0021-972X/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(8):3860–3866

doi: 10.1210/jc.2003-030494

Identification, Characterization, and Regulation of the Canonical Wnt Signaling Pathway in Human Endometrium

S. TULAC, N. R. NAYAK, L. C. KAO, M. VAN WAES, J. HUANG, S. LOBO, A. GERMEYER, B. A. LESSEY, R. N. TAYLOR, E. SUCHANEK, AND L. C. GIUDICE

Department of Gynecology and Obstetrics (S.T., N.R.N., L.C.K., J.H., S.L., A.G., L.C.G.), Stanford University, Stanford, California 94305-5317; Stratagene (M.v.W.), La Jolla, California 92037; Department of Obstetrics & Gynecology (B.A.L.), University of North Carolina, Chapel Hill, North Carolina 27599; Department of Obstetrics, Gynecology, and Reproductive Sciences (R.N.T.), University of California, San Francisco, California 94143; and Department of Obstetrics and Gynecology (E.S.), Zagreb University School of Medicine, 10000 Zagreb, Croatia

Members of the Wnt family of signaling molecules are important in cell specification and epithelial-mesenchymal interactions, and targeted gene deletion of Wnt-7a in mice results in complete absence of uterine glands and infertility. To assess potential roles of the Wnt family in human endometrium, an endocrine-responsive tissue, we investigated in the proliferative and secretory phases of the menstrual cycle, endometrial expression of several Wnt ligands (Wnt-2, Wnt-3, Wnt-4, Wnt-5a, Wnt-7a, and Wnt-8b), receptors [Frizzled (Fz)-6 and low-density lipoprotein receptor-related protein (LRP)-6], inhibitors [FrpHE and Dickkopf (Dkk)-1], and downstream effectors (Dishevelled-1, glycogen synthase kinase-3β, and β-catenin) by RT-PCR, real-time PCR and *in situ* hybridization. No significant menstrual cycle dependence of the Wnt ligands (except Wnt-3), receptors, or downstream effectors, was observed. Wnt-3 increased 4.7-fold in proliferative compared with secretory endometrium ($P < 0.05$). However, both inhibitors showed dramatic changes during the cycle, with 22.2-fold down-regulation ($P < 0.05$) of FrpHE and 234.3-fold up-regulation ($P < 0.001$) of Dkk-1 in the secretory, compared with the proliferative phase. *In situ* hybridization revealed cell-specific expression of different Wnt family genes in human endometrium. Wnt-7a was exclusively expressed in the luminal epithelium, and Fz-6 and β-catenin were expressed in both epithelium and stroma, without any apparent change during the cycle. Both FrpHE and Dkk-1 expression were restricted to the stroma, during the proliferative and secretory phase, respectively. These unique expression patterns of Wnt family genes in different cell types of endometrium and the differential regulation of the inhibitors during the proliferative and secretory phase of the menstrual cycle strongly suggest functions for a Wnt signaling dialog between epithelial and stromal components in human endometrium. Also, they underscore the likely importance of this family during endometrial development, differentiation and implantation. (*J Clin Endocrinol Metab* 88: 3860–3866, 2003)

HUMAN ENDOMETRIUM IS a complex tissue that develops in response to ovarian hormones and undergoes dynamic reorganization during the menstrual cycle in preparation for implantation. Cyclic remodeling of the endometrium is regulated by specific local mesenchymal-epithelial interactions, as well as circulating steroid hormones (1), although the precise mechanisms and mediators of these interactions have not been completely defined. Recent microarray analyses from our group and others (2–4) of human endometrial gene expression in the window of implantation demonstrate expression and regulation of select members of the Wnt family, raising the question of the function of this family in endometrial development and function.

Secreted Wnt proteins comprise a family of highly conserved glycoproteins that exhibit pivotal roles in cell proliferation and differentiation, epithelial-mesenchymal communication, and embryogenesis (5–7). They bind and act through cell surface receptors known as Frizzled (Fz), a large family of seven membrane-spanning domain receptors (8). Wnt/Fz interactions at the cell surface activate Dishevelled (Dvl) leading to the inactivation of glycogen synthase kinase-3β (GSK-3β) by phosphorylation. When activated, GSK-3β phosphorylates adenomatous polyposis coli and Axin, increasing their binding activities for β-catenin, marking it for degradation by N-terminal phosphorylation (9–11). GSK-3β inactivation, in response to Wnt, dissociates this complex formation subsequently leading to cytoplasmic β-catenin accumulation and entry into the nucleus where it regulates gene transcription (12–14). Recent studies have identified several families of inhibitors of Wnt signaling. Secreted frizzled-related proteins share structural similarities to the Frizzled receptor family of proteins and antagonize Wnt actions at the level of receptor-ligand binding (15, 16). Another inhibitor, Dickkopf (Dkk), binds to low-density lipoprotein receptor-related protein-6 (LRP-6), a coreceptor for Wnt, and inhibits the Wnt signaling pathway. Recent work suggests that Dkk interacts with the coreceptors LRP5/6 and inhibits signaling by disrupting the binding of LRP6 to the Wnt/Fz ligand-receptor complex (17, 18). Some of the target genes for Wnt signaling, including Hoxa-10, Hoxa-11, cyclooxygenase-2, and peroxisome proliferator activated receptor-δ, (http://www.stanford.edu/~rnusse/

Abbreviations: Ct, Threshold cycle; Dkk, Dickkopf; Dvl, Dishevelled; Fz, frizzled; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GOI, gene of interest; GSK, glycogen synthase kinase; LRP, low-density lipoprotein receptor-related protein; RT, reverse transcription.

pathways/targets.html) are known to be important in decidualization of the endometrium and in implantation in the mouse (19–21).

Wnt genes are responsive to circulating sex steroids in several steroid-responsive organs including the female reproductive tract. In adult mouse, Wnt-7a is exclusively expressed in the uterine luminal, but not glandular, epithelium and acts to regulate the boundaries of expression of other Wnt ligands to establish the correct developmental axis of the uterus (22). Wnt-7a−/− mice are infertile and have complete absence of uterine glands. In human endometrium, Wnt-2, Wnt-3, Wnt-4, Wnt-5a, Wnt-7a, and Wnt-7b are constitutively expressed throughout the menstrual cycle, and down-regulation of Wnt-2, Wnt-3, Wnt-4, and Wnt-5a has been observed in endometrial carcinoma (23). These observations, in addition to several microarray studies including a previous report from our laboratory (2–4), indicate regulation of Wnt family genes in human endometrium during the menstrual cycle. Herein, we examine the expression of several Wnt ligands, receptors, inhibitors, and downstream effectors in human proliferative and secretory phase endometrium. Our results on the cell-specific expression and cycle-dependent regulation of select members of the Wnt family in human endometrium suggest important roles in mediating mesenchymal-epithelial interactions during endometrial differentiation and possibly implantation.

Materials and Methods

Tissue specimens

Endometrial biopsies were obtained from normally cycling women after informed consent, under an approved protocol by the Stanford University Committee on the Use of Human Subjects in Medical Research, and the Human Subjects Committees at the University of North Carolina and the University of California, San Francisco. All specimens were obtained in accordance with the Declaration of Helsinki. A total of 12 biopsy samples was obtained from two time points of the menstrual cycle: six in the mid- to late proliferative phase (peak circulating estradiol levels) and six in mid-secretory phase (peak estradiol and progesterone). Due to limitation in tissue availability, six of the samples (three in the mid- to late proliferative and three in the mid-secretory phase endometrium) were used for RT-PCR and real-time PCR studies, whereas six additional samples were used for *in situ* hybridization. All subjects in the age group between 28 and 39 yr had regular menstrual cycles (26–35 d), were documented not to be pregnant, had no history of endometriosis, and had not been on hormonal treatment for at least 3 months before biopsy.

RNA extraction and reverse transcription (RT)

Total RNA from endometrial tissue (in the proliferative and secretory phases of the cycle) was isolated using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Isolated total RNA was then treated with deoxyribonuclease and purified by RNeasy Spin Columns (QIAGEN, Valencia, CA). RNA integrity was verified by agarose gel electrophoresis/ethidium bromide staining and by $OD_{260/280}$ absorption ratio greater than 1.95. Total RNA (1 μg) was reverse transcribed using Omniscript kit (QIAGEN) according to the manufacturer's instructions with a 1:1 ratio of oligo $(deoxythymidine)_{16-18}$ and random hexamers (Invitrogen).

PCR

RT products were used in PCRs containing $MgCl_2$ (2.5 mM), deoxynucleotide triphosphates (0.1 mM), 1× PCR buffer, *Taq* polymerase, or HotStarTaq (QIAGEN) (2.5 U), and specific primer pairs (30 pmol) using the Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Primer sequences (Table 1) were designed from public databases and synthesized at the Stanford University School of Medicine Protein and Nucleic Acid (PAN) Facility. Wnt-7a, FrpHE, Fz-6, Dkk-1, and β-catenin PCR products were subcloned into pDrive Cloning Vectors (QIAGEN) by TA cloning (using primer pairs from Table 1) to generate specific probes for *in situ* hybridization. The identity of each PCR product was confirmed by sequencing at the Stanford PAN facility.

Real-time PCR

Primers for target and reference genes were designed using the PCR design software at http://labtools.stratagene.com and synthesized by QIAGEN (Table 2). Real-time PCRs were performed in quadruplicates using the QuantiTect SYBR Green PCR Kit (QIAGEN) following the manufacturer's instructions and carried out in the Mx4000 Q-PCR system (Stratagene, La Jolla, CA). All assays were optimized for primer concentration and PCR product specificity based on melting curve analysis and gel electrophoresis. Each real-time PCR was comprised of 250 ng of template cDNA and optimized primers at the concentrations of 50–250 nM. The thermal cycling conditions included an initial activation step at 95 C for 10 min, followed by 40 cycles of denaturation, annealing and amplification (95 C for 30 sec, 60 C for 1 min, 72 C for 30 sec). PCR products were analyzed by thermal dissociation (55–95 C) with a fluorescence measurement at every 1-degree increment. For each assay, a no-template and no-RT controls were included to verify the quality and cDNA specificity of the primers. Reactions were verified by agarose gel electrophoresis of PCR products and showed single correctly sized bands for each sample or no products for the negative controls. The PCR amplification efficiency of the reactions and correlation coefficients were determined from serially diluted human endometrial cDNA (250, 62.5, 15.6, and 3.9 ng), using the slope of the best-fit curve for Ct *vs.* concentration, and were calculated by the Mx4000 software. Investigated transcripts showed high efficiency of amplification and a linear response. No primer-dimer formation was observed during the 40 PCR amplification

TABLE 1. List of primers used for RT-PCR with corresponding amplicon sizes

Gene	Forward primer	Reverse primer	Amplicon (bp)	Accession no.
Wnt-2	5′-ccagccttttggcagggtc-3′	5′-gcatgtcctgagagtccatg-3′	380	NM_003391
Wnt-3	5′-tgaacaagcacaacaacgag-3′	5′-cagtggcatttttccttcc-3′	439	AB067628
Wnt-4	5′-ccttctcacagtcgtttg-3′	5′-cacagccgtcgatggcctt-3′	429	NM_030761
Wnt-5a	5-′gggaggttggcttgaacata-3′	5′-gaatggcacgcaattacctt-3′	141	NM_003392
Wnt-7a	5′-gctgcctgggccacctctttctca-3′	5′-cccggtggtacaggccttgcttct-3′	411	D83175
Wnt-8b	5′-atgtctttggggttggttcctag-3′	5′-ttgctaggaggaagaaggtcag-3′	272	Y11094
GSK-3β	5′-ggtcgccatccaagccagt-3′	5′-gtgccctatagtaccgagacac-3′	431	NM_002093
β-Catenin	5′-ggtgggctgcagaaaatggtt-3′	5′-gatggcaggctcagtgatgtcttc-3′	567	X87838
Fz-6	5′agtcttcagcggcttgtatcttgt-3′	5′-gctccgtccgctttcacctct-3′	561	AF072873
FrpHE	5′-ccgtgctgcgcttcttcttctgtg-3′	5′-gcgggacttgagttcgagggatgg-3′	461	AF026692
LRP-6	5′-catcctcgtctttcactcatc-3′	5′-ggctcgaggtctgtcctgct-3′	550	NM_002336
Dkk-1	5′-aggcgtgcaaatctgtctcg-3′	5′-tgcatttggatagctggtttagtg-3′	502	AF177394
Dvl-1	5′-cggggcggacgtggtggactg-3′	5′-ctggccggccggacgctctc-3′	532	AF006011
GAPDH	5′-accacagtccatgccatc-3′	5′-tccaccacctgcgctg-3′	452	BC001601

TABLE 2. List of primer used for real-time PCR with corresponding amplicon sizes

Gene	Forward primer	Reverse primer	Amplicon (bp)	Accession no.
Wnt-2	5′-actctcaggacatgctggct-3′	5′-acgaggtcatttttcgttgg-3′	161	NM_003391
Wnt-3	5′-tgtgaggtgaagacctgctg-3′	5′-aaagttgggggagttctcgt-3′	207	AB067628
Wnt-4	5′-catgcaacaagacgtccaag-3′	5′-aagcagcaccagtggaattt-3′	121	NM_030761
Wnt-5a	5-′gggaggttggcttgaacata-3′	5′-gaatggcacgcaattacctt-3′	141	NM_003392
Wnt-7a	5′-ggagggtccttttcctgggt-3′	5′-atattgctgtgatgaggccc-3′	190	D83175
Wnt-8b	5′-ttcccaagaatcttgaatgc-3′	5′-actccagagctccctcttcc-3′	142	Y11094
GSK-3β	5′-aactgcccgactaacaccac-3′	5′-attggtctgtccacggtctc-3′	253	NM_002093
β-Catenin	5′-tgcagttcgccttcactatg-3′	5′-actagtcgtggaatggcacc-3′	162	X87838
Fz-6	5′-atgagagaggtgaaagcgga-3′	5′-tcagatacactgcctgcctg-3′	143	AF072873
FrpHE	5′-ggacagcctatgtcaggcca-3′	5′-tctgtaccaaagggcaaacc-3′	155	AF026692
LRP-6	5′-cccatgcccctggttctact-3′	5′-ccaagccacagggatacagt-3′	114	NM_002336
Dkk-1	5′-catcagactgtgcctcagga-3′	5′-ccacagtaacaacgctggaa-3′	145	AF177394
Dvl-1	5′-ccaccctgaacctcaacagt-3′	5′-ccttcactctgctgactccc-3′	202	AF006011
GAPDH	5′-cgaccactttgtcaagctca-3′	5′-aggggtctacatggcaactg-3′	228	BC001601

cycles. No significant difference was observed in the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) between proliferative and secretory phases using the same starting amount of template, indicating that this is an appropriate reference gene.

The corresponding efficiency of amplification (E) during the exponential phase was calculated according to the equation $E = 10^{[-1/slope]} - 1$ (24), where the slope term corresponds to the slope of the least squares fit of Ct values relative to the logarithm (base 10) of the amount of RNA used. Quantitative analysis was based on the relative quantification of each gene of interest (GOI) in the secretory phase relative to the proliferative phase tissues. This ratio was corrected by the corresponding ratio of the reference gene (GAPDH). The relative expression ratio (R) of the gene of interest was calculated based on the corresponding efficiencies of amplification for each gene and the differences in threshold cycle values (ΔCt) using the following mathematical model (25):

$$R = \frac{(1 + E_{GOI})^{\Delta Ct_{GOI}(\text{proliferative-secretory})}}{(1 + E_{Ref})^{\Delta Ct_{Ref}(\text{proliferative-secretory})}}$$

In this equation, R represents the ratio at which a given gene of interest is expressed in secretory (n = 3) relative to proliferative (n = 3) phase endometrium, when normalized by a reference gene or normalizer. E_{GOI} and E_{Ref} correspond to the measured amplification efficiencies of the respective gene of interest and the normalizer (GAPDH). ΔCt_{GOI} and ΔCt_{Ref} are the differences in threshold values between proliferative and secretory phase samples for the gene of interest and the normalizer. Ct values were calculated by the Mx4000 software based on fluorescence intensity values after normalization with an internal reference dye and baseline correction.

Statistical analysis of the real-time data were done using a comparison (z-test of the difference between two means) of the corrected Ct means for the three specimens in the proliferative and three specimens in the secretory phases for each gene (26).

In situ hybridization

Out of thirteen different genes studied by RT-PCR and real-time PCR, five genes were randomly chosen for *in situ* hybridization. *In situ* hybridization of endometrial frozen sections (three in the mid-late proliferative and three in the mid-secretory phase endometrium) was conducted with ^{35}S-UTP-labeled (DuPont NEN Life Science Products, Boston, MA) sense and antisense riboprobes for: Wnt-7a (ligand), Fz-6 (receptor), FrpHE, and Dkk-1 (inhibitors) and β-catenin (downstream modulator), as described previously (27) with minor modifications. Briefly, 10-μm frozen sections of endometrium were fixed in 4% paraformaldehyde in PBS, treated with pronase E (0.125 μg/μl) in 50 mM Tris (pH 7.5) and 5 mM EDTA, and postfixed in 4% paraformaldehyde. Then the endometrial sections were acetylated with 0.25% acetic anhydride/0.1 M triethanolamine (pH 8.0), dehydrated through an ascending series of alcohols, and were incubated at 55 C overnight (16 h) in the hybridization solution containing the appropriate concentration of the sense and antisense probe (10^5 cpm/μl). After hybridization all slides were treated with RNase A, washed, and dehydrated in an ascending series of alcohols. The slides were coated with NTB2 autoradiographic emulsion (Eastman Kodak, Rochester, NY), stored at 4 C for 10 d, developed in D-19 (Eastman Kodak), and lightly counterstained with hematoxylin.

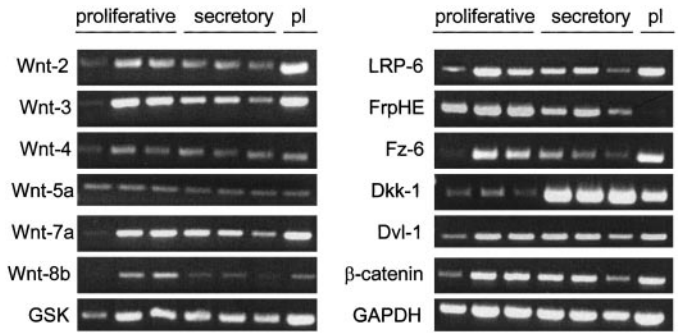

FIG. 1. RT-PCR products showing expression of selected genes relevant to the Wnt signaling pathway during the mid- to late proliferative (n = 3) and mid-secretory (n = 3) phase of endometrium and in placenta (pl). Placental total RNA was used as a positive or negative control for expression of different Wnt genes. GAPDH was used as a constitutively expressed marker to show integrity and relative amounts of RNA in each sample.

Results

Expression of Wnt family genes in human endometrium

RT-PCR experiments using specific primers (Table 1 in *Materials and Methods*) demonstrate (Fig. 1) the expression of Wnt-2, Wnt-3, Wnt-4, Wnt-5a, Wnt-7a, Wnt-8b, Fz-6, LRP-6, FrpHE, Dkk-1, Dvl-1, GSK-3β, and β-catenin in human endometrium. Wnt-2, Wnt-4, Wnt-5a, Wnt-7a, GSK-3β, β-catenin, LRP-6, Fz-6, and Dvl-1 were equally expressed in proliferative and secretory phase endometrium. However, Dkk-1 expression was dramatically increased during the secretory phase, and there was consistent increase in FrpHE expression in most of the samples during the proliferative phase (Fig. 1). Placenta was used as a positive control for Wnt-2, Wnt-3, Wnt-4, Wnt-5a, Wnt-7a, GSK-3β, β-catenin, LRP-6, Fz-6, and Dvl-1 and as a negative control for FrpHE. The integrity and relative amounts of these mRNAs were confirmed using GAPDH as a constitutively expressed marker.

Quantitative estimation of Wnt family genes

Regulation of expression of Wnt family members in human endometrium in the proliferative and secretory phases was quantified using real-time RT-PCR. The data confirmed that all studied Wnt members are expressed in human endometrium. Specificity of each RT-PCR product was documented using gel electrophoresis (data not shown) and resulted in a single product with appropriate length (Table 2 in *Materials and Methods*). Melting curve analysis was performed after every run and resulted in a single product for each target gene. Mean Ct values (n = 3 for mid- to late proliferative phase and n = 3 for mid-secretory phase run in quadruplicates) with corresponding SDs are shown in Table 3.

TABLE 3. Real-time PCR data showing mean Ct values in the proliferative and secretory phase endometrium with corresponding standard deviations for different Wnt genes

Gene	Proliferative (n = 3) Mean Ct ± SD	Secretary (n = 3) Mean Ct ± SD
GAPDH	16.46 ± 0.29	15.74 ± 0.34
Wnt-2	23.31 ± 0.23	23.47 ± 0.31
Wnt-3	29.66 ± 0.50	31.25 ± 0.39
Wnt-4	23.45 ± 0.23	22.04 ± 0.21
Wnt-5a	23.41 ± 0.46	24.69 ± 0.55
Wnt-7a	23.74 ± 0.27	24.56 ± 0.26
Wnt-8b	32.95 ± 1.44	36.20 ± 1.66
GSK-3β	22.00 ± 0.33	21.49 ± 0.56
β-Catenin	23.46 ± 0.43	23.76 ± 0.37
Fz-6	23.82 ± 0.34	23.91 ± 0.34
FrpHE	17.75 ± 0.40	21.55 ± 0.44
LRP-6	23.94 ± 0.26	24.20 ± 0.33
Dkk-1	28.05 ± 0.42	19.91 ± 0.45
Dvl-1	25.82 ± 0.27	25.57 ± 0.23

Among the thirteen genes analyzed, all were expressed in both secretory and proliferative endometrium, and there were no statistically significant differences in expression levels of Wnt-2, Wnt-4, Wnt-5a, Wnt-7a, Fz-6, LRP-6, Dvl-1, GSK-3β, and β-catenin between the two phases. However, the expression levels of FrpHE and Wnt-3 were significantly higher in the proliferative than in the secretory phase by a factor of 22.2 and 4.7, respectively ($P < 0.05$), whereas Dkk-1 was significantly up-regulated in secretory endometrium by a factor of 234.3 ($P < 0.001$). Wnt-8b was found to be up-regulated in the proliferative phase by a factor of 16.3, although this difference was not statistically significant due to high variability among tissue replicates (Fig. 2).

Cellular localization of Wnt family genes in human endometrium

Wnt-7a is expressed exclusively by luminal epithelial cells in human endometrium. In situ hybridization data (Fig. 3, A–D), using a 411-bp antisense riboprobe for the Wnt-7a cDNA fragment, reveal localization of Wnt-7a expression exclusively to the luminal epithelium of the endometrium. Wnt-7a is not expressed by glandular epithelium or stroma in normal human endometrium, and there is no noticeable difference in expression between proliferative and secretory endome-

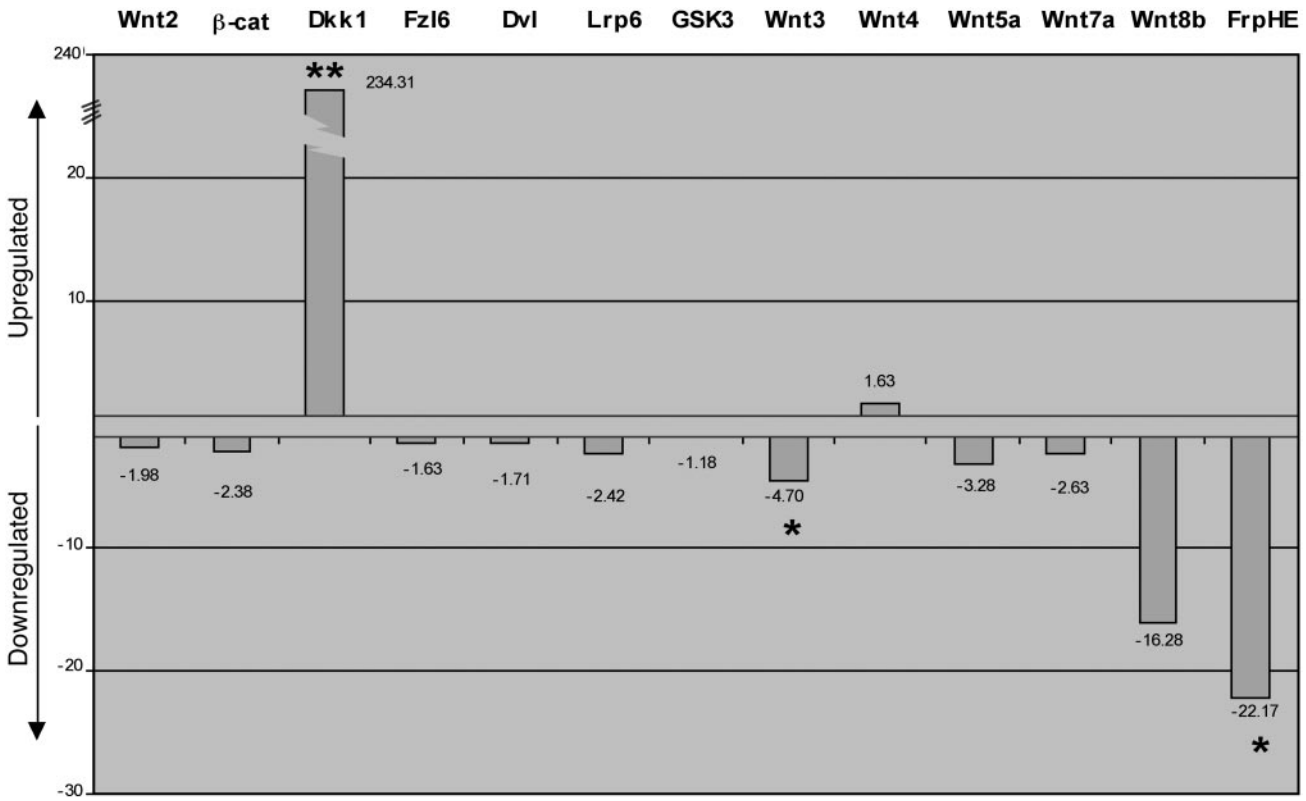

FIG. 2. Relative expression ratios of different Wnt genes showing relative changes in secretory (n = 3) compared with proliferative (n = 3) phase endometrium after normalization to GAPDH. Dkk-1 is significantly up-regulated ($P < 0.001$), and FrpHE and Wnt-3 are significantly down-regulated in the secretory phase. *Asterisks* (**, $P < 0.001$; *, $P < 0.05$) indicate significant changes in expression during secretory compared with proliferative phase endometrium the glands and stromated in the secretory phase.

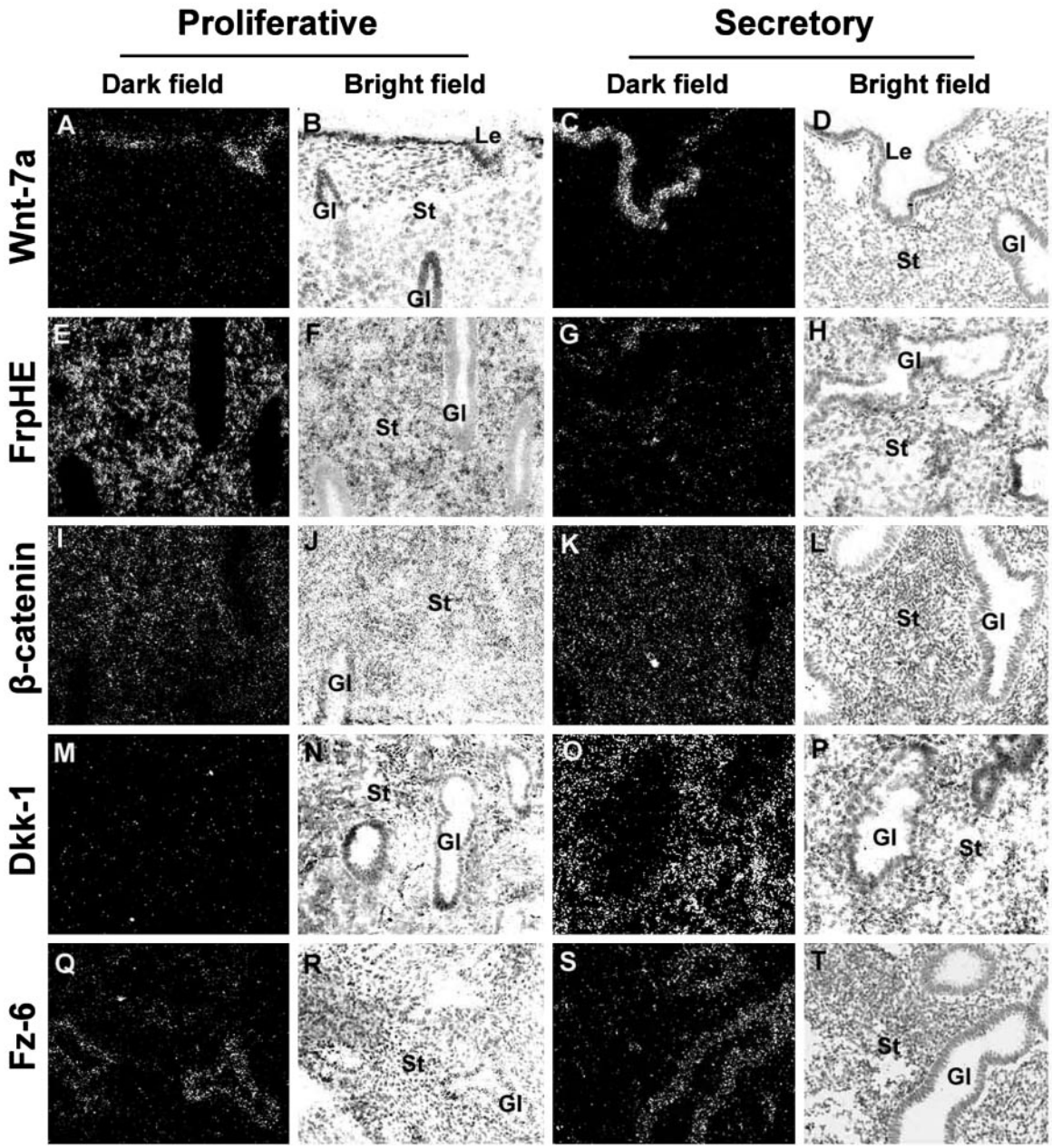

FIG. 3. Representative photomicrographs showing cellular localization of Wnt-7a (A–D), FrpHE (E–H), β-catenin (I–L), Dkk-1 (M–O), and Fz-6 (Q–T) mRNA expression by *in situ* hybridization in the proliferative and secretory phase endometrium. Column 1 (A, E, I, M, and Q) and column 3 (C, G, K, O, and S) represent the dark-field images during proliferative and secretory phase endometrium, respectively; and column 2 (B, F, J, N, and R) and column 4 (D, H, L, P, and T) represent the corresponding bright-field images. Note that Wnt-7a (A and C) is exclusively expressed in the luminal epithelium (Le) and FrpHE (E) in the stroma; Dkk-1 and Fz-6 are predominantly expressed in the stroma and glands respectively; and β-catenin is expressed in both the glands and stroma. Le, Luminal epithelium; Gl, glands; St, stroma; Original magnification, ×120.

trium. The sense probe did not show any signal in any of the cell types (data not shown).

FrpHE is abundantly expressed by proliferative endometrium. By using an antisense riboprobe derived from the cloned 461-bp cDNA fragment of FrpHE, its expression was detected in stromal cells, with no evidence of expression by glandular cells. The pattern of expression was dependent on the phase of the menstrual cycle, with abundant expression in proliferative endometrium, whereas secretory endometrium showed little or no signal (Fig. 3, E–H), consistent with the RT-PCR and quantitative PCR data. The sense probe did not show any specific signal (data not shown).

β-Catenin, Dkk-1, and Fz-6 expression. β-Catenin was found to be expressed in glandular, as well as stromal cells, and is abundant during both the proliferative and secretory phases of endometrial cycle (Fig. 3, I–L), consistent with the PCR data. Dkk-1 was highly expressed by stromal cells in the secretory phase of the cycle, with no or little expression in the proliferative phase, also consistent with the PCR data. Low expression of Dkk-1 was found in glandular cells (Fig. 3,

M–P). Frizzled-6 expression is predominantly located in glands, compared with stromal cells, in both proliferative and secretory endometrium. There was no appreciable difference between the proliferative and secretory phases (Fig. 3, Q–T). No signal was observed with the sense probes for all the studied genes (data not shown).

Discussion

The data presented herein demonstrate dynamic regulation and unique cellular expression of Wnt family members in human endometrium. Our finding of Wnt-7a expression exclusively in the luminal epithelium raises important questions regarding the function of this Wnt ligand in epithelial function, epithelial-stromal interactions, and during embryonic attachment to the epithelium during implantation. In mice, Wnt-7a is exclusively expressed in luminal, but not glandular epithelium (6), consistent with restricted expression found in adult human endometrium in the current study. Mutant mice lacking Wnt-7a do not form uterine glands and are infertile (6). The uterine epithelium in homozygous null animals (−/−) becomes stratified and demonstrates a lack of responsiveness to uterine mesenchyme, suggesting an important role for Wnt-7a in these interactions. These animals have loss of Hoxa-10 and Hoxa-11 expression in the stroma, further demonstrating that Wnt-7a in luminal epithelium is required for maintenance of stromal hoxa gene expression (22). Because Hoxa-10 and Hoxa-11 are up-regulated in human endometrium during endometrial stromal decidualization (28, 29), and targeted disruption of Hoxa-10 or Hoxa-11 genes in mice results in implantation failure and poor decidualization, it is possible that Wnt-7a signaling from the luminal epithelium maintains factors that are important in glandular functions and stromal decidualization.

We did not find significant regulation of Wnt-2, Wnt-4, Wnt-5a, and Wnt-7a mRNA expression in proliferative and secretory phase endometrium, which is consistent with another study in human endometrium (23). In contrast, we observed significant up-regulation of Wnt-3 (4.7 fold, $P < 0.05$) in proliferative endometrium that was not found in a previous study (23), likely due to different experimental methods for detection with different sensitivities. Some Wnt ligands are involved in reproductive processes and participate in mesenchymal-epithelial interactions in mouse endometrium. Wnt-4 is crucial for sex-specific development of the female reproductive tract (30). Wnt-5a is expressed in the uterine mesenchyme but not in the epithelium, and tissue recombinant experiments demonstrate that expression of the homeobox-containing gene Msx1 in the epithelium depends on expression of Wnt-5a in the underlying mesenchyme of uterine origin (31). In turn, Wnt-5a expression in the stroma is maintained by expression of Wnt-7a in the luminal epithelium. These studies together indicate that Wnt signaling plays important roles in mediating mesenchymal-epithelial interactions in the endometrium. Furthermore, Msx1 is highly expressed in mouse uterine epithelial cells that undergo pronounced changes in morphology in response to embryo implantation during early pregnancy, suggesting a possible role of Wnt signaling in this process. Whether these mechanisms are operational in human endometrium is currently under investigation in our laboratory.

Reports on the expression and regulation of Wnt receptors and downstream effectors are very limited in reproductive tissues, and to our knowledge this is the first study on the pattern of mRNA expression and regulation of these molecules in the endometrium. There was no significant change in the expression of studied Wnt receptors (Fz-6 and LRP-6) and the downstream effectors (Dvl-1, GSK-3β, and β-catenin) in proliferative and secretory phase human endometrium, suggesting that their expression is not directly regulated by ovarian steroid hormones in this tissue. β-catenin, which can be found in the plasma membrane, in the cytoplasm and the nucleus, is a key mediator of Wnt signaling and has an important role as a cell-cell adhesion molecule allowing association of cadherins with gap junction proteins (32). In the cytoplasm, β-catenin can associate with the adenomatous polyposis coli-axin-complex, leading to its degradation (33), whereas the free, unbound, and nonphosphorylated form of β-catenin enters the nucleus and activates Wnt target genes. Our data showing no statistically significant difference in β-catenin expression between the proliferative and secretory phase of the cycle are consistent with the status of β-catenin phosphorylation, rather than the intracellular protein levels *per se*, as the key variable in activating downstream transcription (34), although mechanistically the actions of Wnt signaling and β-catenin in endometrial function remain to be elucidated.

Inhibitors of the Wnt signaling pathway are important in regulating Wnt actions (5, 6). Our results are in agreement with those from a previous study (35), and studies on global gene profiling of human endometrium from our laboratory (3) and others (2, 4) that indicate dramatic changes in expression of FrpHE and Dkk-1 during the cycle. In the current study, there was 22.2-fold down-regulation of FrpHE and 234.3 fold up-regulation of Dkk-1 in the secretory phase (Fig. 2), suggesting that progesterone stimulates Dkk-1 expression while inhibiting FrpHE expression in human endometrium and/or that estrogen up-regulates FrpHE. Supporting this are observations that FrpHE is markedly up-regulated in estrogen-dependent endometrial and breast carcinomas, but not other cancers (35). These observations are consistent with a role for FrpHE in endometrial proliferation and overexpression with neoplastic development in estrogen-responsive tissues. Down-regulation of FrpHE and up-regulation of Dkk-1 in the secretory phase are associated with concomitant endometrial differentiation and preparing the endometrium for receptivity to embryonic implantation. Recent studies suggest that Dkk-1 interacts with the coreceptors LRP5/6 and inhibits Wnt signaling by disrupting binding of LRP5/6 to the Wnt/Fz ligand-receptor complex (16) and that FrpHE inhibits Wnt action by competitive binding to Wnt ligand(s) (15). However, the precise mechanisms of how the two inhibitors differentially regulate endometrial function, as well as mechanisms underlying Wnt regulation and action in human endometrium, are not well understood and are currently under investigation in our laboratory.

Acknowledgments

Received March 21, 2003. Accepted May 12, 2003.

Address all correspondence and requests for reprints to: Linda C. Giudice, M.D., Ph.D., Center for Research on Women's Health and Reproductive Medicine, Division of Reproductive Endocrinology and Infertility, Department of Gynecology and Obstetrics, Stanford University, Stanford, California 94305-5317. E-mail: giudice@stanford.edu.

This work is supported by the National Institutes of Health (NIH) Specialized Cooperative Centers Program in Reproductive Research (National Institute of Child Health and Human Development HD 31398) and the NIH Office of Women's Health Research (to L.C.G., B.A.L., R.N.T.), the NIH Women's Reproductive Health Research Career Development Program (to L.C.K.), and the German Research Foundation [Deutsche Forschungsgemeinschaft GE 1173/1-1 (to A.G.)] and the Scientific Research Fund of the Croatian Ministry of Science (Grant No. 108-250).

References

1. **Cunha GR** 1976 Epithelial-stromal interactions in development of the urogenital tract. Int Rev Cytol 47:137–194
2. **Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, Vernon M, Yuan L, Fritz MA, Lessey B** 2002 Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. Mol Hum Reprod 8:871–879
3. **Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA, Giudice LC** 2002 Global gene profiling in human endometrium during the window of implantation. Endocrinology 143:2119–2138
4. **Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC, Smith SK** 2003 Determination of the transcript profile of human endometrium. Mol Hum Reprod 9:19–33
5. **Cadigan KM, Nusse R** 1997 Wnt signaling: a common theme in animal development. Genes Dev 11:3286–3305
6. **Nusse R, Varmus HE** 1992 Wnt genes. Cell 69:1073–1087
7. **Wodarz A, Nusse R** 1998 Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 14:59–88
8. **Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R** 1996 A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 382:225–230
9. **Nakamura T, Hamada F, Ishidate T, Anai K, Kawahara K, Toyoshima K, Akiyama T** 1998 Axin, an inhibitor of the Wnt signalling pathway, interacts with β-catenin, GSK-3β and APC and reduces the β-catenin level. Genes Cells 3:395–403
10. **Nusse R** 1999 WNT targets. Repression and activation. Trends Genet 15:1–3
11. **Willert K, Nusse R** 1998 β-Catenin: a key mediator of Wnt signaling. Curr Opin Genet Dev 8:95–102
12. **van Noort M, Meeldijk J, van der Zee R, Destree O, Clevers H** 2002 Wnt signaling controls the phosphorylation status of β-catenin. J Biol Chem 277: 17901–17905
13. **van Noort M, Clevers H** 2002 TCF transcription factors, mediators of Wnt-signaling in development and cancer. Dev Biol 244:1–8
14. **Woodgett JR** 2001 Judging a protein by more than its name: GSK-3. Sci STKE 2001:RE12
15. **Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J** 1997 A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. Proc Natl Acad Sci USA 94:2859–2863
16. **Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A, Aaronson SA** 1999 Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. J Biol Chem 274:16180–16187
17. **Semenov MV, Tamai K, Brott BK, Kuhl M, Sokol S, He X** 2001 Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr Biol 11:951–961
18. **Li L, Mao J, Sun L, Liu W, Wu D** 2002 Second cysteine-rich domain of Dickkopf-2 activates canonical Wnt signaling pathway via LRP-6 independently of dishevelled. J Biol Chem 277:5977–5981
19. **Lim H, Song H, Paria BC, Reese J, Das SK, Dey SK** 2002 Molecules in blastocyst implantation: uterine and embryonic perspectives. Vitam Horm 64:43–76
20. **Paria BC, Lim H, Das SK, Reese J, Dey SK** 2000 Molecular signaling in uterine receptivity for implantation. Semin Cell Dev Biol 11:67–76
21. **Taylor HS** 2000 The role of HOX genes in human implantation. Hum Reprod Update 6:75–79
22. **Miller C, Sassoon DA** 1998 Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. Development 125:3201–3211
23. **Bui TD, Zhang L, Rees MC, Bicknell R, Harris AL** 1997 Expression and hormone regulation of Wnt2, 3, 4, 5a, 7a, 7b and 10b in normal human endometrium and endometrial carcinoma. Br J Cancer 75:1131–1136
24. **Rasmussen R** 2001 Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K, eds. Rapid cycle real-time PCR. Methods and applications. Heidelberg: Springer Press; 21–34
25. **Pfaffl MW** 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:2002–2007
26. **Sokal RR, James Rohlf F** 1994 Biometry: the principles and practice of statistics in biological research. 3rd ed. New York: Freeman, Co.
27. **Nayak NR, Brenner RM** 2002 Vascular proliferation and vascular endothelial growth factor expression in the rhesus macaque endometrium. J Clin Endocrinol Metab 87:1845–1855
28. **Taylor HS, Arici A, Olive D, Igarashi P** 1998 HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium. J Clin Invest 101:1379–1384
29. **Taylor HS, Igarashi P, Olive DL, Arici A** 1999 Sex steroids mediate HOXA11 expression in the human peri-implantation endometrium. J Clin Endocrinol Metab 84:1129–1135
30. **Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP** 1999 Female development in mammals is regulated by Wnt-4 signalling. Nature 397:405–409
31. **Pavlova A, Boutin E, Cunha G, Sassoon D** 1994 Msx1 (Hox-7.1) in the adult mouse uterus: cellular interactions underlying regulation of expression. Development 120:335–345
32. **Potter E, Bergwitz C, Brabant G** 1999 The cadherin-catenin system: implications for growth and differentiation of endocrine tissues. Endocr Rev 20: 207–239
33. **Willert K, Shibamoto S, Nusse R** 1999 Wnt-induced dephosphorylation of axin releases β-catenin from the axin complex. Genes Dev 13:1768–1773
34. **Staal FJ, Noort Mv M, Strous GJ, Clevers HC** 2002 Wnt signals are transmitted through N-terminally dephosphorylated β-catenin. EMBO Rep 3:63–68
35. **Abu-Jawdeh G, Comella N, Tomita Y, Brown LF, Tognazzi K, Sokol SY, Kocher O** 1999 Differential expression of frpHE: a novel human stromal protein of the secreted frizzled gene family, during the endometrial cycle and malignancy. Lab Invest 79:439–447