REPRODUCTION
REVIEW

The role of WNT signaling in adult ovarian folliculogenesis

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Abstract

Wingless-type mouse mammary tumor virus integration site (WNT) signaling molecules are locally secreted glycoproteins that play important role in regulation of ovarian follicle maturation and steroid production. Components of the WNT signaling pathway have been demonstrated to impact reproductive functions, including embryonic development of the sex organs and regulation of follicle maturation controlling steroidogenesis in the postnatal ovary. Emerging evidence underscores the complexity of WNT signaling molecules in regulation of dynamic changes that occur in the ovary during the reproductive cycle. While disruption in the WNT signaling cascade has been recognized to have deleterious consequences to normal sexual development, more recent studies are beginning to highlight the importance of these molecules in adult ovarian function related to follicle development, corpus luteum formation, steroid production and fertility. Hormonal regulation of WNT genes and expression of members of the WNT signaling network, including WNT ligands, frizzled receptors, and downstream signaling components that are expressed in the postnatal ovary at distinct stages of the estrous cycle suggest a crucial role in normal ovarian function. Similarly, FSH stimulation of T-cell factor-dependent gene expression requires input from β-catenin, a lynchpin molecule in canonical WNT signaling, further indicating β-catenin participation in regulation of follicle maturation. This review will focus on the multiple functions of WNT signaling in folliculogenesis in the adult ovary.

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Introduction

The adult ovary is a dynamic organ undergoing constant changes throughout the estrous cycle as follicles progress from immature preantral follicles to more developed preovulatory follicles and eventually formation of the corpus luteum following ovulation. The multifaceted process of folliculogenesis relies on synchronized input of hormones exchanged between the hypothalamus, the pituitary, and the gonads. While the initial stages of follicle development occur largely in the absence of gonadotropin input, the transition from preantral to a preovulatory follicle occurs as a result of increased follicle-stimulating hormone (FSH) and luteinizing hormone (LH) responsiveness (Richards 1980) along with involvement of numerous other local hormones and growth factors (Findlay 1993, Monget & Bondy 2000).

The actions of the gonadotropins are also dependent on other signaling pathways and a diverse set of intraovarian factors expressed in a cell-specific manner at defined stages of follicular growth (Richards *et al*. 2002*a*). One more recently identified regulator of ovarian function is the wingless-type mouse mammary tumor integration site family (WNT) of signaling molecules. WNTs are highly conserved signaling molecules that act through β-catenin dependent and β-catenin independent pathways to regulate important processes of cellular growth and differentiation including cell proliferation, cell fate specifications, embryonic induction, and the generation of cell polarity (Cadigan & Nusse 1997, Miller *et al*. 1999, Komiya & Habas 2008). Misregulation of WNT signal transduction can lead to a variety of pathologies, including the development of carcinomas of the breast, colon, skin, and ovary (Polakis 2000, Giles *et al*. 2003, Logan & Nusse 2004, Boerboom *et al*. 2005). The foundational study establishing a requirement of WNT signaling molecules in the female ovary was performed by Vainio *et al*. (1999). This group utilized mice null for *Wnt4* to demonstrate a role for this molecule in early ovarian development and suppression of the male reproductive tract. *Wnt4* null females have sex-reversed ovaries that express genes associated with testicular development, along with a reduced number of oocytes at birth. Evaluation of *Wnt4* in the postnatal ovary using this mouse model was not possible, as the homozygous mutation results in death shortly after birth due to renal failure. Subsequent work aimed at elucidating the importance of WNT signaling in the postnatal ovary has identified multiple *Wnt*/*WNT* family member transcripts expressed at specific stages of follicle

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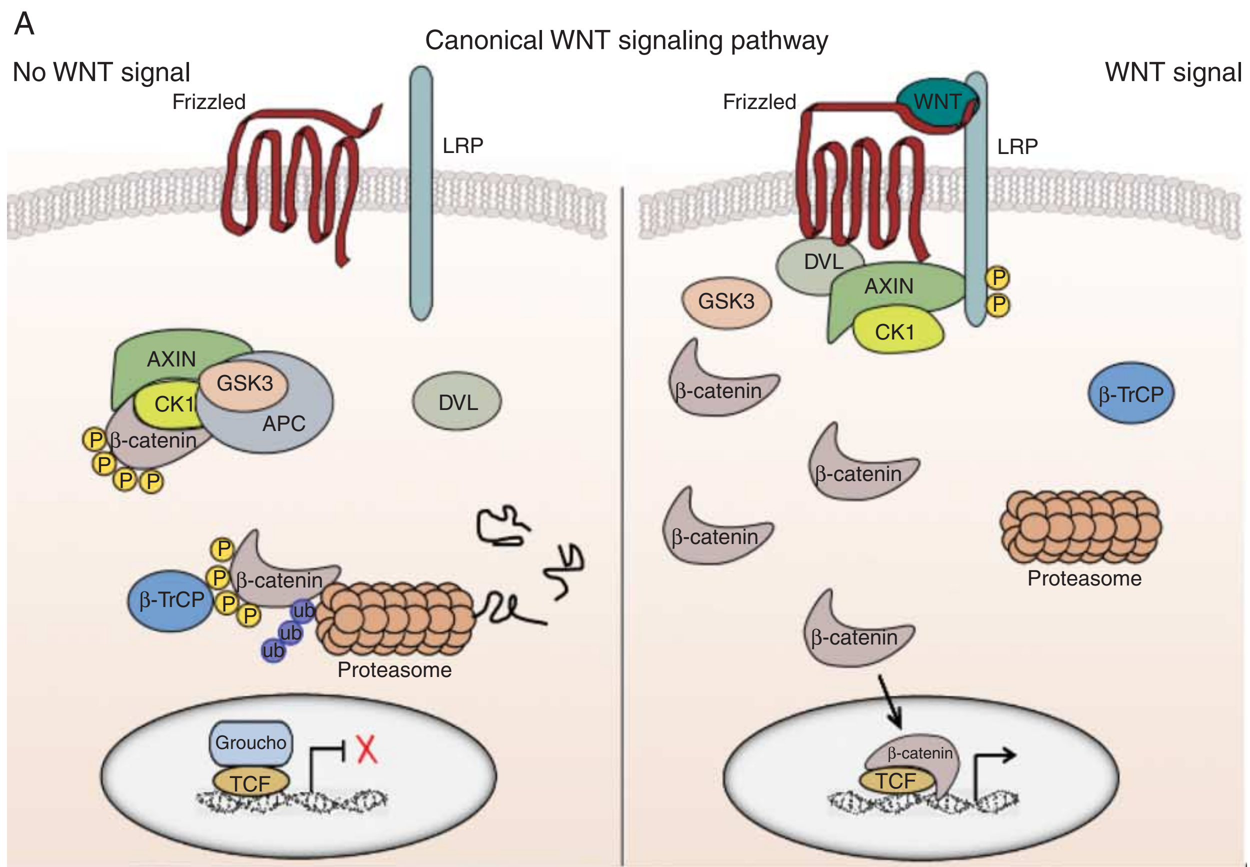

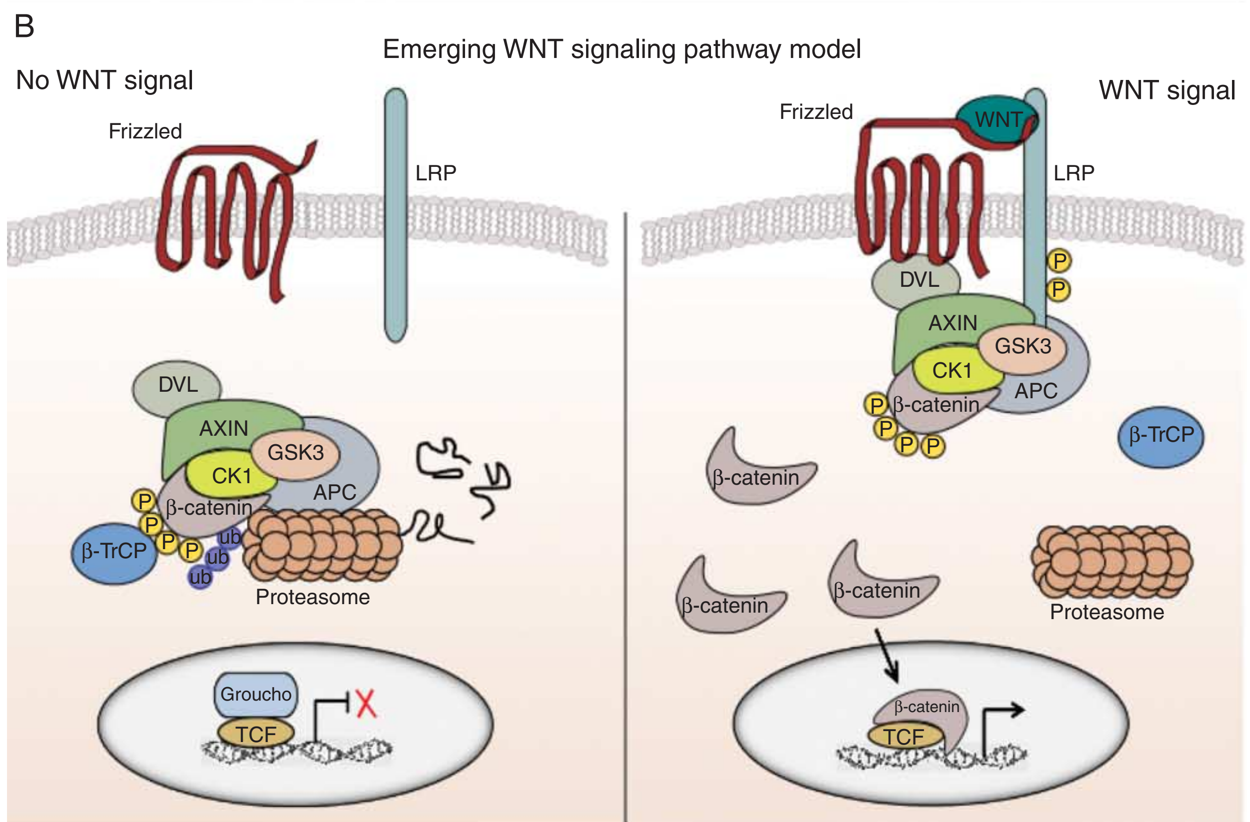

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development within the adult ovary of mice, rats, humans, and cattle (Hsieh *et al.* 2002, Ricken *et al.* 2002, Wang *et al.* 2009, Gupta *et al.* 2014). In addition, functional studies in the adult ovary have shown a fundamental requirement of WNT signaling for normal ovarian function and fertility. Though our understanding of contributions of WNT signaling to the regulation of folliculogenesis has grown tremendously in recent years, much still remains unknown about the broader physiological involvement of WNT signaling in the adult ovary. This review will focus on the role of WNT ligands, downstream signaling molecules, and their interaction with various hormones in the maturation of the ovarian follicle.

WNT signaling

The WNT signaling pathway is a conserved pathway among many species that controls numerous developmental processes as well as disease states. WNTs can initiate three separate signal transduction cascades through interaction of the ligand with their cognate frizzled (FZ) receptor. Most mammalian genomes are comprised of 19 structurally related *Wnt* genes (Logan & Nusse 2004) that encode secreted glycoproteins, which interact with a large extracellular cysteine-rich domain (CRD) on FZ seven-transmembrane receptors (Bhanot *et al.* 1996, Dann *et al.* 2001). In general, WNT proteins range in length from 350 to 400 amino acids, and are ∼40 kDa in size (Cadigan & Nusse 1997, Clevers & Nusse 2012). WNTs contain 20–85% identity among species and are defined by their nearly identical primary sequence that contains 23–24 specifically spaced cysteine residues (Cadigan & Nusse 1997, Miller 2002). While WNTs have been classified as morphogens capable of specifying cell fate in a concentration-dependent manner, in most contexts they are short-range molecules acting predominately on cells that are close to each other (Christian 2000, Sato *et al.* 2011, Strand & Micchelli 2011). The paracrine or autocrine quality of WNTs is likely reflective of the low (∼200 ng/ml) expression levels of these proteins (Willert *et al.* 2003).

The activity of WNT signaling is dependent on the cellular context and the particular combination in which the more than 15 receptors and co-receptors are expressed (reviewed in Niehrs (2012)). The ten FZ proteins are membrane-bound receptors belonging to the G-protein coupled receptor family (Slusarski *et al.* 1997, Liu *et al.* 2001, Foord *et al.* 2005, Bjarnadottir *et al.* 2006) and are thought to bind to WNT proteins promiscuously. FZ proteins contain a conserved 120-amino acid CRD that mediates the binding of WNT ligands (MacDonald & He 2012) with nanomolar affinity (K_d of 1–10 nM) (Hsieh *et al.* 1999, Rulifson *et al.* 2000, Wu & Nusse 2002). Differences in affinity of specific WNTs with different FZ may determine which signaling branch is activated (He *et al.* 1997). Transduction of a WNT signal involves an interaction between WNT and FZ as well as cooperation with single-pass co-receptors, LDL receptor-related protein 5 or 6 (LRP5/6) or receptor tyrosine kinase-like orphan receptor 1 or 2, to direct β-catenin-dependent or β-catenin-independent pathways respectively. The main WNT signaling pathways include the canonical WNT/β-catenin (β-catenin-dependent) and non-canonical (β-catenin-independent) planar cell polarity, and WNT/Ca^{2+} pathways. Upon binding of WNT to the FZ/co-receptor complex, the signal is relayed to the downstream cytoplasmic phosphoprotein dishevelled (DVL) that is pivotal in all three pathways (Boutros & Mlodzik 1999, Sheldahl *et al.* 2003).

The most extensively dissected and therefore the best understood WNT pathway is the canonical WNT signaling cascade that signals through the transcriptional co-factor, β-catenin to regulate gene expression. In addition to the WNT/FZ complex, the canonical WNT/β-catenin pathway also requires the presence of a single-span transmembrane molecule identified in vertebrates as LRP5/6 (Pinson *et al.* 2000) to relay a signal. The prevailing view regarding the mechanism regulating cytoplasmic β-catenin has been that in the absence of WNT ligand, constitutively active casein kinase 1 (CK1) and glycogen synthase kinase 3 beta (GSK3β) phosphorylate β-catenin, captured by the degradation complex, at four specific serine and

Figure 1 A new model for regulation of β-catenin in canonical WNT signaling pathway is emerging. This overview provides comparisons and contrasts between the current model and the emerging model. (A) The prevailing dogma for canonical WNT signaling denotes that in absence of a WNT signal, β-catenin is phosphorylated at N-terminal sites by the multi-protein degradation complex. Phosphorylated β-catenin is targeted for ubiquitination and subsequent degradation by the proteasome. WNT binding to the frizzled (FZ)/LRP co-receptor complex promotes association of AXIN1 to the phosphorylated tail of LRP, resulting in the disassociation of the degradation complex and the stabilization of β-catenin. Unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus where it acts as a coactivator of TCF/LEF to restore transcriptional activity of genes normally bound by repressor complexes. (B) An emerging view of canonical WNT signaling relies on an intact degradation complex to regulate β-catenin. In the absence of a WNT signal, the degradation complex binds β-catenin, and subsequent phosphorylation, ubiquitination, and proteosomal degradation occur within the AXIN1/GSK3β/APC complex. In the presence of a WNT signal, activation of the FZ/LRP co-receptors promotes association of the intact AXIN1 degradation complex with the phosphorylated tail of LRP and the disassociation of β-TrCP. The degradation complex still binds and phosphorylates β-catenin, but ubiquitination by β-TrCP fails to occur. Phosphorylated β-catenin saturates the complex, effectively inactivating the complex and allowing newly synthesized β-catenin to initiate gene transcription. Figure modified from Clevers & Nusse (2012), for details see Li *et al.* (2012).

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threonine residues (Ser33, Ser37, Thr41, and Ser45) in the N-terminal region (Liu *et al*. 2002) targeting β-catenin for ubiquitination and degradation by the proteasome (Fig. 1A) (Aberle *et al*. 1997). Interaction of WNT and FZ/LRP receptors promotes hyperphosphorylation of DVL, and inhibits the β-catenin degradation complex made up of adenomatous polyposis coli (APC), GSK3β, and the scaffold protein AXIN1, effectively blocking phosphorylation and degradation of cytoplasmic β-catenin. Activation of the receptor complex promotes recruitment of AXIN1 protein to the phosphorylated tail of LRP (Tamai *et al*. 2004). The 200 amino acid LRP5/6 cytoplasmic domain contains five PPPSPxS motifs that are conserved from invertebrates to humans (MacDonald & He 2012). WNT-mediated phosphorylation of LRP5/6 at the PPPSPxS motif occurs via GSK3β and CK1 to provide a docking site for AXIN1 (Davidson *et al*. 2005, Zeng *et al*. 2005). Association of AXIN1 with LRP was thought to facilitate disassociation of the degradation complex, resulting in stabilized β-catenin. Emerging data evaluating endogenous destruction complex components changes this view slightly (Li *et al*. 2012). In the absence of a WNT signal, the cytoplasmic degradation complex binds and phosphorylates β-catenin. Within the complex, β-TrCP subsequently ubiquitinates phosphorylated β-catenin, thereby removing it from the complex by proteosomal degradation. In this model, β-catenin phosphorylation, ubiquitination and degradation by the proteasome are all occurring within the AXIN1 degradation complex without physical disassociation of the complex (Li *et al*. 2012). This alternate model also demonstrates that in the presence of a WNT ligand, the complex remains largely intact, showing only the disassociation of β-TrCP as AXIN1 binds to phosphorylated LRP. The degradation complex continues to bind and phosphorylate β-catenin but ubiquitination and degradation do no occur without the presence of β-TrCP. Phosphorylated β-catenin within the complex saturates and inactivates the degradation complex, allowing newly synthesized, non-phosphorylated β-catenin to accumulate (Li *et al*. 2012; Fig. 1B). Interestingly, others have also suggested that only newly synthesized β-catenin is able to transduce a signal (Staal *et al*. 2002). Cytoplasmic β-catenin then translocates to the nucleus to activate transcription by displacing transcriptional repressors such as Groucho (Cavallo *et al*. 1998, Cinnamon & Paroush 2008) and associating with the T-cell factor (TCF)/lymphoid enhancer binding factor (LEF) family of transcription factors to alter target gene transcription (Molenaar *et al*. 1996, Riese *et al*. 1997, Behrens *et al*. 1998). Though the presence of many WNT signaling pathway components have been identified in the adult ovary of rodents and more recently in bovine, many questions remain regarding their mechanistic role in ovarian follicle development.

The role of WNT in follicle development

The presence and activity of WNT signaling components in the ovary is not unexpected given the variety of physiological processes known to be regulated by the WNT family of proteins. Members of the WNT family are divided into two functional groups, with the canonical WNTs (Wnt1, Wnt2, Wnt3A, and Wnt8) classified by their ability to induce secondary dorsal–ventral axis in *Xenopus* embryos and to transform mammary epithelial cell lines (Wong *et al*. 1994, Shimizu *et al*. 1997). Canonical WNT signaling is governed by the interaction of β-catenin with other molecules to regulate cellular decisions related to proliferation, differentiation, and morphogenesis (Willert & Jones 2006, Komiya & Habas 2008, Angers & Moon 2009). A series of studies have identified the expression and regulation of WNT ligands and downstream WNT signaling components in the developing follicle and corpus luteum of rats, mice, humans, and cattle (Hsieh *et al*. 2002, Ricken *et al*. 2002, Harwood *et al*. 2008, Wang *et al*. 2009, Castanon *et al*. 2012, Gupta *et al*. 2014; Table 1). However, characterization of specific WNT molecules during folliculogenesis has been focused primarily on *Wnt2*/WNT2 and *Wnt4*/WNT4 in mice, rats, and humans, although recent studies have unveiled contributions of FZ receptor agonist, WNT3A in follicular development and steroid production of mice and rats (Li *et al*. 2014, Stapp *et al*. 2014).

Wnt2 expression is detected in granulosa cells of immature rat ovaries at all stages of follicle development (Ricken *et al*. 2002) with the greatest WNT2 immunoreactivity in mouse cumulus and mural granulosa cells and in large, healthy preantral and antral follicles (Wang *et al*. 2010). Supporting a role of WNT2 during these distinct stages of follicle growth is the demonstrated increased expression of *WNT2* mRNA in response to FSH-treatment in cultured bovine granulosa cells (Castanon *et al*. 2012) and WNT2 in human cumulus cells collected after gonadotropin stimulation (Wang *et al*. 2009). Likewise, RNAi-mediated knockdown of *Wnt2* inhibits granulosa cell proliferation as indicated by reduced 5-ethynyl-2′-deoxyuridine (EdU) incorporation into DNA and a marked decrease in proliferating cell nuclear antigen (PCNA) accumulation (Wang *et al*. 2010). Overexpression of WNT2 via transduction of granulosa cells with a WNT2 encoding retrovirus conversely increased the proportion of EdU-positive cells and abundance of PCNA, events that are expected to promote cell proliferation (Wang *et al*. 2010). Additionally, WNT2/*Wnt2* overexpression increases cytoplasmic and nuclear accumulation of β-catenin in mouse granulosa cells (Wang *et al*. 2010) and in a rat granulosa cell line (DC3) that displays characteristics of early-stage follicle development (Finnson *et al*. 2012). The mechanism by which WNT2 controls β-catenin is seemingly by regulating cytoplasmic accumulation of

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Table 1 Expression of WNT ligand and FZ receptor in adult mammalian ovaries.

Gene	Descriptions of location	Species, references
Wnt1/WNT1	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Luteinized granulosa cells from healthy and endometrial afflicted ovaries	Human, Sanchez *et al.* (2014)
Wnt2/WNT2	Granulosa cells of all growing follicles collected from eCG/hCG stimulated ovaries	Rat, Ricken *et al.* (2002)
	Granulosa cells of all stages of follicles	Mouse, Wang *et al.* (2010)
	Cultured granulosa cells treated with FSH	Bovine, Castanon *et al.* (2012)
	Whole ovary following PMSG/hCG stimulation	Mouse, Hsieh *et al.* (2002)
	Cumulus cells obtained from oocytes collected for IVF	Human, Wang *et al.* (2009)
Wnt2b/WNT2B	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Ovarian surface epithelium from gonadotropin stimulated ovaries	Rat, Ricken *et al.* (2002)
	Granulosa cells from dominant follicles	Bovine, Abedini *et al.* (2015)
	Theca interna from large and small antral follicles	Bovine, Hatzirodos *et al.* (2014)
Wnt3/WNT3	Whole ovary immediately *postpartum* and on days 8–12 *postpartum*	Mouse, Harwood *et al.* (2008)
	Luteinized granulosa cells from healthy and endometrial afflicted ovaries	Human, Sanchez *et al.* (2014)
Wnt3a	Whole ovary on days 6–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Whole ovary following PMSG/hCG stimulation	Mouse, Hsieh *et al.* (2002)
Wnt4/WNT4	Granulosa cells throughout follicular development as well as luteal cells	Mouse, Hsieh *et al.* (2002) and Harwood *et al.* (2008)
	Granulosa and luteal cells from hormone stimulated ovaries	Rat, Hsieh *et al.* (2002)
	Luteinized granulosa cells from healthy and endometrial afflicted ovaries	Human, Sanchez *et al.* (2014)
	Luteal cells	Porcine, Kiewisz *et al.* (2011)
	Granulosa cells from primary, secondary, and antral follicles and theca cells from antral follicles	Human, Jaaskelainen *et al.* (2010)
	Cumulus cell–oocyte complex	Mouse, Hernandez-Gonzalez *et al.* (2006)
Wnt5a/WNT5A	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Luteinized granulosa cells from healthy and endometrial afflicted ovaries	Human, Sanchez *et al.* (2014)
	Granulosa cells from dominant follicles	Bovine, Abedini *et al.* (2015)
	Luteal cells	Porcine, Kiewisz *et al.* (2011)
	Theca cells from normal and PCOS ovaries	Human, Wood *et al.* (2003)
	Whole ovary following PMSG/hCG stimulation	Mouse, Hsieh *et al.* (2002)
Wnt5b/WNT5B	Whole ovary on days 6–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Granulosa cells from dominant follicles	Bovine, Abedini *et al.* (2015)
Wnt6	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
Wnt7a/WNT7A	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Luteal cells	Porcine, Kiewisz *et al.* (2011)
	Whole ovary following PMSG/hCG stimulation	Mouse, Hsieh *et al.* (2002)
Wnt7b	Whole ovary on days 6–12 *postpartum*	Mouse, Harwood *et al.* (2008)
Wnt8	Whole ovary following PMSG/hCG stimulation	Mouse, Hsieh *et al.* (2002)
WNT8B	Granulosa cells from dominant follicles	Bovine, Abedini *et al.* (2015)
Wnt9b	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
Wnt10a		
Wnt10b		
Wnt11/WNT11	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Granulosa cells from dominant follicles	Bovine, Abedini *et al.* (2015)
	Whole ovaries following PMSG/hCG stimulation	Mouse, Hsieh *et al.* (2002)
Wnt16/WNT16	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Granulosa cells from dominant follicles	Bovine, Abedini *et al.* (2015)
Fzd1	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Cumulus cell–oocyte complex	Mouse, Hernandez-Gonzalez *et al.* (2006)
	Granulosa cells of pre-ovulatory follicles from ovaries following PMSG/hCG	Mouse, Hsieh *et al.* (2002)
Fzd2	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Cumulus cell–oocyte complex	Mouse, Hernandez-Gonzalez *et al.* (2006)
	Whole ovary following PMSG/hCG stimulation	
Fzd3	Whole ovary following PMSG/hCG stimulation	Mouse, Hsieh *et al.* (2002)
Fzd4	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	PMSG/hCG stimulated, pregnant and *postpartum* ovaries as well as CL	Mouse and rat, Hsieh *et al.* (2002)
Fzd5	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
Fzd6/FZD6	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
	Granulosa cells from follicles at the emergence, predeviation, onset of deviation, and early dominance stage	Bovine, Gupta *et al.* (2014)
	Whole ovary following PMSG/hCG stimulation	Mouse, Hsieh *et al.* (2002)
Fzd7	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
Fzd8		
Fzd9	Whole ovary on days 0–21 *postpartum*	Mouse, Harwood *et al.* (2008)
Fzd10		

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GSK3β as WNT2 knockdown granulosa cells have increased cytoplasmic GSK3β that results in reduced β-catenin. Moreover, siRNA knockdown of β-catenin reduced granulosa cell expression of PCNA and prevents WNT2 overexpression to enhance DNA synthesis of mouse granulosa cells (Wang *et al*. 2010). These data indicate that regulation of granulosa cell proliferation relies on intact WNT2/β-catenin signaling.

Additional recent data also indicate that in mouse granulosa cells WNT2 can regulate gap junction signaling pathways important for ovarian folliculogenesis (Wang *et al*. 2013). In WNT2 siRNA treated mouse granulosa cells, connexin 43, a gap junction protein required for follicular development beyond the early preantral stages, and gap junctional intercellular communication between cells was reduced (Wang *et al*. 2013). While WNT2 appears to be important for follicle maturation and granulosa cell proliferation, female mice null for *Wnt2* are reported to be fertile (Monkley *et al*. 1996), suggesting compensatory activity of other molecules, possibly other WNTs. Though defects in placental vascularization are observed in *Wnt2*-null females, no data specifically related to ovarian function have been reported (Monkley *et al*. 1996). Together these data suggest that *Wnt2* expression is regulated by FSH and contributes to preantral to antral maturation of the follicle through granulosa cell proliferation mediated by β-catenin.

Wnt4 expression is found in rat and murine granulosa cells throughout follicle development (Hsieh *et al*. 2002) and in mouse cumulus–oocyte complexes (Hernandez-Gonzalez *et al*. 2006). Conversely, *WNT4* is not detected in human cumulus granulosa cells obtained from oocytes prior to IVF (Wang *et al*. 2009). In adult rodent granulosa cells *Wnt4* is elevated in response to human chorionic gonadotropin (hCG) stimulation and remains elevated in the corpora lutea (Hsieh *et al*. 2002). Likewise, estrus synchronization of gilts utilizing PGF2α/pregnant mares serum gonadotropin (PMSG)/hCG increased expression of *WNT4* in luteal tissue compared to control females (Kiewisz *et al*. 2011). Targeted deletion of *Wnt4* in mouse granulosa cells resulted in subfertile females with smaller ovaries and fewer healthy antral follicles at 42 days of age compared with control mice (Boyer *et al*. 2010). These results suggest that WNT4 originating from the granulosa cells is necessary for follicle maturation. Adenoviral overexpression of WNT4 in cultured granulosa cells from equine CG (eCG)-treated mice results in increased expression of ovarian β-catenin target genes, *Cyp11a1*, *Cyp19a1*, and *StAR* (Boyer *et al*. 2010). Furthermore, WNT4 was shown to regulate the expression of steroidogenic genes *in vivo* as granulosa cells isolated from *Wnt4*-null mice treated for 48 h with eCG, followed by an ovulatory dose of hCG had lower expression of *Cyp11a1*, *Cyp19a1*, and *StAR*, compared to controls (Boyer *et al*. 2010). Similarly, eCG-treated *Wnt4*-null mice had lower serum progesterone at 0, 12, and 24 h after hCG compared to controls. Further evidence of WNT4 signaling via β-catenin is found in the fetal mouse ovary where constitutively active β-catenin is able to prevent germ cell loss in *Wnt4* KO ovaries (Liu *et al*. 2010). Data suggests that β-catenin can mediate the events of WNT4 that are important in regulation of antral follicle maturation and steroidogenesis.

Similar to WNT ligands, FZ receptors have been shown to be expressed at specific stages during ovarian follicular maturation, ovulation, and luteinization (Table 1). A number of FZ receptors have been detected in granulosa cells; however, little is known about the physiological relevance of FZ in adult folliculogenesis. In the mouse ovary, *Fz1* expression is selectively and transiently induced in large ovulatory follicles by an ovulatory dose of hCG (Hsieh *et al*. 2002). Evaluation of *Fz1* expression in progesterone receptor (PR) knockout mice, which fail to ovulate when hormonally stimulated, show an altered expression of *Fz1* compared with PR heterozygotes. In this model, the initial increase of *Fz1* expression is comparable in ovaries of PR knockout and PR heterozygotes, however, by 12 h after LH-stimulation (a time point just prior to ovulation), the expression of *Fz1* was reduced in PR knockout ovaries compared to PR heterozygotes (Hsieh *et al*. 2002). While these data indicate that LH-mediated induction of *Fz1* appears to depend on PR, *Fz1*-deficient mice are fertile (Yu *et al*. 2010) with only marginal differences in litter size reported (Lapointe *et al*. 2012). Therefore, *Fz1* does not appear to be necessary in processes related to rupture. In contrast to *Fz1*, *Fz4* displays distinct expression in the adult rodent corpus luteum of gonadotropin-treated and pregnant mice and is required for fertility. Mice lacking *Fz4* receptor demonstrate follicle development that is responsive to hormone stimulation, and results in the expected genes expression profiles involved in early follicle development (Hsieh *et al*. 2005). Furthermore, adult female *Fz4*-null mice exhibit normal ovulation and ability to produce fertilized oocytes but are sterile as a consequence of failure of embryo implantation. This inability to establish a successful implantation is due to the impaired formation of the corpora lutea and the associated reduction of luteal-specific gene expression and progesterone production (Hsieh *et al*. 2005).

Of note, *Lrp4*, a member of the LDL receptor family implicated in a number of diverse biological functions has been detected in follicular cells of the adult mouse ovary (Yamaguchi *et al*. 2006). While the ligand for LRP4 remains unknown, it is closely related to the WNT co-receptors LRP5/6 (Zong *et al*. 2012). Expression of *Lrp4* specific to the migratory primordial germ cells and adult gonad but not in embryo or germ cell-derived stem cells suggest *Lrp4* may be a marker distinguishing germ cells from embryo-derived pluripotent stem cells (Yamaguchi *et al*. 2006).

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Gonadotropin regulation of WNT gene expression

There is also evidence that select *Wnt* family gene expression is hormonally regulated in rodent ovaries. For example, *Wnt4* expression is elevated in rat granulosa cells following hCG stimulation, and high expression of *Wnt4* is detected in terminally differentiated luteal cells (Hsieh *et al.* 2002). Additionally, genetically modified mice that hypersecrete LH (Tg(Cga-LHB/CGB)94Jhn/J) also develop granulosa cell tumors that display alterations in members of the WNT signaling pathway (Owens *et al.* 2002). Specifically, *Wnt4* and secreted frizzled related protein 4 (*SFRP4*), a proposed inhibitor of the WNT pathway, are dramatically decreased in granulosa cell tumors, while a WNT receptor, *Fz10*, was increased in these same granulosa cell tumors. However, it was the work of Parakh *et al.* (2006) that provided the first direct indication that β-catenin was required for FSH/cAMP-induction of *Cyp19a1* expression in a human granulosa tumor cell line (KGN), and in primary cultures of rat granulosa cells. This increased expression of *Cyp19a1* in response to FSH was determined in KGN cells to be mediated by functional interactions of β-catenin with steroidogenic factor 1 (NR5A1). In subsequent studies, conditional deletion of β-catenin in primary cultures of mouse granulosa cells similarly resulted in a compromised ability of FSH to stimulate *Cyp19a1* expression as well as consequent estradiol (E_2) production, reinforcing a role for β-catenin in steroid production from the ovary (Hernandez Gifford *et al.* 2009). A requirement for β-catenin in FSH regulation of steroid production has more recently been identified in granulosa cells of large bovine antral follicles, as high estrogen-producing follicles demonstrate an increase in β-catenin protein accumulation compared to follicles with low intrafollicular E_2 concentrations (Castanon *et al.* 2012). Consistent with β-catenin's role in regulation of steroidogenesis is the demonstrated ability of FSH to directly increase β-catenin protein accumulation (Castanon *et al.* 2012, Stapp *et al.* 2014) and β-catenin/TCF dependent transcriptional activity in granulosa cells (Fan *et al.* 2010, Stapp *et al.* 2014). In addition, Law *et al.* (2013) showed that FSH via PKA stimulates phosphorylation of β-catenin on Ser552 and Ser675, leading to its activation. FSH stimulation of transcriptionally active β-catenin promotes NR5A1 and TCF-regulated gene expression, including *Lhcgr* (Law *et al.* 2013). Together these data confirm that activation of β-catenin facilitates FSH-mediated actions in ovarian follicular cells.

β-catenin's participation in the regulation of steroidogenesis has also been linked to LH-mediated production of progesterone from bovine corpora lutea. In cultured bovine luteal cells, LH stimulation of cAMP/PKA results in phosphorylation and inhibition of GSK3β allowing stabilization of β-catenin (Roy *et al.* 2009). Increased levels of transcriptionally active β-catenin interact with the proximal promoter of the *StAR* gene and successively increase *StAR* mRNA expression and progesterone synthesis. However, it appears that β-catenin alone is insufficient to modulate steroid pathways and that contributions of the gonadotropins are integral for β-catenin to maximally impact steroidogenesis in ovarian cells. Overexpression of adenoviral Δ90 β-catenin, a β-catenin mutant lacking N-terminal GSK3β phosphorylation sites involved in its targeted degradation, resulted in only modest regulation of *Cyp19a1* and *Cyp11a2* mRNA in granulosa cells (Parakh *et al.* 2006) and had no effect on progesterone concentrations in media from cultured luteal cells (Roy *et al.* 2009).

Negative feedback loops regulate WNT/β-catenin

Whereas previous studies utilizing overexpression systems indicate β-catenin participates in gonadotropin induction of steroidogenic enzyme expression and steroid output, a recent study from Stapp *et al.* (2014) revealed a previously unappreciated inhibition of steroidogenesis with concomitant stimulation of FSH and canonical WNT signaling pathways. Exposure of primary rat granulosa cells to recombinant WNT3A at a minimal effective dose of 50 ng/ml caused specific induction of canonical WNT signaling as determined by increased expression of the WNT target gene, *Axin2* and stimulation of the β-catenin/TCF promoter reporter TOPflash (Stapp *et al.* 2014). Unexpectedly, WNT3A induction of β-catenin resulted in downregulation of FSH-mediated expression of key steroidogenic enzymes (*StAR*, *Cyp11a1*, and *Cyp19a1*) and ovarian differentiation factors (*Lhcgr* and inhibin alpha). Co-incubation of FSH and WNT3A repressed FSH-induced steroidogenic enzyme expression that further translated to a reduction in E_2 and progesterone production (Stapp *et al.* 2014). In agreement with these findings, WNT pathway agonist/GSK3β inhibitor, LiCl, and WNT3A significantly decreased E_2 concentration in cultured mouse follicles, while treatment with a WNT inhibitor increased culture media concentrations of E_2 (Li *et al.* 2014).

The noted upregulation of *Axin2*, a negative regulator of WNT signaling, in response to co-stimulation of granulosa cells with WNT3A and FSH allowed for detection of a negative feedback mechanism whereby FSH regulates canonical WNTs in an effort to control TCF responsive genes. These data provide valuable insight into the physiological functions of β-catenin in the adult ovary. The notion of creating a negative feedback loop to ensure β-catenin remains controlled is consistent with the detection of WNT/β-catenin signaling antagonists WNT inhibitory factor 1 (*Wif1*), naked cuticle homolog 1 (*Nkd1*), dickkopf 4 (*Dkk4*), and *Axin2* in ovaries of mice that constitutively express β-catenin (Boerboom *et al.* 2006). Similarly,

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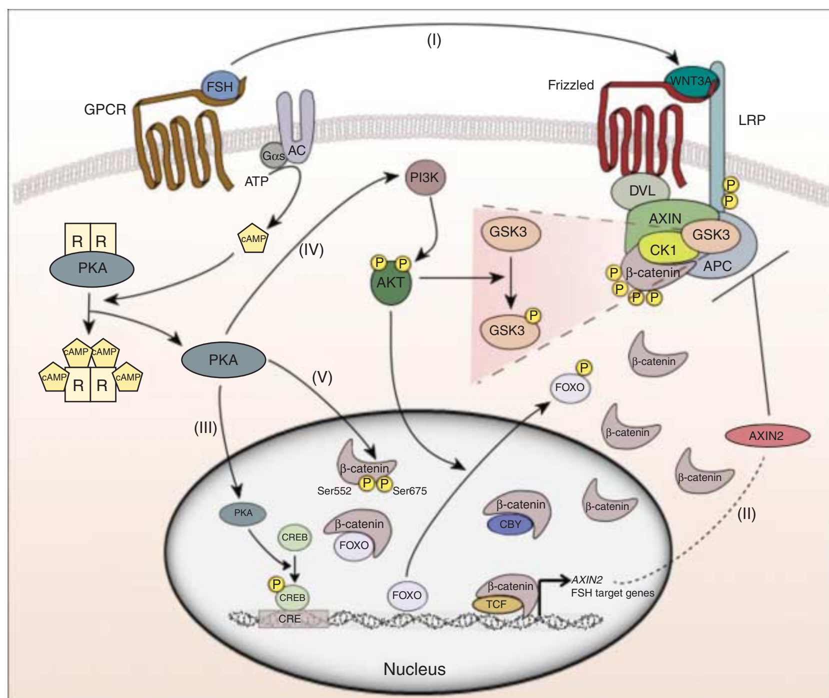

Figure 2 FSH regulation of WNT contributes to a negative feedback mechanism to regulate TCF responsive genes in the granulosa cells. (I) FSH regulates induction of several WNT ligands, any of which may contribute to negative feedback regulation. Recent data provide evidence that FSH regulates transduction of a WNT signal which in turn upregulates *Axin2* and FSH target genes via the β-catenin/TCF pathway. (II) *Axin2* induction may subsequently exert an inhibitory effect on β-catenin to effectively shut down β-catenin/TCF gene transcription. Alternative negative modulators including FOXO1/3A and Chibby can prevent β-catenin transcriptional activity by binding it in the nucleus. (III) FSH binding to the G-protein coupled FSH receptor stimulates adenylyl cyclase and promotes cAMP dependent PKA activity. This active kinase phosphorylates CREB to regulate expression of PKA target genes in granulosa cells. (IV) PKA also enhances the activity of PI3K leading to AKT phosphorylation. AKT phosphorylates FOXO leading to its export from the nucleus and releasing its inhibition on transcriptional activity of genes regulating granulosa cell proliferation and steroid production. Additionally, FOXO may bind to β-catenin in the nucleus to repress its transcriptional activity. This negative regulation could work to ensure that β-catenin remains controlled and its target genes are not overexpressed. (V) In addition, PKA regulates stability and activity of β-catenin by phosphorylation on Ser552 and Ser675 in granulosa cells providing a new layer of complexity to the intracellular mechanisms regulating follicle development.

overactivation of β-catenin has negative effects on LH-induced cumulus–oocyte complex expansion, ovulation, luteinization, and progesterone production (Fan *et al.* 2010). Granulosa cells from mice expressing dominant stable β-catenin have muted expression of *StAR*, *Cyp11a1*, and *Lhcgr* following forskolin and phorbol myristate acetate (PMA)-treatment that is meant to mimic the effects of LH *in vitro* (Fan *et al.* 2010).

Modulators of β-catenin suppression

Negative feedback mechanisms that limit the duration of a signaling event following initial stimulus are present in most signal transduction pathways. The data mentioned above provide evidence that FSH via β-catenin/TCF pathway upregulates FSH target genes involved in granulosa cell maturation and differentiation. WNT ligands appear to be another FSH target that may function in a feedback manner by upregulating *Axin2* mRNA expression. *Axin1* is a known negative regulator of the canonical WNT signaling pathway; however, the significance of the *Axin1* homologue *Axin2* in granulosa cells remains to be characterized. AXIN2 is thought to act as a scaffold protein to facilitate phosphorylation of β-catenin by GSK3β resulting in its consequent degradation (Jho *et al.* 2002). Induction of *Axin2*, therefore, may exert an inhibitory effect on β-catenin to effectively shut down β-catenin/TCF gene transcription (Fig. 2). Numerous FSH target genes in granulosa cells are TCF-responsive, including but not limited to *Cyp19a1*, *Inha*, *Foxo1*, and *Lhcgr* (Law *et al.* 2013).

Additional alternative scenarios for limiting a WNT signal exist including β-catenin's interaction with a nuclear molecule that could prevent it from binding transcriptional targets. One such candidate is Chibby (CBY1), a conserved nuclear associated antagonist of the WNT pathway that associates with the C-terminal domain of β-catenin and blocks its interaction with TCF/LEF transcription factors (Takemaru *et al.* 2003). The expression of *Cby1* has been detected in a variety of adult human tissues (Takemaru *et al.* 2003). In COS7 cells, the CBY1 protein is largely nuclear and its

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localization is unaffected by expression of WNT1, WNT5a, or β-catenin (Takemaru *et al.* 2003). While characterization and gonadotropin control of CBY1 in the ovary remains to be demonstrated, a recent study Finnson *et al.* (2012) identified the expression of CBY1 in a SV-40 transformed rat granulosa cell line (DC3). Overexpression of *Wnt2* in DC3 cells led to β-catenin accumulation in the nucleus but failed to stimulate β-catenin/TCF-dependent transcription, likely as a consequence of CBY1 association and suppression of endogenous β-catenin (Finnson *et al.* 2012).

Another molecule that may modulate follicular development is the Forkhead box O (FOXO) family of transcription factors that are recognized for their involvement in the regulation of apoptosis, proliferation, and cell cycle arrest (Burgering & Medema 2003). FOXOs are downstream targets of PI3K/AKT pathway, and direct phosphorylation by AKT inhibits transcriptional activation of FOXO by causing their exclusion from the nucleus into the cytoplasm and subsequent degradation. FOXO transcription factors are found in the rodent ovary and are regulated by gonadotropins. In granulosa cells, FSH enhances *Foxo1* gene expression in granulosa cells of the preovulatory follicle, and is rapidly downregulated following hCG induced ovulation (Richards *et al.* 2002*b*, Fan *et al.* 2010) a pattern consistent with FOXO1 repression of granulosa cell proliferation and steroidogenesis (Park *et al.* 2005, Liu *et al.* 2009). Likewise, FOXO1 represses *Lhcgr* expression in granulosa cells and is present on the promoter of vehicle-treated cells, but is removed from the promoter after FSH stimulation (Law *et al.* 2013). A study by Hoogeboom *et al.* (2008) proposed β-catenin to be a link between the WNT signaling and FOXO pathways, given the ability of FOXO3A to inhibit TCF-transcription by binding to β-catenin. To elucidate the role of WNT/β-catenin in regulation of early follicle development, a recent study employed an *in vitro* follicle culture system utilizing isolated secondary follicles that were cultured in the presence or absence of WNT pathway activators and inhibitors (Li *et al.* 2014). In this study, WNT pathway activators, LiCl and WNT3A were found to decrease phosphorylation of FOXO3A while the WNT inhibitor, IWR-1, increased FOXO3A phosphorylation. In addition, FOXO3A targets, *Bim*, *Puma*, and *p27* were increased by WNT3A and LiCl and decreased by WNT inhibition (Li *et al.* 2014). Furthermore, activation of WNT/β-catenin resulted in a large number of abnormal follicles, while suppression of this pathway promoted follicle growth (Li *et al.* 2014). Consistent with negative feedback results of WNT inhibiting FSH signaling responses, these data suggest that β-catenin signaling may be necessary for keeping follicle growth in check by negatively controlling early follicle development and that several different mechanisms may participate in this regulation.

Future considerations

A large body of data definitively recognizes WNT signaling as an essential factor for proper development of the female mammalian gonad (Vainio *et al.* 1999, Heikkila *et al.* 2001, Biason-Lauber & Konrad 2008, Maatouk *et al.* 2008); however, the contribution of WNT family signaling components to ovarian folliculogenesis in the adult remains to be fully elucidated. It is suspected that the divergent roles or even opposing effects of WNT signaling is likely attributed to the different stages of follicle development and hormonal milieu present during the development of the ovarian follicle. It is clear that pituitary gonadotropins regulate ovarian events during the estrous cycle through the convergence of multiple signaling pathways. One newly recognized pathway is the canonical WNT signaling pathway that regulates levels of the downstream transcriptional co-factor, β-catenin shown to impact gonadotropin-responsive target gene expression and steroid production. Identification of WNT signaling in gonadotropin-mediated events in the adult ovary highlights the role of this pathway in regulation of normal follicle maturation, ovulation and corpus luteum formation and function, but many questions in this field remain to be explored.

Functional studies in granulosa cells have evaluated the influences of only a few WNTs, namely WNT2, WNT4, and more recently WNT3A. A need therefore remains to determine if other WNTs known to be present in the adult ovary are involved in ovarian function. Although the non-canonical WNTs have been less characterized than the canonical WNT/β-catenin pathway, it is possible that these WNTs contribute to folliculogenesis and ovarian steroidogenesis. This idea is emphasized by the apparent discordant data in the literature regarding the effect of co-stimulation of the extracellular WNT and FSH signaling pathways on steroidogenic enzyme expression in granulosa cells. This difference is conceivably due to the use of two different WNT ligands employed in each study. Indeed, WNT3A and WNT4 have differing biological activities and as such are classified into two separate functional groups that can trigger distinct developmental outcomes (Wong *et al.* 1994, Du *et al.* 1995). However, the lines between these prototypical classifications are becoming blurred as data now suggests that WNT signaling is not strictly regulated by the ligand itself but that the receptor context dictates the signal output (Mikels & Nusse 2006). Furthermore, a single WNT protein has been shown to simultaneously activate different branches of the WNT signaling pathway in the same cell dependent on WNT concentration (Nalesso *et al.* 2011). Together, these findings underscore the significance of evaluating the specific receptors present during the different stages of follicular development, along with defining which WNTs may be binding. Since WNT proteins have been

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shown to activate different pathways with distinct and independent outcomes depending on the concentration of WNT (Nalesso *et al*. 2011), it will be interesting to evaluate dose-dependent treatment paradigms at different stages of follicle development such as in granulosa, granulosa–lutein, and differentiated luteal cells. Investigating changes that occur in the FZ and co-receptor complexes in follicular cells co-incubated with gonadotropin and WNT ligands has not been evaluated but would also be of value.

Follicles are exposed to various WNTs during follicle maturation that target β-catenin to the nucleus via the canonical WNT/β-catenin pathway to regulate target gene expression. Recent studies also identify a unique PKA-dependent regulation of β-catenin in response to FSH stimulation (Law *et al*. 2013) that regulates granulosa cell gene expression. It is interesting to consider whether PKA activated β-catenin regulates a similar set of genes as β-catenin that is regulated by GSK3β. Additionally, it remains to be determined if PKA-activation of β-catenin by both LH and FSH occurs in an equivalent fashion. Evaluation of WNT promoters for steroid response elements or other important regulatory regions may provide insight into the factors that may play a role in their function. In conclusion, the WNT signaling pathway encompasses multiple layers of complexity, and while our understanding of the role of WNTs in regulation of postnatal ovarian function and steroidogenesis continues to expand, there are many important questions that need to be answered in order to gain a complete understanding of the contribution of this large family of signaling molecules to folliculogenesis.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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