



Dynamics of WNT signaling components in the human ovary from development to adulthood

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Abstract

WNT signaling has been shown to play a pivotal role in mammalian gonad development and sex differentiation; however, its role in the developing human ovary has not been investigated. We analyzed a quantitative mass spectrometry dataset to determine the expression of WNT signaling components between 47 and 137 days of development and in adult ovarian cortex tissue. WNT signaling was identified within the top ten canonical pathways of proteins detected at every developmental stage examined. We further examined the specific localization of WNT signaling components glycogen synthase kinase 3 (GSK3B), frizzled 2 (FZD2), and β -catenin (CTNNB1) within ovarian tissue. GSK3B was nearly ubiquitously expressed during fetal development, while FZD2 was specific to germ cell nests during early development. β -catenin exhibited translocation from primarily membrane bound during early ovarian development to cytoplasmic and nuclear staining specifically in early primordial follicles in the fetal ovary. This cytoplasmic and nuclear β -catenin persisted in primordial follicles in adult ovarian tissue, but returned to membrane-bound localization in secondary follicles. We conclude that WNT signaling components are expressed in the human ovary from early to mid-gestation and remain in the adult ovary, and observed evidence for canonical WNT signaling only in the oocytes of primordial follicles. Together, these data are indicative of a role for canonical WNT signaling via β -catenin nuclear translocation during human follicle formation and follicle maintenance.

Keywords WNT signaling · Development · Ovary · β -Catenin

Introduction

The processes governing human ovarian development have been largely described based on histological examination and comparison to model organisms. In humans, primordial germ cells (PGCs) are specified and are identifiable at approximately 3 weeks of development (Witschi 1948) (Truman et al. 2017). These PGCs proliferate and migrate between 3 and 4 weeks of development (Witschi 1948) (Politzer 1933) to colonize the developing gonad at approximately 6 weeks (Makabe and Motta 1989). In mice, germ cell cysts break down and follicle assembly occurs in the

postnatal period, from 20.5 to 22.5 days post-coitum (dpc), during which time pregranulosa cells form close associations with single oocytes and primordial follicles are formed (Pepling and Spradling 2001). The process of germ cell nest breakdown and follicle formation is similar in humans; however, human ovarian follicle formation occurs entirely during fetal development between 17 and 20 weeks (Kurilo 1981; Konishi et al. 1986; Satoh 1991; Motta et al. 1997). Because human follicle assembly occurs prenatally, the signaling mechanisms required for human oocyte meiotic arrest, granulosa cell specification, and follicle assembly are still unknown.

Wingless-type mouse mammary tumor virus integration site (WNT) signaling is a key signaling network implicated in numerous processes including embryonic development, tissue regeneration, and cancer (Clevers 2006; Gordon and Nusse 2006). In canonical WNT signaling, the excreted WNT ligand binds a frizzled receptor resulting in the accumulation of non-phosphorylated β -catenin; as β -catenin accumulates, it translocates to the nucleus where it binds with lymphoid enhancer factor/T-cell factor (LEF/TCF)

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transcription factors (Behrens et al. 1996). Downstream targets of WNT signaling include genes that promote proliferation or cell fate determination and differentiation in stem cells (Reya and Clevers 2005). In addition to the canonical pathway, WNT signaling via frizzled receptors has been demonstrated to activate non-canonical signaling, independent of β -catenin. Non-canonical WNT signaling encompasses the planar cell polarity pathway, resulting in regulation of the cytoskeleton, as well as mechanisms mediated by intracellular calcium release, Rho family GTPases, heterotrimeric G proteins, and the JNK pathway (Veeman et al. 2003). Aside from WNT signaling, β -catenin also forms associations with cadherin complexes to control cell-to-cell contacts and cell migration (Nelson and Nusse 2004).

With respect to gonadogenesis, WNT signaling has been implicated at several stages of development in the mammalian gonad. With respect specifically to the establishment of the germline, *Wnt3* is required in mice for appropriate responsiveness to *Bmp4* in the early embryo and subsequent PGC differentiation (Ohinata et al. 2009; Aramaki et al. 2013). Later in development, during sex determination, WNT4 is required in both sexes for Mullerian duct formation and XX gonads lacking either R-spondin 1 (*Rspo1*) or *Wnt4* result in partial sex reversal (Vainio et al. 1999; Tomizuka et al. 2008; Chassot et al. 2008). Both *RSPO1* and WNT4 activate β -catenin signaling, and accordingly, stabilized β -catenin acts as a pro-ovarian signaling molecule capable of inducing male to female sex reversal in XY gonads and rescues the sex reversal observed in *Wnt4*-null XX gonads (Maatouk et al. 2008).

It has become clear that the impact of WNT signaling on the bipotential ovary impacts both germline and somatic cells. Pregranulosa cells within *Wnt4*-null and *Rspo1*-null ovaries undergo failure to maintain mitotic arrest and exhibit signs of precocious differentiation and differentiation to Sertoli cells (Maatouk et al. 2013). In humans, loss of function mutations in both *WNT4* and *RSPO1* results in female to male sex reversal (Mandel et al. 2008; Parma et al. 2006; Tomaselli et al. 2008). Beyond development, WNT signaling has also been implicated in cancer and tumor growth; in human granulosa cell tumors, cells had a higher rate of nuclear β -catenin than in healthy granulosa cells (Boerboom et al. 2005). WNT signaling components were also identified as one of several pathways misregulated in polycystic ovary syndrome (PCOS) patients (Jansen et al. 2004).

Despite the critical role for WNT signaling in ovarian development and function, the dynamics of WNT signaling have not been thoroughly examined during gonadogenesis, including critical time points associated with germline differentiation and follicle assembly. Currently, it is unknown which components of WNT signaling are present in the human ovary during development, and it is also not established whether canonical and/or non-canonical

mechanisms of WNT signaling participate in human follicle assembly. Utilizing a comprehensive quantitative mass spectrometry data set, we identified WNT signaling components in the human ovary during stages of PGC differentiation to primordial follicle formation. We then characterized β -catenin localization to provide insight into the role for canonical vs non-canonical WNT signaling in the formation of the human ovary. We conclude that β -catenin was primarily membrane bound throughout development, but that there was evidence for nuclear β -catenin in oocytes during primordial follicle formation, indicating canonical WNT signaling may play a role in follicle assembly and maintenance.

Materials and methods

Ovarian tissue sample collection

All procedures described herein have been reviewed and approved by the Institutional Review Board (IRB) at Northeastern University, Saitama Medical University, and the University of Washington. Human fetal ovarian tissue was collected after medical termination by the Laboratory of Developmental Biology at the University of Washington. Samples were collected between 30 min and 24 h post-procedure. Developmental ages were determined from prenatal intakes, foot length, Streeter's stage, and crown-rump length. Whole ovary tissues were snap frozen for proteomics analysis or fixed in 4% paraformaldehyde in PBS and then stored at 4 °C until being processed for immunohistochemistry. Human adult ovarian cortical tissue from reproductive aged women was generously provided to us for our use by Dr. Yasushi Takai at Saitama Medical University.

Quantitative proteomics analysis

Ovarian tissue samples were analyzed by LC-MS/MS as described previously (Bothun et al. 2018). Proteomics data were retrieved from Mendeley Data (<https://doi.org/10.17632/x7zbyj6xh7.1>) and analyzed for WNT signaling components based on intensity-based absolute quantitation (iBAQ) values provided by Scaffold (Schwanhausser et al. 2011). Common proteins were identified as those with expression at 47, 108, 122, and 137 days of development in addition to in adult ovarian cortex tissue. PANTHER Gene Ontology Pathway Analysis was performed to determine protein pathways present in human ovarian tissue samples and to identify proteins associated with each pathway (Mi et al. 2010).

Immunohistochemistry and immunofluorescence

Paraformaldehyde-fixed ovarian tissue was dehydrated, embedded in paraffin, and 5-micron sections were prepared. Antigen retrieval was performed in 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker, sections were cooled, washed, and endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min at room temperature. Sections were blocked for 1 h in TNK Buffer (0.1 M Tris-HCl, 0.55 M NaCl, 0.1 mM KCl, 0.5% BSA, 0.1% Triton-x100, and 1% normal goat serum). Slides were stained with primary antibody overnight at 4 °C according to Table 1. Secondary antibody labeling was performed and DAB Plus Chromagen was applied prior to nuclear counterstaining with Weigert's iron hematoxylin. Secondary only controls were included to assess background staining of the DAB substrate (Supplementary Fig. 1a). Slides were cleared through xylene and mounted with Permount Mounting Medium prior to imaging.

For immunofluorescence analysis of β -catenin, slides were processed as described above and blocked in PBS-blocking buffer (PBS, 2% bovine serum albumin, 0.1% Triton X-100, 1% normal goat serum) for 1 h at room temperature. Slides were incubated at 4 °C overnight with mouse anti- β -catenin (1:100, Santa Cruz sc-7963) in PBS-blocking buffer. Slides were washed with PBS and incubated in anti-mouse Alexa Fluor 647 (1:500). Slides were counterstained with Hoechst 33342, trihydrochloride, trihydrate (Invitrogen) to visualize cell nuclei. Secondary only controls were included to assess background signal from the secondary antibody (Supplementary Fig. 1b). Slides were washed with PBS and cover-slipped with ProLong Gold Antifade Reagent (Invitrogen) prior to imaging. All antibodies were validated by the manufacturer for specificity, utilizing western blotting for size validation, and, for glycogen synthase kinase-3 beta (GSK3B), in WT cells and (GSK3B) knockout mice.

Image collection and analysis

Images were taken on an Axiovert Inverted Fluorescence Microscope (Zeiss) fitted with filters for 640/30-nm excitation and 690/50-nm emission or 365-nm excitation and 445/50-nm emission.

All reagents were purchased from Thermo Fisher Scientific unless otherwise noted.

Table 1 Antibodies for immunohistochemical analyses

Primary antibody	Dilution	Detection
Rabbit anti-glycogen synthase kinase-3 beta (CST 12456)	1:500	Rabbit signal stain boost detection reagent
Goat anti-frizzled 2 (Abcam ab109094)	1:100	Anti-goat biotin (1:100), 30 min Streptavidin poly-HRP (1:250) 30 min

Results and discussion

WNT signaling components were detected throughout development in human ovarian tissue

Gene Ontology (GO) analysis of quantitative mass spectrometry identified protein pathways represented in human ovarian tissue at 47, 108, 122, and 137 days of development in addition to in adult ovarian cortex tissue (Supplementary Data). The protein pathways identified in this analysis represent the most abundant proteins throughout early to mid-gestation in the human ovary and were maintained in adult ovarian tissue. WNT signaling was identified within the top ten pathways observed with components present in every developmental stage of ovarian tissue analyzed (Fig. 1a). 24 proteins were identified by GO terms to be related to WNT signaling (Table 2). In addition to proteins detected at all stages of development, additional components of WNT signaling were individually screened for detection in ovarian tissues from specific stages. Among the family of frizzled receptors, three were identified throughout the dataset: Frizzled 1 (FZD1), Frizzled 2 (FZD2), and Frizzled 7 (FZD7) had varying degrees of expression based on iBAQ quantitation at each stage of development or adult tissue (Fig. 1b). In addition to frizzled receptors, glycogen synthase kinase-3 beta (GSK3B), a protein kinase and negative regulator of WNT signaling, was detected at developmental days 47, 108, 122, and 137, but not in adult tissue (Fig. 1b). Finally, β -catenin (catenin beta-1, CTNNB1) was detected in all tissues tested (Fig. 1b).

WNT signaling component localization in early ovarian development

Based on the proteomics analysis, we performed immunohistochemical staining to determine localization of several specific proteins to identify the cell types that expressed each protein throughout development. GSK3B was detected in human ovarian tissue at 56 days of development and expression was detected in germ cell nests as well as in somatic cells surrounding the germ cell nests (Fig. 2a). At 76, 116, and 137 days of development, GSK3B localization became increasingly cytoplasmic, with more intense staining evident in larger oocytes and the surrounding granulosa cells

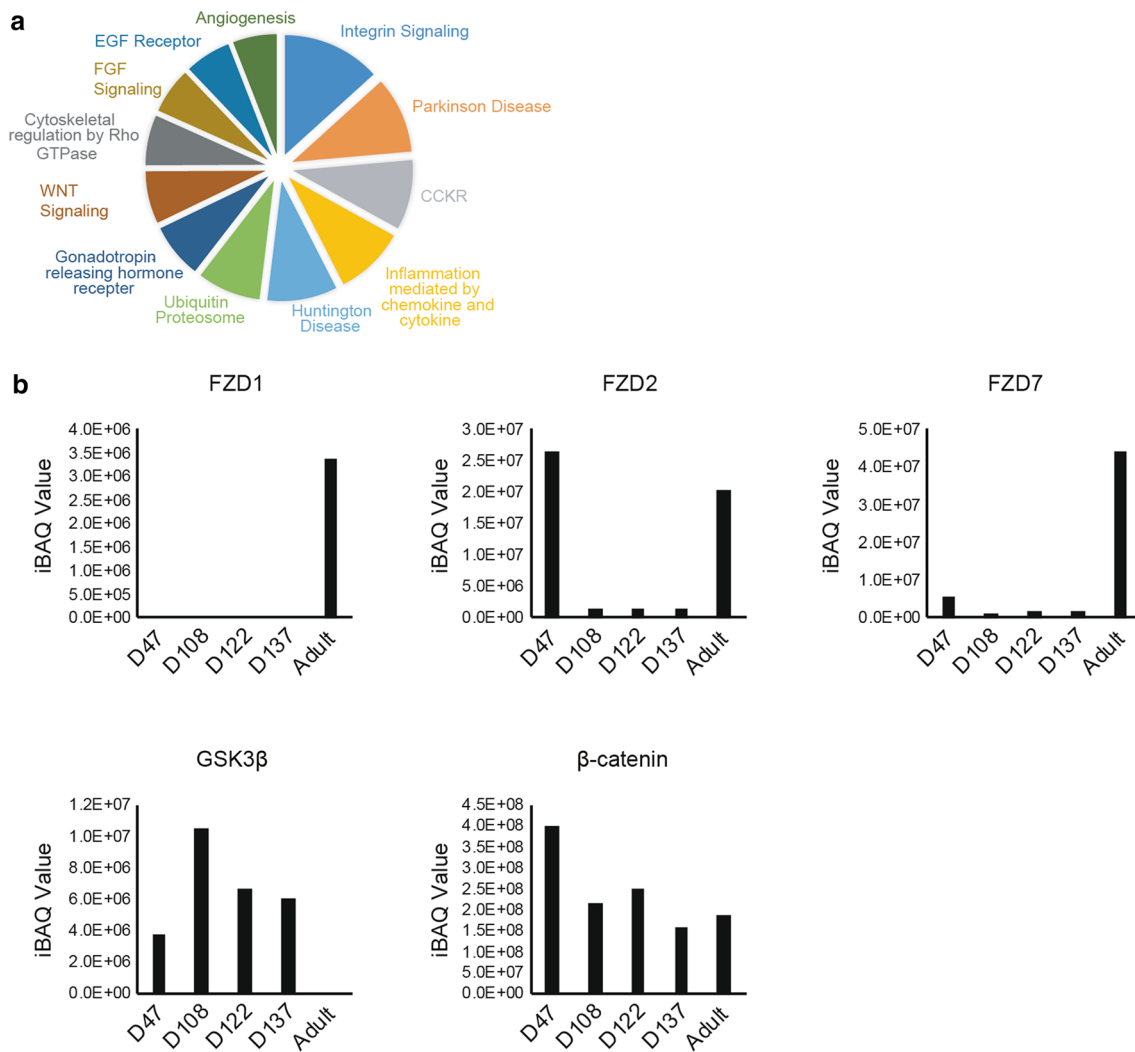


Fig. 1 Proteomic analysis of WNT signaling components. Of the core proteins identified in all tissues by quantitative mass spectrometry, the top ten pathways by number of components identified are displayed (a). Individual protein components of WNT signaling were assessed individually to identify quantitation across tissues. Frizzled 1 (FZD1),

Frizzled 2 (FZD2), Frizzled 7 (FZD7), glycogen synthase kinase 3b (GSK3B) and β -catenin (CTNNB1) were detected at four stages of fetal ovarian development (developmental days 47, 108, 122, and 137) and in a representative adult ovarian cortex. Values presented are intensity-based absolute quantification (iBAQ) values (b)

of primordial follicles (Fig. 2a, arrowhead). In adult ovarian cortex tissue, GSK3B was only faintly detected, with cytoplasmic expression in oocytes of primordial follicles. GSK3B plays a role in multiple signaling cascades, and is responsible for phosphorylation and subsequent destabilization of β -catenin (Yost et al. 1996). Because of its nearly ubiquitous expression and localization to all cell types of the ovary during development, it is likely that GSK3B in the ovary is participating in several signaling cascades. However, in adult ovarian tissue, GSK3B was more specifically detected in oocytes of primordial follicles and was only faintly detected throughout somatic cells, indicating a more specialized function in the maintenance of primordial follicles into adulthood.

While there were multiple frizzled receptors detected in the proteomics analysis, FZD2 was detected at all time points of ovarian development assessed. FZD2 was localized specifically within germ cell nests early in development at day 56 (Fig. 2b). FZD2 expression at developmental days 76, 122, and 137 maintained specificity to germ cell nests and was expressed in oocyte cytoplasm in primordial follicles (Fig. 2b, arrowheads). Adult tissue had only faint cytoplasmic staining of FZD2 throughout the tissue. Because WNT signaling depends on both the WNT ligand present in addition to the specific frizzled receptor it is paired with, we concluded that likely there are multiple frizzled receptors participating in WNT signaling in the human ovary. Because WNT signaling via frizzled receptors is generally understood

Table 2 GO term analysis identified proteins involved in WNT signaling present in ovarian tissue from 47 days, 108 days, 122 days, and 137 days of development and in adult tissue

Gene name	Protein description
<i>SMARCA5</i>	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5; SMARCA5; ortholog
<i>ARID1A</i>	AT-rich interactive domain-containing protein 1A; ARID1A; ortholog
<i>GNG12</i>	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12; GNG12; ortholog
<i>SMARCC1</i>	SWI/SNF complex subunit SMARCC1; SMARCC1; ortholog
<i>CTNNA1</i>	Catenin alpha-1; CTNNA1; ortholog
<i>GNB1</i>	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1; GNB1; ortholog
<i>SMARCC2</i>	SWI/SNF complex subunit SMARCC2; SMARCC2; ortholog
<i>FZD7</i>	Frizzled-7; FZD7; ortholog
<i>TBL1XR1</i>	F-box-like/WD repeat-containing protein TBL1XR1; TBL1XR1; ortholog
<i>PPP2R5D</i>	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform; PPP2R5D; ortholog
<i>GNAQ</i>	Guanine nucleotide-binding protein G(q) subunit alpha; GNAQ; ortholog
<i>CSNK2B</i>	Casein kinase II subunit beta; CSNK2B; ortholog
<i>PLCB3</i>	1-Phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3; PLCB3; ortholog
<i>CAD11</i>	Cadherin-11; CDH11; ortholog
<i>ADSS</i>	Adenylosuccinate synthetase isozyme 2; ADSS; ortholog
<i>CTNNB1</i>	Catenin beta-1; CTNNB1; ortholog
<i>SMARCB1</i>	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1; SMARCB1; ortholog
<i>ACTB</i>	Actin, cytoplasmic 1; ACTB; ortholog
<i>CTBP1</i>	C-terminal-binding protein 1; CTBP1; ortholog
<i>CSNK2A2</i>	Casein kinase II subunit alpha'; CSNK2A2; ortholog
<i>PPP2CA</i>	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform; PPP2CA; ortholog
<i>FZD2</i>	Frizzled-2; FZD2; ortholog
<i>CTBP2</i>	C-terminal-binding protein 2; CTBP2; ortholog
<i>SMARCA2</i>	Probable global transcription activator SNF2L2; SMARCA2; ortholog

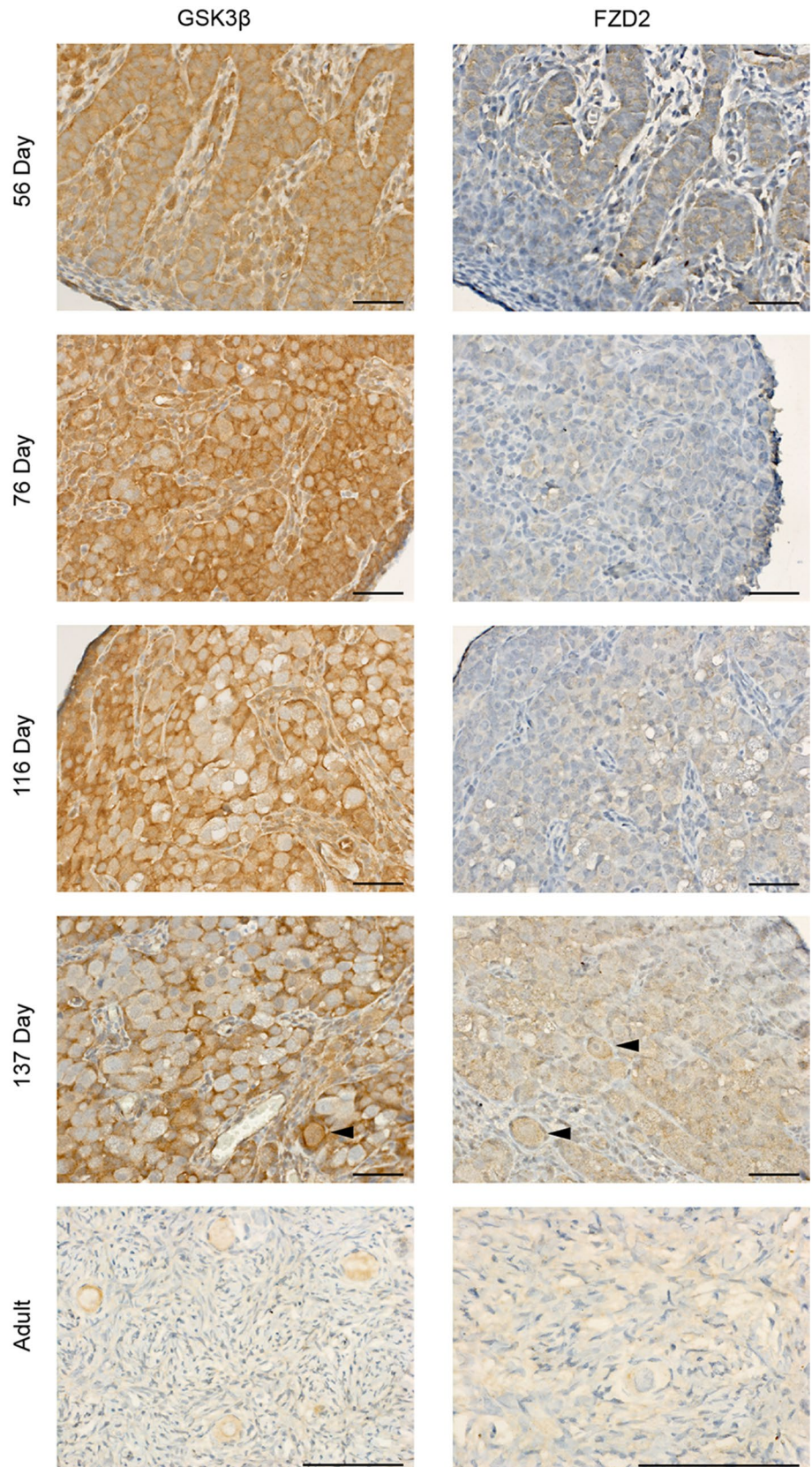
to lead to either canonical signaling via β -catenin- or to calcium-mediated signaling, it is likely that WNT signaling is occurring during these stages through one of these mechanisms.

β -Catenin localization suggests canonical WNT signaling is prominent at follicle formation in oocytes

As canonical WNT signaling is dependent on β -catenin translocation to the nucleus, we examined β -catenin localization throughout development. Immunofluorescence analysis of β -catenin in ovarian tissue from developmental day 56 revealed localization primarily to cell membranes within germ cell nests, in addition to expression at the surface epithelium (Fig. 3a). In tissue at 137 days of development, β -catenin was detected in the cytoplasm and nucleus of oocytes within primordial follicles (Fig. 3b, arrow), but it was not detected in somatic cells (Fig. 3b, arrowhead) or it was detected only at the membrane in germ cells that were not yet formed into follicles (Fig. 3b, asterisk). In adult ovarian cortex, β -catenin was localized specifically to primordial follicles, where expression was detected at cell membranes

between pregranulosa cells and the oocyte, in the oocyte cytoplasm, and the oocyte nucleus (Fig. 3c). Expression was similar in primary follicles (Fig. 3d). Within secondary follicles, β -catenin was localized at the cell membrane junctions between granulosa cells and also at the oocyte cell membrane (Fig. 3e). At this stage, little to no β -catenin was detected in the oocyte cytoplasm or nucleus (Fig. 3e). This pattern of β -catenin localization reveals that β -catenin is nuclear in oocytes primarily during follicle formation and maintenance of follicles to the primary stage, but becomes exclusively membrane bound in secondary follicles. Importantly, we did not observe nuclear β -catenin localization in somatic cells at any developmental age. There was no nuclear β -catenin observed in pregranulosa cells, nor of granulosa cells in primary or secondary follicles. This suggests that during human ovarian development after sex determination, β -catenin-dependent canonical WNT signaling is only active in the oocyte specifically during follicle formation. The primarily membrane-bound β -catenin and presence of FZD2 and other WNT signaling proteins prior to folliculogenesis and in secondary follicles in adulthood indicate that WNT signaling is occurring through non-canonical pathways during these developmental stages. The

Fig. 2 Immunohistochemical analysis of WNT signaling components from developing and adult human ovarian tissue. GSK3B and FZD2 were detected in fetal ovarian tissue from 56, 76, 116, and 137 developmental days; staining was primarily localized to germ cell nests and was mostly cytoplasmic. FZD2 showed the highest expression at 56 days of development, where localization was specific to germ cell nests. GSK3B and FZD2 were detected in primordial follicles at 137 days of development (arrowheads). In adult cortex tissue, GSK3B was detected in the cytoplasm of oocytes within primordial follicles (arrows), while FZD2 was detected throughout tissue (arrow indicating a primordial follicle). Primary antibodies were labeled and detected with DAB substrate (brown). Nuclei were counterstained with hematoxylin (blue). Scale bar = 50 μ m



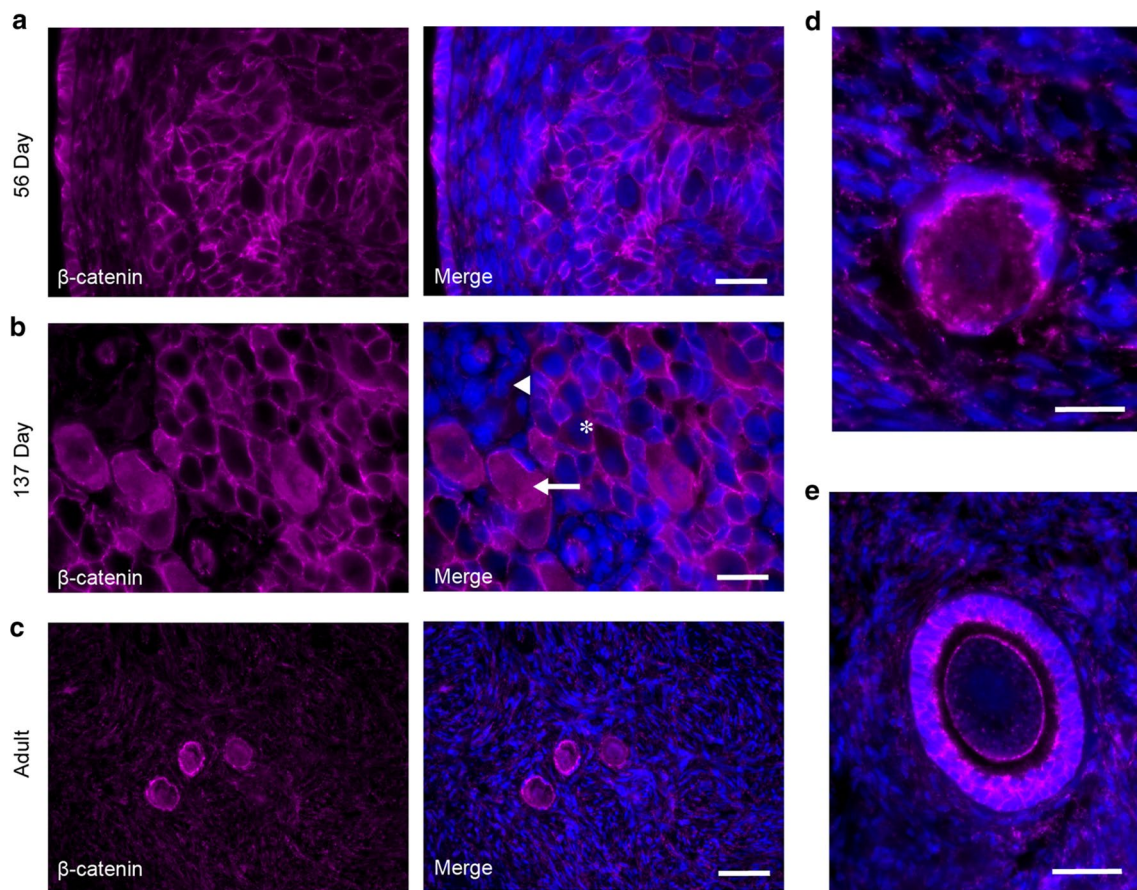


Fig. 3 β -catenin expression and localization throughout ovarian development. Immunofluorescence analysis reveals β -catenin localization is cell type and development stage specific. β -catenin was exclusively membrane bound throughout all cell types at 56 days of development. Scale bar 20 μ m (a). At 137 days of development, β -catenin was undetected in some somatic cells (arrowhead), membrane bound in PGCs and somatic cells (asterisk) and was cytoplasmic and nuclear in the oocytes of newly assembled primordial fol-

licles (arrow). Scale bar 20 μ m (b). In adult tissue, β -catenin was detected in the cytoplasm and nucleus within oocytes of primordial follicles and at cell membranes between granulosa cells. Scale bars 50 μ m and 20 μ m respectively (c, d). In secondary follicles in adult tissue, β -catenin was only detected at the membranes between granulosa cells, at the oocyte membrane, and showed some punctate staining in the cytoplasm of the oocyte. Scale bar 50 μ m (e)

identification of β -catenin-dependent WNT signaling coincident with follicle formation in humans is consistent with its role in mouse development, where stabilized β -catenin disrupts male XY development and induces follicle formation (Maatouk et al. 2008).

Conclusion

Proteins involved in WNT signaling were detected in the human ovary from early to mid-gestation and remained detectable in follicles of adult ovaries. The detection of frizzled receptors indicated that either canonical WNT signaling via β -catenin or non-canonical calcium signaling may be occurring; and as we detected multiple frizzled receptors (FZD1, FZD2, and FZD7) the ligand-receptor pairs likely change throughout development or are cell-type specific.

We observed evidence for canonical WNT signaling during follicle formation based on the nuclear translocation of β -catenin, the signal transducer of WNT signaling, shifting from membrane localization to cytoplasmic and nuclear localization in the oocytes of newly formed primordial follicles. There was no indication of canonical WNT signaling occurring in somatic cells of the ovary; however, the adult tissue studied did not contain follicles of more advanced stage than secondary. Additionally, it is possible that WNT signaling is occurring through non-canonical pathways throughout the ovary, based on the abundance of frizzled receptor expression throughout development.

Female germ cell development in humans is a process that, due to its timing during early development, is particularly difficult to study. Establishing the signaling mechanisms that are occurring during germ cell differentiation and follicle development is key to understanding disorders of the

ovary that have far reaching consequences for hormone production and fertility. Determining the physiological role of WNT signaling in human folliculogenesis may uncover new mechanisms for determining pathologies caused by dysregulated WNT signaling (Wood et al. 2004; Aydos et al. 2016). Additionally, as the field of stem cell biology progresses, establishing in vitro models of human ovarian cellular lineages relies on translation from basic biological studies to characterize protein and gene expression cascades needed for these processes (Truman et al. 2017).

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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