CTNNB1 directly at Ser-552 *in vivo* and in *vitro* (Figure 2A). This AKT-mediated phosphorylation of CTNNB1 causes its disassociation from cell-cell contacts and its accumulation in the nucleus, thereby enabling the activation of target genes (61).

Whereas the aforementioned mechanisms of crosstalk between the PI3K/AKT and WNT/CTNNB1 pathways have been demonstrated in various cell types (62-67), it remains to be determined if any are pertinent to the activation of *Cyp19a1* transcription by FSH in ovarian granulosa cells. However, recent evidence suggests that the PI3K/AKT and WNT/CTNNB1 pathways can act in synergy in granulosa cells in the context of granulosa cell tumor development. A strain of transgenic mice was engineered to have granulosa cell-specific expression of a dominant-stable CTNNB1 mutant, as well as constitutive derepression of the PI3K/AKT pathway by conditional inactivation of the PI3K antagonist gene *Pten* (68). These mice developed metastatic granulosa cell tumours with higher penetrance, earlier onset and greater severity than those in mice in which only either WNT/CTNNB1 or PI3K/AKT signaling was altered. This study also showed that *Pten* loss results in loss of FOXO1 expression in granulosa cell tumors, supporting the idea that the relief of antagonism between FOXO1 and CTNNB1 could represent

a mechanism of synergy between the pathways in this context.

4.3. NR5A1 and NR5A2

CTNNB1 lacks a DNA-binding domain, and must therefore interact with transcription factors in order to modulate the transcriptional activity of WNT/CTNNB1 target genes. Although the TCF/LEF family of transcription factors are the best-characterized CTNNB1 binding partners (69), those of the greatest potential relevance to the regulation of ovarian steroidogenic genes are the orphan nuclear hormone receptors NR5A1 and NR5A2 (also known as liver receptor homologue-1, LRH1). NR5A1 and NR5A2 share a high degree of homology in their DNA binding domains, and are thought to bind the same consensus motif that is found in the promoters of a number of steroidogenesis-related genes (70). Descriptive studies in mice have shown that NR5A1 mRNA is present throughout the ovary but mostly in the theca and interstitial regions, whereas NR5A2 is restricted to estrogen-producing cells and to luteal cells (71, 72).

Conditional inactivation of *Nr5a1* in granulosa cells in mice was shown to result in sterility associated with reduced ovary size, lower numbers of growing follicles and absence of corpora lutea (73). These finding suggested that NR5A1 is required for the terminal stages of follicle differentiation and/or ovulation. Results also showed that NR5A1 is necessary to permit FSH-induced transcription of *Cyp19a1*, and that its loss could not be compensated for by NR5A2 (73, 74) (Figure 2A). Coupled with the recent finding that CTNNB1 interaction with NR5A1 is required for FSH-induced CYP19A1 expression (40), NR5A1 would thus appear to be a critical point of convergence between FSH and WNT/CTNNB1 signaling, and a key transcriptional effector of WNT/CTNNB1 regulation of granulosa cell steroidogenesis.

NR5A2 may also bind to and regulate the murine *Cyp19a1* promoter, although this remains a matter of debate (71, 75). Since total deletion of *Nr5a2* results in embryonic lethality, a first study of the role of NR5A2 in the ovary was conducted using mice with a single functional *Nr5a2* allele (76). The results showed that *Nr5a2* haploinsufficiency causes a reduction of female reproductive function due to impaired progesterone production. A more recent study used the Cre-loxP system to ablate *Nr5a2* in granulosa cells, which resulted in complete sterility due to anovulation (75). The latter study also demonstrated that the NR5A2 deficiency resulted in a significant decrease in the ovarian expression of StAR and CYP11A1 and that the *Star* and *Cyp11a1* promoters are direct targets of NR5A2, further indicating a critical role for NR5A2 in progesterone biosynthesis (Figure 2B). *In vitro* studies have also implicated NR5A2 in luteal steroidogenesis in humans, specifically in the regulation of *StAR* and *HSD3B2* (77, 78). Finally, a recent study showed that both NR5A1 and NR5A2 could regulate luteal steroidogenesis, and that both transcription factors bind to the bovine *Star* promoter during the mid-luteal phase (79) (Figure 2B). Taken together, these studies suggest that, despite its strong homology with NR5A1, NR5A2 could have a distinct ovarian function and be of particular importance for luteal steroidogenesis, at least in certain species. Significantly, even if the two proteins share a high degree of homology in their DNA binding domains, they differ in other regions that likely serve to bind distinct co-activators and co-repressors that in turn may vary during the ovarian cycle (80). Unlike NR5A1, NR5A2 has not yet been shown to associate with CTNNB1 in ovarian cells, although this has been demonstrated in another cell type (81). Further studies will therefore be required to determine if gonadotropin and WNT/CTNNB1 signaling converge on NR5A2 as they do for NR5A1.

5. CONCLUDING REMARKS

The identification of WNT signaling as a modulator of gonadotropin action adds a potential new layer of regulatory control of steroid hormone production by follicles and corpora lutea, and is one of the more intriguing recent developments in the field of ovarian physiology. However, it must be emphasized that this field is still in its infancy, and many fundamental questions remain unanswered. For instance, it is not clear if WNT proteins themselves play a significant role in regulating follicular estrogen synthesis, or which WNT(s) may be involved. Indeed, FSH may signal through CTNNB1 without need for a WNT ligand, as detailed above. Which FZD receptor(s) may bind ovarian WNT(s) has not been defined, although available evidence suggests that FZD1, 4 and 9 are strong candidates (29, 35, 36), and these could act during distinct phases of follicle development. The roles of ovarian WNT signaling antagonist molecules such as SFRP4 also remain unclear. Paradoxically, the latter is strongly expressed in the corpus luteum alongside WNT4 and FZD4 (33). As mentioned above, the potential for WNT signaling via NR5A2 will be an important avenue of investigation, as will the elucidation of how CTNNB1 may regulate NR5A1 or NR5A2 activation of (or perhaps binding to) steroidogenic promoters in specific contexts. Whereas the canonical (WNT/CTNNB1) signaling pathway seems to be active in ovarian granulosa/lutein cells, whether or not non-canonical WNT signaling occurs in the ovary has not been investigated, nor has its potential roles in steroidogenesis or other ovarian functions. Finally, WNT/CTNNB1 pathway involvement in transducing the signal from ovarian signaling molecules other than the gonadotropins has not been explored. IGF-1 in particular is well known to modulate steroidogenic gene expression and acts via the PI3K/AKT pathway (82-84), creating the potential for crosstalk with CTNNB1 signaling by the mechanisms discussed above. The answers to these and many other questions will be required to define and substantiate the roles of WNTs in the regulation of ovarian steroidogenesis.

6. ACKNOWLEDGEMENTS

The authors thank Jean Sirois (U de Montréal) for kindly proofreading the manuscript and providing helpful suggestions. EL is supported by a doctoral bursary from the Reseau Quebecois en Reproduction, DB is the holder of

the Canada Research Chair in Ovarian Molecular Biology and Functional Genomics.

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Key Words: ovary, follicle, granulosa cell, steroidogenesis, WNT, CTNNB1

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