Identification of WNT/β-CATENIN signaling pathway components in human cumulus cells

Hong-Xing Wang1, Francis R. Tekpetey2,3, and Gerald M. Kidder1,2,4,5,6

1Department of Physiology and Pharmacology, The University of Western Ontario, London, ON, Canada N6A 5C1 2Department of Obstetrics and Gynaecology, The University of Western Ontario, London, ON, Canada N6A 5C1 3Reproductive Endocrinology and Infertility Program, London Health Sciences Centre, 339 Windermere Road, London, ON, Canada N6A 5A1 4Department of Paediatrics, The University of Western Ontario, London, ON, Canada N6A 5C1 5Children’s Health Research Institute, 800 Commissioners Road East, London, ON, Canada N6C 2V6
6Correspondence address. Tel: +1-519-661-3132; Fax: +1-519-850-2562; E-mail: gerald.kidder@schulich.uwo.ca

Abstract: Signaling via the conserved WNT/β-CATENIN pathway controls diverse developmental processes. To explore its potential role in the ovary, we investigated the expression of WNTs, frizzled (FZD) receptors and other pathway components in human cumulus cells obtained from oocytes collected for in vitro fertilization. Proteins were detected in cultured cells using immunofluorescence microscopy. Protein–protein interactions were analyzed by means of immunoprecipitation. WNT2, FZD2, FZD3 and FZD9 were identified but WNT1, WNT4 and FZD4 were not detected. WNT2 is co-expressed with FZD2, FZD3 and FZD9. Co-immunoprecipitation using WNT2 antibody demonstrated that WNT2 interacts with both FZD3 and FZD9, but only FZD9 antibody precipitated WNT2. We also identified DVL (disheveled), AXIN, GSK-3β (glycogen synthase kinase-3β) and β-CATENIN. β-CATENIN is concentrated in the plasma membranes. DVL co-localizes with FZD9 and AXIN in the membranes, but GSK-3β has little co-localization with AXIN and β-CATENIN. Interestingly, β-CATENIN is highly co-localized with FZD9 and AXIN. CDH1 (E-cadherin) was also detected in the plasma membranes and cytoplasm, co-localized with β-CATENIN, and CDH1 antibody precipitated β-CATENIN. The results suggest that WNT2 could act through its receptor FZD9 to regulate the β-CATENIN pathway in cumulus cells, recruiting β-CATENIN into plasma membranes and promoting the formation of adherens junctions involving CDH1.

Key words: ovarian follicle / paracrine signaling / folliculogenesis / gene expression / signal transduction

Introduction

WNT genes encode a large family of secreted, cysteine-rich glycoproteins that have been implicated in a variety of cellular processes, such as cell fate specification, cell proliferation, differentiation, survival and apoptosis, polarity and migration (Wodarz and Nusse, 1998; Hoppler and Kavanagh, 2007). Dysregulation of WNT signaling has been found to cause developmental defects and tumorigenesis (Logan and Nusse, 2004). To date, 19 WNT members have been identified in human. Frizzled (FZD) receptors are a family of seven-transmembrane proteins, 10 of which are encoded in the human genome. The N-terminal extracellular cysteine-rich domain (CRD) has been identified as the WNT-binding domain (Dann et al., 2001). The WNT-FZD relationship is well characterized in Drosophila, however, the specificity of WNT-FZD interactions remains largely unresolved in vertebrates because of the large number of WNT and FZD genes and the numerous possibilities for WNT-FZD interactions and functional redundancies (Hsieh et al., 1999; Rulifson et al., 2000).

The canonical WNT pathway is well understood for its ability to regulate cell–cell adhesion and cell cycle control, and β-CATENIN is the central and essential component in this pathway (Mulholland et al., 2005). In the absence of WNT activation, β-CATENIN is tightly regulated through phosphorylation at specific N-terminal residues by a destruction complex that includes casein kinase-1α (CK1α), glycogen synthase kinase-3 (GSK3), along with tumor suppressors adenomatous polyposis coli (APC) and AXIN. Phosphorylated β-CATENIN is targeted for rapid ubiquitylation and degradation in the 26S proteasome (Habas and Dawid, 2005; Gordon and Nusse, 2006). Disheveled (DVL) is, at present, the only known key intermediate connecting FZD and the downstream β-CATENIN pathway, and may bind directly to the FZD C-terminal region via a PDZ domain (Wong et al., 2003). Following WNT...
binding to the receptor complex consisting of FZD and lipoprotein receptor-related protein 5 and 6 (LRP5/6), the activation of the phosphoprotein DVL recruits AXIN and the destruction complex to the plasma membrane. AXIN is then degraded by proteasomes, which decreases β-CATENIN degradation. The activation of DVL also leads to the destabilization of the destruction complex and inhibition of GSK3, which further reduces the phosphorylation and degradation of β-CATENIN (Moon et al., 2004; Gordon and Nusse, 2006). As a result, hypophosphorylated β-CATENIN accumulates in the cytoplasm and is translocated to the nucleus where it interacts with the T-cell factor and lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors to activate the transcription of target genes (Gordon and Nusse, 2006). In addition to its function in the WNT pathway, β-CATENIN also functions as a key component of the cadherin complex, which controls cell–cell adhesion and influences cell migration (Nelson and Nusse, 2004; Bienz, 2005).

Ovarian folliculogenesis requires complex regulatory mechanisms involving both endocrine and intra-ovarian signaling pathways. Recently, WNT signaling has been implicated in ovarian development, oogenesis and early development. Wnt4 deficient mice exhibit sex reversal and a paucity of oocytes in the newborn ovary, while mice oogenesis and early development.

In the WNT pathway, β-CATENIN is involved in target gene transcription (Gordon and Nusse, 2006). In addition to its function (TCF/LEF) family of transcription factors to activate the transcription of target genes (Gordon and Nusse, 2006). In addition to its function in the WNT pathway, β-CATENIN also functions as a key component of the cadherin complex, which controls cell–cell adhesion and influences cell migration (Nelson and Nusse, 2004; Bienz, 2005).

Materials and Methods

Cumulus cell culture

The acquisition and use of human cumulus cells was approved by the Health Sciences Research Ethics Board of the University of Western Ontario and all patients gave informed consent. Cumulus granulosa cells were collected from oocytes being prepared for intracytoplasmic sperm injection (ICSI) as previously described (Wang et al., 2009). The cells were washed twice with culture medium consisting of DMEM/F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were grown on glass coverslips treated with 0.358 mg/ml collagen (BD Biosciences, Mississauga, ON, Canada) and cultured at 37°C, 5% CO2 in air for 24 h. Cumulus cells from individual follicles were cultured separately. All products for this study were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada) unless specifically mentioned.

Immunofluorescence microscopy

Information for all antibodies used in this study is listed in Table I. As a positive control for each antibody, we also used it to stain mouse ovaries by immunohistochemistry. Cells grown on glass coverslips were fixed with pre-chilled methanol/acetone (4:1) at 4°C for 20 min and then rinsed with phosphate-buffered saline (PBS) to prepare them for immunostaining. Briefly, the cells were blocked with washing buffer containing 3% w/v bovine serum albumin for 1 h, immunolabeled with primary antibody for 1 h, washed with PBS and immunolabeled with appropriate secondary antibody for 1 h in the dark. For double-immunolabeling, cells were treated with the first primary antibody for 1 h and then with Alexa Fluor® 594-conjugated secondary antibody for 1 h, followed by treatment with the second primary antibody for 1 h and finally by an Alexa Fluor® 488-conjugated secondary antibody. Several washes were interposed between the different antibody incubations. Cells were washed in PBS and the nuclei stained with 0.1% Hoechst for 10 min followed by washes with PBS and double-distilled H2O. The coverslips were mounted on slides with Airvol (Air Products & Chemicals, Inc., Allentown, PA, USA) before storage at 4°C. At least 50 patients contributed cumulus cells for this study, with at least five coverslips from each patient being analyzed by immunostaining with each antibody. The cells were imaged using a Zeiss (Thornwood, NY, USA) LSM 510 META confocal microscope. Fluorescent signals were captured after excitation with 488, 543 or 730 nm laser lines. Digital images were prepared using Zeiss LSM and Adobe Photoshop 7.0 software.

Co-immunoprecipitation

Cumulus cells from all follicles of one or two patients collected on the same day were pooled for these analyses. Cumulus cells were lysed in immunoprecipitation (IP) buffer [125 mM NaCl, 20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 2 mM EDTA, 1 mM Na3VO4, 1 mM NaF and 1 × protease inhibitor cocktail (Roche, Mannheim, Germany)]. Five-hundred microgram total proteins were incubated with 3–5 μg of antibody against WNT2, FZD2, FZD3, FZD9 or epithelial cadherin (CDH1) in 1.0 ml IP buffer on a rocker at 4°C overnight. The antibodies were precipitated with 50 μl of Dynabeads® Protein G for 2 hours on a rocker at 4°C. The beads were washed twice with washing buffer (25 mM citric acid, 50 mM Na2HPO4, pH 5.0) and then eluted with 0.1 M citrate (pH 2–3). Fifty micrograms of total protein before IP were set as the input sample, while the sample without antibody incubation was considered as negative control.

Immunoblotting

All samples were boiled for 5 min and loaded on 12% SDS–PAGE gels. The proteins were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfout, Buckinghamshire, UK). The membrane was blocked with 5% non-fat milk (w/v) in Tris-buffered saline Tween-20 (TBST) for 1 h, and subsequently probed with specific primary antibody other than the immunoprecipitating antibody overnight at 4°C followed by incubation with infrared fluorescent-labeled secondary antibody (Alexa-680 anti-rabbit, anti-goat or anti-mouse). Antibody binding was detected by use of the Odyssey infrared-imaging system (LI-COR Biosciences, Lincoln, NE, USA). The membrane was stripped and re-probed with the same antibody used for IP, then incubated with the corresponding infrared fluorescent-labeled secondary antibody. The immunoblots were processed using LI-COR. Two washes with TBST were inserted between the antibody treatment steps.
Results

Detection of WNTs and FZDs in human cumulus cells

To begin to explore WNT signaling in human cumulus cells, we looked for the expression of WNT1, WNT2 and WNT4. The results showed that WNT2 is expressed in cumulus cells, but WNT1 and WNT4 were not detected (Fig. 1A). The same WNT1 antibody revealed strong immunoreactivity in oocytes and corpora lutea of mouse ovaries but the WNT4 antibody did not reveal any distinct immunoreactivity (H.-X. Wang and G.M. Kidder, unpublished results). It has been reported that FZD2, FZD3, FZD4 and FZD9 are potential receptors for WNT2 in Drosophila or mammalian cells (Karasawa et al., 2002; Wu and Nusse, 2002; Klein et al., 2008). In our study, FZD2, FZD3 and FZD9 but not FZD4 were detected (Fig. 1B); again, the FZD4 antibody did reveal expression of this protein in mouse oocytes and corpora lutea (H.-X. Wang and G.M. Kidder, unpublished results). FZD2 is localized mainly in the cytoplasm (see also Fig. 2A showing co-localization with cytoplasmic WNT2), whereas FZD3 and FZD9 are localized in the cytoplasm and membranes.

Interaction of WNT2 and its possible receptors

We used double-label immunofluorescence to explore the co-localization of WNT2 and FZDs in human cumulus cells. We found that WNT2 is co-expressed with each of FZD2, FZD3 and FZD9 (Fig. 2). WNT2 co-localizes with FZD2 in the cytoplasm (Fig. 2A) and with FZD3 and FZD9 in both the membranes and cytoplasm (Fig. 2B and C). The possibility of direct interaction of WNT2 with the three receptors was then tested by co-immunoprecipitation experiments. When cell lysates were immunoprecipitated with antibody directed against WNT2, FZD3 and FZD9 (Fig. 3A). WNT2 co-localizes with FZD2 in the cytoplasm (Fig. 2A) and with FZD3 and FZD9 in both the membranes and cytoplasm (Fig. 2B and C). The possibility of direct interaction of WNT2 with the three receptors was then tested by co-immunoprecipitation experiments. When cell lysates were immunoprecipitated with antibody directed against WNT2, FZD3 and FZD9 were also found in the pellet, but FZD2 was not (Fig. 3A–C). Conversely, although WNT2 was precipitated by FZD9 antibody, FZD2 and FZD3 antibodies were ineffective (Fig. 3D). No protein bands were evident in cell lysates not treated with immunoprecipitating antibody (negative controls in Fig. 3A–D).

### Table 1 Details of antibodies used

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-WNT1</td>
<td>Santa Cruz</td>
<td>1:100</td>
</tr>
<tr>
<td>Goat anti-WNT2</td>
<td>Santa Cruz</td>
<td>1:100 1:200</td>
</tr>
<tr>
<td>Goat anti-WNT4</td>
<td>R&amp;D Systems</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit anti-FZD2</td>
<td>Zymed</td>
<td>1:100 1:400</td>
</tr>
<tr>
<td>Rabbit anti-FZD3</td>
<td>Sigma</td>
<td>1:200 1:1000</td>
</tr>
<tr>
<td>Goat anti-FZD3</td>
<td>US Biological</td>
<td>1:500 3.0 µg</td>
</tr>
<tr>
<td>Mouse anti-FZD3</td>
<td>Abnova Corporation</td>
<td>1:1000 3.0 µg</td>
</tr>
<tr>
<td>Rabbit anti-FZD4</td>
<td>Abcam</td>
<td>1:200 1:1000</td>
</tr>
<tr>
<td>Rabbit anti-FZD9</td>
<td>Abcam</td>
<td>1:200 1:1000</td>
</tr>
<tr>
<td>Goat anti-FZD9</td>
<td>Santa Cruz</td>
<td>1:100 1:300 4.0 µg</td>
</tr>
<tr>
<td>Rabbit anti-FZD9</td>
<td>MBL International Corp.</td>
<td>1:100 1:1000</td>
</tr>
<tr>
<td>Goat anti-DVL</td>
<td>Santa Cruz</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit anti-DVL</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td>Goat anti-AXIN</td>
<td>Santa Cruz</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse anti-GSK-3β</td>
<td>Santa Cruz</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit anti-β-CATENIN</td>
<td>Chemicon</td>
<td>1:500 1:1000</td>
</tr>
<tr>
<td>Mouse anti-CDH1</td>
<td>Zymed</td>
<td>1:100 1:500 5.0 µg</td>
</tr>
<tr>
<td>Mouse anti-CDH1</td>
<td>Abcam</td>
<td>1:200 1:1000</td>
</tr>
<tr>
<td>Alexa Fluor® 488 goat anti rabbit</td>
<td>Molecular probes</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor® 488 goat anti mouse</td>
<td>Molecular probes</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor® 488 rabbit anti goat</td>
<td>Molecular probes</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor® 594 goat anti rabbit</td>
<td>Molecular probes</td>
<td>1:300</td>
</tr>
<tr>
<td>Alexa Fluor® 594 goat anti mouse</td>
<td>Molecular probes</td>
<td>1:300</td>
</tr>
<tr>
<td>Alexa Fluor® 680 goat anti rabbit</td>
<td>Molecular probes</td>
<td>1:10 000</td>
</tr>
<tr>
<td>Alexa Fluor® 680 donkey anti rabbit</td>
<td>Molecular probes</td>
<td>1:10 000</td>
</tr>
<tr>
<td>Alexa Fluor® 680 rabbit anti mouse</td>
<td>Molecular probes</td>
<td>1:10 000</td>
</tr>
</tbody>
</table>

IF, immunofluorescence; IB, immunoblotting; IP, immunoprecipitation.
Expression of WNT2/β-CATENIN signaling proteins in human cumulus cells

As reported previously, FZD9 is activated by WNT2 and functions through the WNT/β-CATENIN pathway (Karasawa et al., 2002). DVL, AXIN, GSK-3β and β-CATENIN are four important molecules in this pathway (Gordon and Nusse, 2006). All four proteins were detected in human cumulus cells by immunofluorescence (Fig. 4). The pattern of immunostaining for DVL, AXIN and β-CATENIN indicated their localization in both the cell periphery and the perinuclear cytoplasm, whereas that for GSK-3β was clearly cytoplasmic.

Co-immunolabeling experiments demonstrated that DVL co-localizes with FZD9 and AXIN in the membranes (Fig. 5A and B), while GSK-3β is localized mostly in the cytoplasm, with little co-localization with AXIN or β-CATENIN (Fig. 5C and D). Interestingly, FZD9 is highly co-localized with β-CATENIN in the cell membranes (Fig. 5E).

Localization of β-CATENIN in cumulus cells

As recently reported, activation of the WNT pathway recruits dephosphorylated β-CATENIN into the plasma membrane where it co-localizes with two members of the destruction complex, APC.
and AXIN (Hendriksen et al., 2008). Our results indicate that AXIN is highly localized with β-CATENIN in the cytoplasm of cumulus cells (Fig. 6A). As a component of adherens junctions, β-CATENIN promotes cell adhesion by binding to the intracellular domain of the transmembrane protein, CDH1 (Bienz, 2005). CDH1 was also detected in human cumulus cells, with some junction-like plaques being found between the cells (arrows in Fig. 6B). Co-immunoprecipitation confirmed that β-CATENIN interacts with CDH1 in human cumulus cells (Fig. 6C). Co-immunolabeling further confirmed that CDH1 co-localizes with β-CATENIN to a limited extent in the membranes (Fig. 6D).

**Discussion**

WNTs comprise a large family of secreted glycoproteins that are responsible for key developmental processes including cell proliferation, cell polarity and cell fate determination. Conventional WNT signaling causes β-CATENIN accumulation in a complex with the transcription factor TCF/LEF that regulates target gene expression (Wodarz and Nusse, 1998; Hoppler and Kavanagh, 2007). Dysregulation of WNT signaling is linked to a range of diseases, most notably cancer (Logan and Nusse, 2004). A few reports have indicated that WNT signaling plays a key role in development of the ovary, and WNT pathway components are normally expressed in ovarian granulosa cells (Vainio et al., 1999; Hsieh et al., 2002, 2005; Ricken et al., 2002). Aberrant WNT signaling leads to granulosa cell tumorigenesis (Boerboom et al., 2005). These findings suggest that WNT signaling is important for follicular development. However, we know little about WNT signaling in human ovarian follicles and its roles in folliculogenesis. It was thus important to explore the expression of WNT signaling components in human cumulus cells.

Previous experiments revealed that WNT2 mRNA is concentrated in the cumulus granulosa cells of rat ovaries and that WNT4 mRNA is expressed in small growing follicles and corpora lutea of mouse ovaries (Hsieh et al., 2002; Ricken et al., 2002). In the present study, we only detected the expression of WNT2 in human cumulus cells; WNT1 and WNT4 were not detected. Neither of the two different WNT4 antibodies we used, both of which have been successfully used by others, revealed the presence of this protein in mouse ovaries either. We are unaware of any report confirming expression of WNT4 in adult ovaries at the protein level. Therefore, we think it unlikely that WNT4 is present in our human cumulus cell samples, although some uncertainty still exists. Since WNT4 might function
FZD2 and FZD3 interact with WNT2 in transfected 293 cells, FZD9 is activated by WNT2 and functions through the β-CATENIN pathway (Karasawa et al., 2002). β-CATENIN is a key effector of WNT/β-CATENIN signaling and is regulated by the cytoplasmic destruction complex formed by AXIN, GSK-3β and APC. In this study, we detected the expression of DVL, AXIN, GSK-3β and β-CATENIN in human cumulus cells. DVL is co-localized with FZD9 and AXIN in the plasma membranes, indicating that FZD9 can activate the cytoplasmic protein DVL and recruit it into the membrane, where it then interacts with AXIN. This interpretation is supported by the observations that overexpression of FZD9 induces the hyperphosphorylation and re-location of mouse DVL-1 from the cytoplasm to the cell membrane, and DVL-2 promotes the recruitment of AXIN to the plasma membrane during WNT signaling (Karasawa et al., 2002; Schwarz-Romond et al., 2007). Once AXIN is recruited into the membrane, it is then degraded by proteasomes leading to inhibition of the activity of the destruction complex. The activation of DVL also leads to inhibition of GSK-3β, which further contributes to β-CATENIN accumulation (Moon et al., 2004; Schwarz-Romond et al., 2007). These results directly support our finding that GSK-3β is not co-localized with AXIN and β-CATENIN in human cumulus cells.

An interesting finding of our study is that β-CATENIN is mostly localized in the membrane of cumulus cells, and that FZD9 is highly co-localized with β-CATENIN in the plasma membrane. These data indicate that FZD9 will activate β-CATENIN and recruit it into the membrane. In line with this, Hendriksen et al. (2008) have recently shown that endogenous dephosphorylated β-CATENIN appears on the membrane upon WNT3A treatment. This translocation occurs independently of CDH1, and β-CATENIN is co-localized with phospho-LRP6, AXIN and APC. We also found co-localization of AXIN and β-CATENIN in human cumulus cells. However, the mechanism and function of the recruitment of β-CATENIN into membranes remain unclear. Aside from its function in WNT signaling, β-CATENIN was initially discovered by virtue of its role in forming adherens junctions with CDH1 (Nelson and Nusse, 2004; Brembeck et al., 2006). The expression of CDH1/β-CATENIN complexes had been identified in the rat ovary, suggesting that this complex might play roles in follicle development and luteinization (Sundfeldt et al., 2000). In our study, CDH1 was detected in junction-like plaques between cumulus cells and CDH1 antibody precipitated β-CATENIN; furthermore, CDH1 co-localized with β-CATENIN in some cells. Thus, β-CATENIN in cumulus cells may play a role not only in WNT signaling, but also in forming adherens junctions with CDH1.

In summary, our results are consistent with the possibility that WNT2 signals through its receptor FZD9 to regulate the β-CATENIN pathway in human cumulus cells, potentially recruiting β-CATENIN into the plasma membrane and promoting the formation of adherens junctions involving CDH1. Our recent work with cultured mouse granulosa cells (Wang and Kidder, unpublished results) has revealed that WNT2 stimulates proliferation, suggesting that it may also act as a mitogen to promote follicle growth. Thus, WNT2/β-CATENIN signaling might play an important role in human folliculogenesis.

Figure 6 Localization of β-CATENIN in human cumulus cells. (A) Co-localization of AXIN and β-CATENIN. (B) Expression of CDH1 (arrows indicate junction-like plaques). (C) Immunoprecipitation of β-CATENIN with CDH1 antibody. (D) Limited co-localization of CDH1 and β-CATENIN. Hoechst dye was used for nucleus staining. The same magnification was used for all micrographs; scale bar = 50 μm.
Acknowledgements

We gratefully acknowledge the technical advice and assistance of Lynda Hughes, Kevin Barr and Dr Tony Li.

Funding

This work was funded by a grant (No. MOP-14150 to G.M.K.) from the Canadian Institutes of Health Research.

References


Submitted on September 16, 2008; resubmitted on November 11, 2008; accepted on November 17, 2008.