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WNTs in the Ovine Uterus: Potential Regulation of Periimplantation Ovine Conceptus Development

K. Hayashi, R. C. Burghardt, F. W. Bazer, and T. E. Spencer

Center for Animal Biotechnology and Genomics (K.H., R.C.B., F.W.B., T.E.S.), Department of Animal Science (K.H., F.W.B., T.E.S.), and Image Analysis Laboratory and Department of Veterinary Integrative Biosciences (R.C.B.), Texas A & M University, College Station, Texas 77843

WNTs (Wingless-type MMTV integration site family member) are involved in critical developmental and growth processes in animals. These studies investigated WNT pathways in the ovine uterus and conceptus during the periimplantation period of pregnancy. *WNT2* and *WNT2B* mRNAs were detected in endometrial stroma. *WNT5A* and *WNT5B* mRNAs were most abundant in the stroma and less so in the luminal epithelium, whereas *WNT11* mRNA was detected primarily in the glands. *WNT7A* mRNA was present in the luminal epithelium on d 10, absent on d 12 and 14, and increased between d 16 and 20. Only *WNT2*, *WNT2B*, and *WNT4* were detected in conceptus trophectoderm. *FZD6/8* (frizzled receptor) and *GSK3B* (glycogen synthase kinase 3β) mRNAs were detected primarily in endometrial epithelia and conceptus trophectoderm, whereas the *LRP5/6* (low-density lipoprotein receptor-related proteins 5 and 6) coreceptor was present in all endometrial cells and the trophectoderm. *DKK1* (Dickkopf), a WNT signaling inhibitor, increased in the endometrium from d 16–20. *CTNNB1* [catenin (cadherin associated protein) β1] and *CDH1* (E-cadherin) mRNAs were most abundant in the endometrial epithelia and trophectoderm. *LEF1* (lymphoid enhancer-binding factor 1) mRNA was expressed primarily in uterine epithelia, whereas TCF7L2 [(transcription factor 7-like 2 (T-cell specific, HMG-box)] was primarily in the conceptus. CTNNB1 and TCF7L2 proteins were both abundant in the nuclei of trophoblast giant binucleate cells. WNT7A stimulated a TCF/LEF-luciferase reporter activity in ovine trophectoderm cells that was inhibited by dominant-negative TCF and Sfrp2 (secreted FZD-related protein 2). WNT7A increased trophectoderm cell proliferation as well as *MSX2* (msh homeobox 2) and *MYC* (myelocytomatosis oncogene) mRNA levels. Wnt5a increased trophectoderm cell migration in a Rho kinase-dependent manner. These results support the hypotheses that canonical and noncanonical WNT signaling pathways are conserved regulators of conceptus-endometrial interactions in mammals and regulate periimplantation ovine conceptus development. (*Endocrinology* 148: 3496–3506, 2007)

WNT (WINGLESS-TYPE MMTV integration site family member) genes are homologous to the *Drosophila* segment polarity gene wingless (*wg*). In humans and mice, the WNT family of genes encodes a group of 19 highly conserved secreted signaling molecules that are critical regulators of cell fate, growth, and differentiation, as well as cell-cell interactions (1). Autocrine or paracrine signaling by WNTs involves a family of 10 frizzled receptors (FZD) (2), which are seven transmembrane G protein-coupled receptors that possess an extracellular cysteine-rich domain for WNT binding (3, 4). LRP5/6 (low-density lipoprotein receptor-related proteins 5 and 6) are coreceptors with FZD (5). Secreted FZD-related proteins (SFRPs) are forms of FZDs that contain the cysteine-rich domain but no transmembrane or intracellular segments and can bind WNTs to inhibit their activity (6, 7). Other antagonists of the WNT signaling pathway are products of four *DKK* (Dickkopf) genes that encode secreted proteins that bind the LRP coreceptors (8).

Signaling pathways activated by WNTs are generally divided into the canonical and noncanonical or planar cell polarity pathways (9). In the canonical WNT signaling pathway, FZD receptors transduce a signal to several intracellular proteins that include DVL (disheveled), GSK3B (glycogen synthase kinase 3 β), axin, adenomatous polyposis coli, and the transcriptional regulator CTNNB1 [or catenin (cadherin associated protein) β1] (9, 10). Cytoplasmic CTNNB1 levels are normally low because of continuous proteasome-mediated degradation controlled by GSK3B. When cells receive WNT signals, the degradation pathway is inhibited, resulting in accumulation of CTNNB1 in the cytoplasm and nucleus. In the cytoplasm, CTNNB1 interacts with CDH1 (E-cadherin). Adherens junctions formed by classical cadherin/catenin complexes mediate epithelial organization and function. Nuclear CTNNB1 interacts with transcription factors, most notably members of the TCF7/LEF1 [transcription factor 7 (T-cell specific, HMG-box)/lymphoid enhancer-binding factor one] family, to regulate transcription of genes involved in cell proliferation and differentiation, such as MYC (myelocytomatosis oncogene) and MSX2 (msh homeobox 2). Other WNTs, such as WNT5A and WNT11, appear to primarily activate the noncanonical or planar cell polarity pathway involved in cell movement, proliferation, and migration (9) and may also antagonize the canonical WNT pathways in *Xenopus* embryos and mammalian cells (11–13).

WNTs are important regulators of uterine morphogenesis, uterine receptivity to the embryo, and blastocyst implantation in mice (14–16). Many *WNT* genes are expressed in

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Abbreviations: BNC, Binucleate cell; C_T, threshold cycle; dnTCF, dominant-negative human TCF7L2; FZD, frizzled receptor; IFNT, interferon τ; LE, luminal epithelium; oTr cells, ovine trophectoderm cells; ROCK, Rho kinase; SFRP, secreted frizzled-related protein; sGE, superficial glandular epithelium.

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human endometrium during the proliferative and secretory phases of the menstrual cycle (17), and the canonical WNT signaling pathway is implicated in trophoblast differentiation in humans (18) and cattle (19). Of particular note, we found that *WNT7A* is a novel gene induced in the endometrial luminal epithelium (LE) and superficial ductal glands of the ovine uterus by interferon τ (IFNT) (19), the pregnancy recognition signal produced by the mononuclear trophectoderm cells of the conceptus (embryo/fetus and associated extraembryonic membranes) from d 11 to 20 (20). No other information is available on WNT signaling pathways in the ruminant uterus and conceptus.

Early pregnancy in sheep is characterized by blastocyst elongation that is concomitant with pregnancy recognition signaling and the onset of trophoblast growth and differentiation (21, 22). The blastocyst hatches from the zona pellucida on d 8, develops into a tubular form by d 11, and then elongates on d 12 to 10 cm or more in length by d 14, and achieves a length of 25 cm or more by d 17. Elongation of the blastocyst is critical for developmentally regulated production of IFNT and conceptus implantation (21). In the sheep conceptus, trophoblast giant binucleate cells (BNCs) begin to differentiate on d 14 (23) and are thought to arise from mononuclear trophectoderm cells by consecutive nuclear divisions without cytokinesis or endoreduplication (24). The BNCs have at least two critical functions, including fusion with uterine epithelial cells to form a hybrid fetomaternal syncytium for successful implantation and subsequent cotyledonary growth in the placentome, and synthesis and secretion of protein, *e.g.* placental lactogen, and steroid, *e.g.* progesterone, hormones (24). WNT signaling via CTNNB1 has recently been implicated in bovine trophoblast giant BNC differentiation (19).

Our working hypothesis is that the WNT signaling pathway is a conserved and critical regulator of conceptus-endometrial interactions during establishment of pregnancy in sheep and that canonical and noncanonical WNT signaling is important for trophectoderm growth, differentiation, and function. As a first step in testing our hypothesis, we conducted studies to determine the following: 1) which WNTs and WNT signaling pathways were present in the ovine uterus and conceptus during the periimplantation period of pregnancy; 2) effects of WNT7A on trophectoderm proliferation and gene expression; and 3) effects of WNT5A on trophectoderm migration.

Materials and Methods

Animals

Crossbred Suffolk ewes (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and were used in the experiments after they had exhibited at least two estrous cycles of normal duration (16–18 d). All experiments and surgical procedures were in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A & M University. Ewes were maintained according to normal animal husbandry practices.

Experimental design

At estrus (d 0), ewes were mated to an intact ram and then hysterectomized (n = 5 ewes/d) on d 10, 12, 14, 16, 18, or 20 of pregnancy. On d 10 to 16, the uterine lumen was flushed with 20 ml of sterile 10 mM Tris-HCl (pH 8.0) to confirm the presence of one or more morphologically normal conceptuses. Uterine flushes were not obtained on either d 18 or 20 because the conceptus was firmly adhered to the endometrial LE and basal lamina. At hysterectomy, several sections (~0.5 cm) from the midportion of each uterine horn ipsilateral to the corpus luteum were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the corpus luteum. No tissues from the contralateral uterine horn were used in this study. After 24 h, fixed tissues were changed to 70% ethanol and then embedded in Paraplast Plus (Oxford Labware, St. Louis, MO). The remainder of the endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at −80 C for RNA extraction.

RT-PCR analysis

Partial cDNAs for components of the WNT signaling pathway were generated by RT-PCR using methods described previously (25) and total RNA isolated from d 16 pregnant endometrium and/or d 18 conceptuses. Primers for each target gene were derived from conserved sequences of human and bovine genes using primer 3 (26). Primer and annealing temperatures used for PCR are summarized in Table 1. The amplified PCR products were subcloned into the pCRII cloning vector using a T/A Cloning kit (Invitrogen, Carlsbad, CA) and sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing kit and ABI PRISM automated DNA sequencer (Applied Biosystems, Foster City, CA) to confirm identity.

In situ localization of mRNA

In situ hybridization analyses was performed in cross-sections (5 μm) of paraffin-embedded uterine sections (n = 5 ewes/d of pregnancy) using methods described previously (27). Briefly, deparaffinized, rehydrated, and deproteinated cross-sections (5 μm) of the uterine horns from each ewe were hybridized with radiolabeled sense or antisense cRNA probes generated from linearized plasmid DNA templates using *in vitro* transcription with [α-^{35}S] UTP. After hybridization, washing, and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY), stored at 4 C for 4 d to 1 month, and developed in Kodak D-19 developer. Slides were then counterstained with Gill's modified hematoxylin, dehydrated through a graded series of alcohol to xylenes, and protected with a coverslip.

Immunohistochemistry

Immunolocalization of CTNNB1 and TCF7L2 [(transcription factor 7-like 2 (T-cell specific, HMG-box)] proteins was performed in cross-sections (5 μm) of paraffin-embedded uterine sections (n = 5 ewes/d of pregnancy) using specific antibodies and a Vectastain ABC Mouse IgG kit (PK-6102; Vector Laboratories, Burlingame, CA). Mouse monoclonal antibodies to mouse CTNNB1 (610153; BD Biosciences, San Jose, CA) and human TCF7L2 antibody (05-511; Upstate Biotechnology, Lake Placid, NY) were used for immunohistochemistry. The working antibody concentrations for immunohistochemistry were 1.0 μg/ml for CTNNB1 and 0.5 μg/ml for TCF7L2. Negative controls were performed in which the primary antibody was substituted with the same concentration of normal mouse IgG (Sigma, St. Louis, MO). Antigen retrieval using a boiling citrate buffer was performed as described previously (28). Multiple tissue sections from each ewe were processed as sets within an experiment. Sections were not counterstained before affixing the coverslip.

Photomicroscopy

Images of representative fields of sections hybridized with antisense or sense cRNAs were recorded under bright-field or dark-field illumination using a Nikon (Tokyo, Japan) Eclipse 1000 photomicroscope fitted with a Nikon DXM1200 digital camera. Representative photomicrographs of protein immunolocalization were also recorded. Constant image acquisition parameters were used across slides to ensure accurate comparisons. Digital photomicrographs were assembled and labeled using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

TABLE 1. Sequences of primers for RT-PCR

Primer	Sequence of forward and reverse primers (5′–3′)	GenBank accession no.	Annealing temperature (C)	Product size (bp)
WNT2	GCAAAGGCATTTTTGATTGG	BT019608	53	338
	ACGAGGTCATTTTTCGTTGG			
WNT2B	CACTATGGTGTTCGCTTTGC	XM_612293	54	552
	GACGTCCACAGTGTTTCTGC			
WNT4	GCTGGGCTCCAAGTACACC	CA997682	54	241
	GGCTATCCTGACACACATGC			
WNT5A	CATGAACTTGCACAACAACG	BC064694	53	370
	ACAGCACATGAGGTCACAGC			
WNT5B	GAGCACATGGCCTACATCG	CN435723	54	358
	CGAAGTTCTTCTCCCTTTCG			
WNT7A	CCTGGAGGAGAACATGAAGC	CA997684	52	261
	CAGTAATTGGGTGACTTCTCG			
WNT11	TTCCCGATGCTCCTATGAAGG	CB452990	55	430
	ACAGCACATGAGGTCACAAGC			
FZD6	AGTCTTCAGCGGCTTGTATC	BC060836	56	561
	GCTCCGTCCGCTTTCACCTCT			
FZD8	CCCAGCGTCAAGTCTATCG	CK977318	54	325
	TGTGCTGCTCGTAGAAGAGG			
LRP5	AGCTGTACTGGACGGACTCG	CK769878	54	480
	GCGAGTAGAGAGCGCTAAGG			
LRP6	TTGGGCTGATTCAGATCTCC	NM_002336	55	511
	CTGGAACTGGGACTCTGAGC			
DKK1	CACTCCAGATTTTCGGAAGG	BF107232	54	301
	AAGTCAAGTGTGCACCAAGC			
GSK3B	TCCATTCCTTTGGAATCTGC	CB465245	55	448
	AGCAGACGGCTACAAAGTGC			
CDH1	GGCTGAGTTGGACAGAGAGG	AY508164	55	723
	TCATTGCGAGTCACTTCAGG			
CTNNB1	GGTGGGCTGCAGAAAATGGTT	NM_001904	59	567
	GATGGCAGGCTCAGTGATGTCTTC			
LEF1	CTCATCCAGCCATTGTAACG	XM_582726	53	228
	TGCCTAGAATCTGGTTGATAGC			
MYC	TCTGTGGAAAAGAGGCAACC	Z68501	54	486
	CAACTGTTCTCGCCTCTTCC			
MSX2	ACACAAGACCAATCGGAAGC	BF043395	55	331
	GGGGAGCACAGGTCTATGG			

Cell culture

Ovine trophectoderm cells (oTr) were isolated and cultured from d 15 ovine conceptuses and cultured as described previously (29, 30). Briefly, the embryonic disc was removed from the conceptuses, and the remaining tissues were carefully minced, pooled, and placed in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine (BioSource, Camarillo, CA), 700 nM insulin (Invitrogen), 1 mM pyruvate (BioSource), 0.1 mM nonessential amino acids (BioSource), and antibiotics (100 U penicillin, 100 μg streptomycin, and 0.25 μg/ml amphotericin B; Invitrogen). Cultures were maintained in an atmosphere of 5% CO_2 in air at 37 C. Fluid-filled trophoblastic vesicles, which developed spontaneously in culture, were physically ruptured to enhance generation of monolayers. One isolate of cells (oTr-1) was used for these studies and displayed typical epithelial cell morphology with both mononuclear cells and a small population of BNCs for more than 20 passages.

Plasmids

The reporter constructs, Super8XTOPFlash that contained eight copies of TCF/LEF binding sites and Super8XFOPFlash that contained eight mutant LEF/TCF binding sites, were generously provided by Dr. Randall Moon (University of Washington, Seattle, WA) (31). Expression constructs for a constitutively active form of human CTNNB1 (codon 33 substitution of tyrosine for serine, S33Y) (32) and a dominant-negative human TCF7L2 (dnTCF) (33) were generously provided by Dr. Bert Vogelstein (Johns Hopkins University Medical School, Baltimore, MD). A full-length human *WNT7A* cDNA was purchased from Invitrogen. A mouse *Sfrp2* cDNA was generously provided by Dr. Sanjoy Das (Vanderbilt University Medical Center, Nashville, TN) (34). Both human *WNT7A* and mouse *Sfrp2* cDNAs were subcloned into the pcDNA3.1 mammalian expression vector from Invitrogen.

Transient transfections and luciferase assays

The oTr-1 cells were subcultured into 12-well plates and maintained in growth medium. At 70% confluency, cells were transiently transfected using ExGen 500 (Fermentas, Hanover, MD) following the instructions of the manufacturer. Luciferase reporter constructs (1 μg/well) were cotransfected with either pcDNA3.1 vector alone or expression plasmids for WNT7A, S33Y CTNNB1, dnTCF, or Sfrp2 (1 μg total per well). After transfection, cells were maintained in growth medium. Some cells were treated with LiCl (20 mM; Sigma), an inhibitor of GSK3B, at 12 h after transfection. Cells were harvested at 24 h after transfection, and luciferase activity was measured using a Promega (Madison, WI) luciferase assay system. Each experiment was independently repeated three times with different batches of oTr-1 cells between passages 7 and 10.

Cell proliferation assay

The oTr-1 cells were subcultured into 12-well plates (50% confluent) in growth medium for 6–8 h and then switched to serum and insulin-free DMEM for 24 h. The cells were then transfected with either pcDNA3.1 vector alone or expression plasmids for WNT7A, Sfrp2, or their combination (1 μg each per well in quadruplicate) using the Exgen 500 (Fermentas) transfection reagent according to the instructions of the manufacturer. The transfected cells were maintained in serum and insulin-free DMEM for 48 h. Cell number was determined as described previously (35). Briefly, DMEM was removed from cells by vacuum aspiration, and cells were fixed in 50% ethanol for 30 min, followed by vacuum aspiration of the fixative. Fixed cells were stained with a Janus Green B in PBS (0.2% w/v) for 3 min at room temperature. The stain was immediately removed using a vacuum aspirator, and the whole plate was sequentially dipped into water and destained by gentle shaking. The remaining water was removed by shaking, stained cells were immediately lysed in 0.5 N HCl, and absorbance

readings were taken at 595 nm using a microplate reader. As described previously (35), cell numbers were calculated from absorbance readings using the following formula: cell number = (absorbance − 0.00462)/0.00006926. The entire experiment was independently repeated three times with different batches of oTr-1 cells between passages 7 and 10. Cell number data are expressed as a percentage of cells transfected with the empty pcDNA3.1 vector as a control.

Real-time PCR analysis

The oTr-1 cells were cultured and transfected as described above for the cell proliferation assay. At 48 h after transfection, total cellular RNA (n = 3 wells per construct) was isolated from transfected oTr using the Trizol reagent (Invitrogen) according to the recommendations of the manufacturer. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively. The cDNA was synthesized from total RNA (5 μg) using random primers (Invitrogen), oligo-dT primers, and SuperScript II Reverse Transcriptase (Invitrogen) as described previously (36). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 μl of water, and stored at −20 C. PCR analysis of mRNA expression was performed using an ABI PRISM 7900HT Fast Real-Time PCR (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems) as the detector according to recommendations of the manufacturer. Primers were designed to amplify cDNAs of less than 100 bp to maximize efficiency and are summarized in Table 2. PCR cycle parameters were 95 C for 15 sec and 60 C for 1 min for 40 cycles. Data were analyzed using GeneAmp 7900 SDS software (version 2.2.2). The threshold line was set in the linear region of the plots above the baseline noise, and threshold cycle (C_T) values were determined as the cycle number at which the threshold line crossed the amplification curve. PCR without template or template substituted with total RNA were used as negative controls to verify experimental results. The results are shown as 40 − C_T values.

Cell migration assay

The oTr-1 cells (50,000 cells/100 μl serum and insulin-free DMEM) were seeded on 8-μm pore transwell inserts (catalog no. 3422; Corning Costar, Corning, NY). Treatments were then added to each well (n = 3 wells per treatment) including the following: serum and insulin-free DMEM (600 μl) as a negative control; recombinant mouse Wnt5a (catalog no. 645-WN; R & D Systems, Minneapolis, MN) at 100, 500, and 1000 ng/ml; 500 ng/ml recombinant mouse Wnt5a with 10, 50, or 100 μM Y27632 [Rho kinase (ROCK) inhibitor; catalog no. 688001; Calbiochem, San Diego, CA]; 500 ng/ml recombinant mouse Wnt5a with 10, 50, or 100 μM JNK (c-Jun N-terminal protein kinase) inhibitor (catalog no. 159-600-R100; Alexis, San Diego, CA); or growth medium including serum and insulin as a positive control. After 8 h, cells on the upper side of the inserts were removed with a cotton swab. For evaluation of migrated cells on the lower surface, inserts were fixed in 4% paraformaldehyde for 3 min. Then, transwell membranes were removed on the slide and counterstained with 4′,6′-diamidino-2-phenylindole. The migrated cells were systematically counted in five nonoverlapping locations, which covered approximately 70% of the insert membrane growth area, using a Zeiss (Thornwood, NY) Axioplan 2 fluorescence microscope with an Axiocam HR digital camera and Axiovision 4.3 software.

TABLE 2. Sequences of primers for real-time PCR

Primer	Sequence of forward and reverse primers (5′–3′)	GenBank accession no.
MSX2	GCAAGTTCCGCCAGAAACAG GAGGTTCAGAGAGCTGGAGAACTC	XM_592489
MYC	TCAGCAACAACCGCAAATGT TTGTGTGTCCGCCTCTTGTC	Z68501
CST3	GGCGCTGTCCTTTGCG CGGCTCTGGTAAGCGTCG	BC109629
CTSL	CGGGAGAAGGCCCTTATGAA GGCCTGCATCAATAGCAACA	BC102312
ACTB	TCACGGAGCGTGGCTACAG CCTTGATGTCACGGACGATTT	U39357

The entire experiment was independently repeated three times with different batches of oTr-1 cells between passages 7 and 10.

Statistical analyses

All quantitative data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Real-time PCR data were corrected for differences in sample loading using the *ACTB* data as a covariate. Tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P value ≤ 0.05 was considered significant. Data are presented as least-square means with SES.

Results

Cloning and analysis of WNT signaling pathways

RT-PCR analyses detected expression of only 7 of the 19 *WNT* genes (*WNT2*, *WNT2B*, *WNT4*, *WNT5A*, *WNT5B*, *WNT7A*, and *WNT11*) and only 2 of the 10 *FZD* genes (*FZD6* and *FZD8*) in endometria and/or conceptuses. Expression of *LRP5*, *LRP6*, *CDH1*, *GSK3B*, *CTNNB1*, *LEF1*, *TCF4*, *MSX2*, and *MYC* genes were detected in endometria and/or conceptuses. Only one of the four *DKK* genes (*DKK1*) was detected in sheep endometrium by RT-PCR, and none of the six *SFRP* genes were detected in either endometria or conceptuses.

In situ hybridization and immunohistochemical analyses revealed which cell types in the uterus and conceptus expressed specific WNT signaling pathway components (Figs. 1–3). As illustrated in Fig. 1, *WNT2* mRNA was detected primarily in the endometrial stroma and at lower abundance in conceptus trophectoderm and increased after d 16. *WNT2B* mRNA was observed at low abundance primarily in trophectoderm. *WNT4* mRNA was particularly abundant in conceptus trophectoderm and at lower abundance in the endometrial stroma, which increased between d 16 and 20 of pregnancy. Between d 10 and 20 of pregnancy, *WNT5A* mRNA was most abundant in the stratum compactum stroma underlying the LE but was also detected in endometrial epithelia at lower abundance. A similar pattern of expression was found for *WNT5B*. Neither *WNT5A* nor *WNT5B* mRNAs were detected in conceptuses. As expected, *WNT7A* mRNA was detected only in LE and superficial glandular epithelium (sGE) of the endometrium but was not detected in conceptuses. Distinct temporal alterations in expression of *WNT7A* mRNA in endometrial LE and sGE were observed. Although *WNT7A* mRNA was detected in the endometrial LE/sGE of d 10 pregnant ewes, it was absent on d 12 and 14, reappeared on d 16, and then increased to maximal abundance in LE/sGE on d 20. In contrast, *WNT11* mRNA was detected predominantly in the endometrial glands and at lower abundance in the LE but not in trophectoderm. Overall abundance of *WNT11* mRNA in the endometrium did not change during early pregnancy.

FZD6, *FZD8*, and *GSK3B* mRNAs were detected in the conceptus trophectoderm and uterine epithelia (Figs. 1 and 2). The coreceptor *LRP5* mRNA was observed in the conceptus trophectoderm and all endometrial cell types (Fig. 2), whereas the *LRP6* mRNA was most abundant in the endometrial epithelia but at much lower abundance in trophectoderm and endometrial stroma (Fig. 2). *GSK3B* mRNA was detected in both the endometrium and conceptus (Fig. 2). *DKK1* mRNA was very low or not detectable in both uteri or conceptus trophectoderm

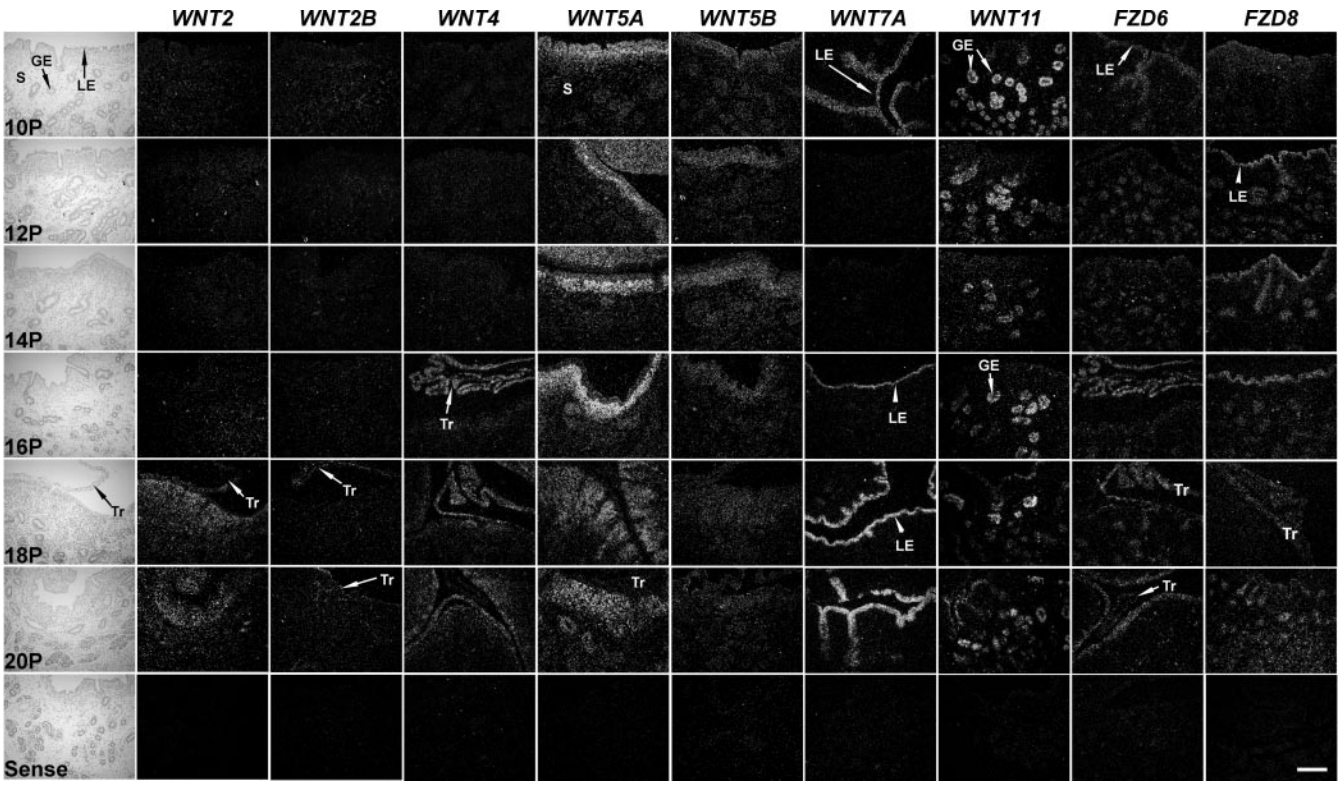

FIG. 1. *In situ* hybridization analysis of WNTs and FZD receptors in endometria and conceptuses from d 10 to 20 of pregnancy (P). In each *column*, representative photomicrographs of *in situ* hybridization results are presented in dark-field illumination. Mel, Melanocyte; S, stroma; Tr, trophectoderm. *Scale bar*, 100 μm.

between d 10 and 14 but appeared on d 16 and was most abundant on d 18 and 20 in endometrial stroma. As expected, *CDH1* mRNA was detected in endometrial epithelia and was particularly abundant in conceptus trophectoderm. The overall levels of *CDH1* mRNA in endometrial LE declined from d 10 to 14 of pregnancy and then increased thereafter. *CTNNB1* mRNA was detected in all uterine cell types but was most abundant in endometrial epithelia and conceptus trophectoderm. *LEF1* mRNA was detected in endometrial epithelia but not in conceptus trophectoderm. Abundant *MSX2* mRNA was detected in conceptus trophectoderm and in endometrial LE and sGE at much lower levels on d 18 and 20 of pregnancy. *MYC* mRNA was detected at low abundance in all uterine cell types and conceptus trophectoderm and was particularly abundant in trophoblast giant BNCs.

As expected from the *in situ* hybridization results, immunoreactive CTNNB1 protein was most abundant in the epithelia of the endometrium and conceptus (Fig. 2). In the endometrium, CTNNB1 protein decreased in the LE after d 10, whereas CTNNB1 protein was abundant in conceptus trophectoderm on d 18 and 20 of pregnancy. In the mononuclear cells of the trophectoderm, CTNNB1 protein was noticeably present at junctions between the cells. Furthermore, CTNNB1 protein was particularly abundant in the cytoplasm and nucleus of trophoblast giant BNCs and endodermal cells. TCF7L2 protein was abundant in nuclei of trophectoderm and endoderm cells of the conceptus. Collectively, these results suggest that WNT signaling via both canonical and noncanonical pathways is present and potentially active in cells of the endometrium and conceptus during the periimplantation period of pregnancy in sheep.

WNT7A stimulates trophectoderm cell proliferation and gene expression

We next focused on *WNT7A*, because it is a novel IFNT-stimulated gene expressed only in endometrial LE and ductal glands in the uterus of sheep and other mammals. *WNT7A* is hypothesized to act in a paracrine manner on conceptus trophectoderm based on results of the present study and a previous study from our laboratory (19).

oTr-1 cells, isolated from d 15 conceptuses, were found to express *WNT2*, *WNT2B*, *WNT4*, *FZD6*, *FZD8*, *LRP5*, *LRP6*, *GSK3B*, *CTNNB1*, *CDH1*, *MSX2*, *MYC*, and *IFNT* but not *WNT7A* or *DKK1* at several different passages by RT-PCR (data not shown). Furthermore, the oTr-1 cells contained CTNNB1 and TCF7L2 proteins of the expected size in Western blot analyses (data not shown). As illustrated in Fig. 4, transfection analyses were used to study WNT7A signaling in oTr cells. In oTr cells transfected with a WNT7A overexpression vector, activity of the TOP-FLASH reporter, containing eight consensus LEF/TCF response elements, was stimulated ($P < 0.001$) 12-fold relative to cells transfected with the pCDNA3.1 empty vector. Although expression of dnTCF and Sfrp2 alone had no effect ($P > 0.10$) on basal activity of the TOP-FLASH reporter in the oTr cells, cotransfection of those inhibitors with WNT7A inhibited ($P < 0.0001$) reporter activity. Transfection of a constitutively ac-

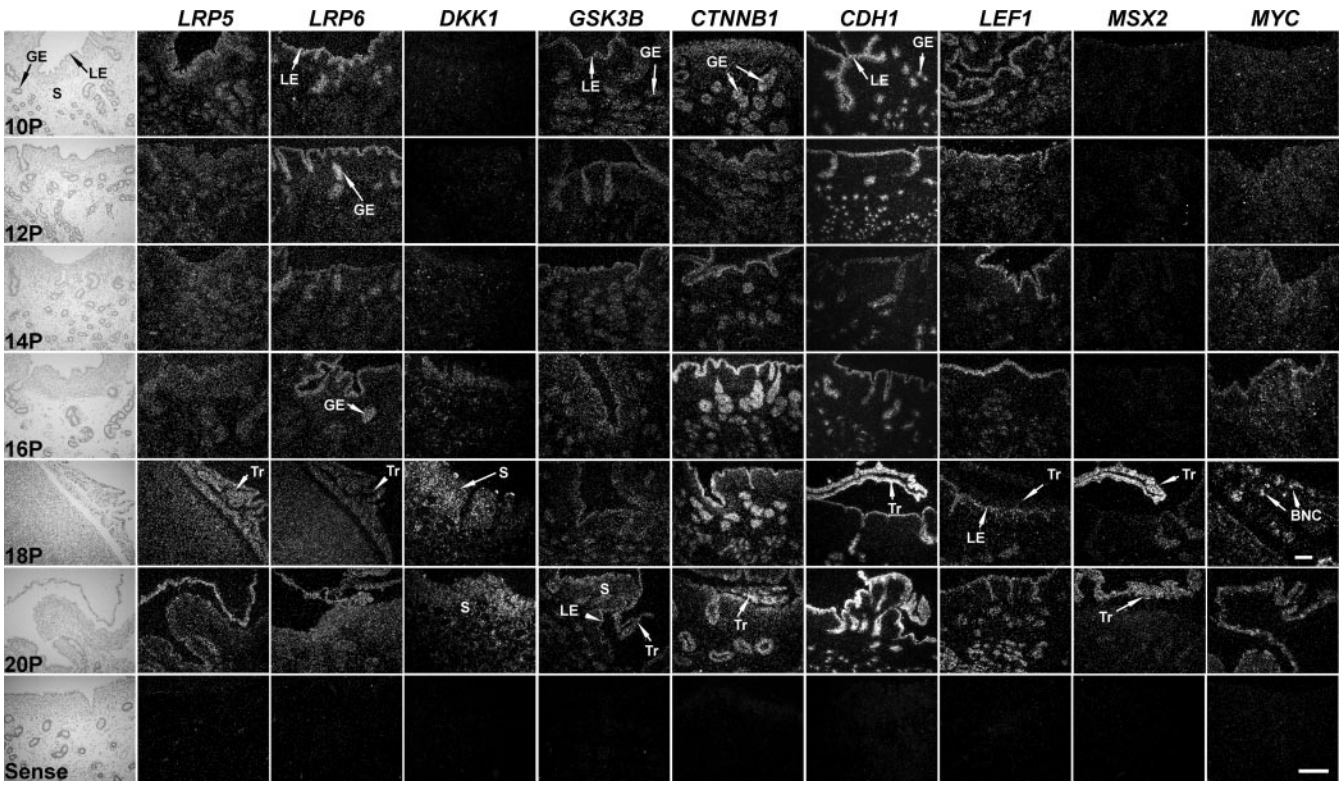

FIG. 2. *In situ* hybridization analysis of WNT signaling pathway components in endometria and conceptuses from d 10 to 20 of pregnancy (P). In each *column*, representative photomicrographs of *in situ* hybridization results are presented in dark-field illumination. Mel, Melanocyte; S, stroma; Tr, trophectoderm. *Scale bars*, 100 μm.

tive CTNNB1 (S33Y) and treatment with LiCl, a GSK3B inhibitor, increased ($P < 0.0001$) TOP-FLASH activity approximately 26- and 29-fold, respectively. Furthermore, transfection of WNT7A had no effect ($P > 0.10$) on a FOP-FLASH reporter containing mutated and inactivated LEF/TCF binding sites (data not shown). Collectively, these results indicate that the oTr cells have an intact canonical WNT signaling pathway that can be activated by WNT7A *in vitro*.

As illustrated in Fig. 5, cell proliferation assays found that transfection of *WNT7A* increased ($P < 0.01$) oTr cell number by approximately 30%. Although *Sfrp2* alone had no effect, *Sfrp2* reduced ($P < 0.05$) *WNT7A*-induced cell proliferation. Next, the effects of *WNT7A* on oTr cell gene expression were studied by transfection and semiquantitative real-time PCR analysis (Fig. 6). The oTr cells were transfected with empty pcDNA3.1 vector, *WNT7A*, *Sfrp2*, or their combination. Transfection with *WNT7A* increased ($P < 0.01$) *MSX2* and *MYC* mRNA levels in oTr cells by 2.1- and 1.6-fold, respectively, compared with cells transfected with vector alone. Although *Sfrp2* alone had no effect, *Sfrp2* inhibited ($P < 0.01$) *WNT7A* stimulation of *MSX2* and *MYC* mRNAs in cotransfected oTr cells. However, neither *WNT7A* nor *Sfrp2* or their combination affected ($P > 0.10$) expression of *CST3* or *CTSL* genes in oTr cells. Collectively, these results indicate that WNT7A from the endometrial LE acts in a paracrine manner on conceptus trophectoderm to stimulate its cell proliferation and transcription of selected genes, such as *MSX2* and *MYC*, via the canonical WNT signaling pathway.

Wnt5a stimulates trophectoderm cell migration

We next focused on *WNT5A*, because it and *WNT11* are expressed constitutively in the endometrium during the periimplantation period of pregnancy and both primarily activate the noncanonical or planar cell polarity pathway involved in cell movement and migration during critical processes such as gastrulation in embryos (9). As illustrated in Fig. 7, treatment of oTr cells in serum- and insulin-free DMEM with 500 and 1000 ng/ml recombinant mouse Wnt5a considerably increased ($P < 0.001$) oTr cell migration. Treatment of oTr cells with Y27632, ROCK inhibitor, and JNK inhibitor did not ($P > 0.10$) affect the basal rate of migration in the absence of Wnt5a. However, the ROCK inhibitor Y27632, but not the JNK inhibitor, reduced ($P < 0.0001$) Wnt5a-stimulated oTr cell migration in a dose-dependent manner. Collectively, these results strongly support the idea that WNT5A, and perhaps WNT11, from the endometrium acts in a paracrine manner on conceptus trophectoderm to stimulate cell movement and migration via activation of the noncanonical WNT signaling pathway involving Rho kinase.

Discussion

This is the first comprehensive analysis of WNTs and their signaling pathway components in the ovine uterus and conceptus during the periimplantation period of pregnancy. Results of the present study indicate that WNTs using the canonical (*WNT2*, *WNT2B*, *WNT4*, and *WNT7A*) and noncanonical (*WNT5A*, *WNT5B*, and *WNT11*) signaling pathways are present

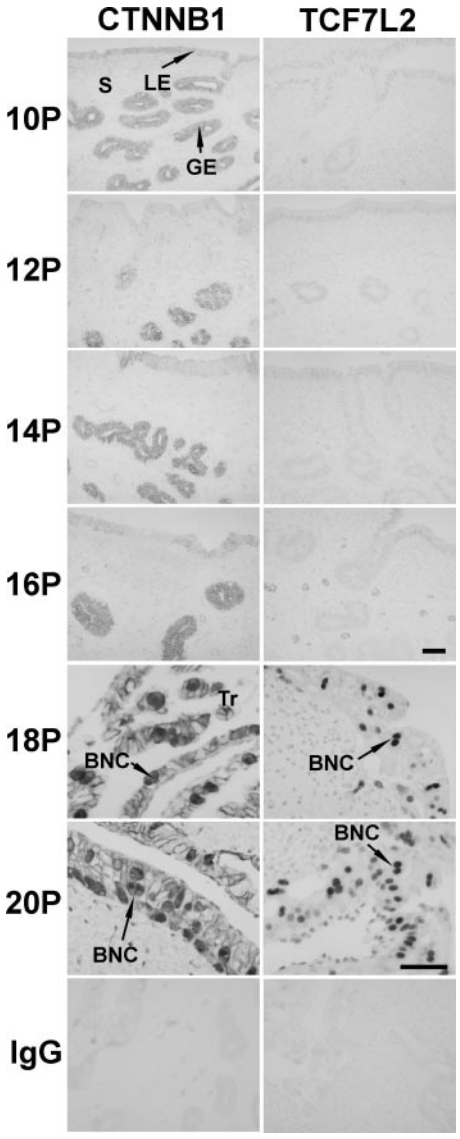

FIG. 3. Immunohistochemical analysis of CTNNB1 and TCF7L2 in endometria and conceptuses from d 10 to 20 of pregnancy (P). Representative photomicrographs of results are presented, and sections were not counterstained. S, Stroma. *Scale bars*, 100 μm.

in endometria and/or conceptuses during early pregnancy. Of particular note, several WNTs (*WNT5A*, *WNT5B*, and *WNT11*) are primarily of endometrial origin and hypothesized to act in a paracrine manner on the developing conceptus. Components of the WNT signaling pathway identified in endometria and/or conceptus trophectoderm include receptors (*FZD6*, *FZD8*, *LRP5*, and *LRP6*), signaling molecules (*GSK3B* and *CTNNB1*), and transcription factors (*LEF1* and TCF7L2). Tulac *et al.* (17) found that WNTs using the canonical (*WNT2*, *WNT3*, *WNT4*, *WNT7A*, and *WNT8B*) and noncanonical (*WNT5A*) signaling pathways were expressed in endometria of women during the secretory phase of the menstrual cycle. In that study, *WNT7A* was detected only in uterine LE, but the spatial expression of the other WNTs was not reported. Few reports of expression of WNTs in uteri during early pregnancy in women or mice are available, but *WNT4* and *WNT5A* are expressed predominantly in uterine stroma of mice during the estrous cycle, whereas *WNT7A* is expressed only in the LE (37). Collectively, available studies demonstrate that *WNT7A* is expressed only in endometrial LE of sheep, mice, and humans (17, 37), implicating the canonical WNT signaling pathway as a conserved regulator of endometrial function across mammals. In human uteri, *WNT7A* is present in the endometrial LE during both the proliferative and secretory phases of the menstrual cycle (17). In the present study, *WNT7A* mRNA was present on d 10, disappeared by d 12, reappeared on d 16, and increased to d 20. In the sheep, the temporal changes in *WNT7A* abundance correlate with loss of progesterone receptors from endometrial LE after d 10 of pregnancy (38) and secretion of IFNT by conceptus trophectoderm on d 16 (39). Indeed, progestins stimulate WNT7A in human endometrial epithelial cells (40), and our previous study determined that *WNT7A* was a novel IFNT-induced gene expressed only by the endometrial LE of the ovine uterus (19). The loss of WNT7A and CTNNB1 in the endometrial LE on d 12 and 14 may allow for synchronization of development of the preimplantation blastocyst and uterus as proposed in mice (41).

All essential components of the canonical WNT signaling pathway were identified in conceptus trophectoderm, including receptors (*FZD6*, *FZD8*, *LRP5*, and *LRP6*), signaling molecules (*GSK3B* and *CTNNB1*) and transcription factors (TCF7L2). Similarly, Pollheimer *et al.* (18) found that WNT ligands, FZD receptors, LRP6, and TCF7L1/TCF7L2 were in human placenta and various trophoblast cell lines. In mice, the canonical Wnt signaling pathway plays a central role in coordinating blastocyst-uterine interactions required for implantation (16). In the former study, canonical Wnt/Ctnnb1 signaling appeared first in circular smooth muscle cells of the myometrium on the antimesometrial side of the uterus early on d 4 of pregnancy and then became restricted to endometrial LE directly apposed to the blastocyst at the time of implantation. Activation of the canonical Wnt signaling pathway required the presence of the blastocyst and inhibition of Wnt/Ctnnb1 signaling interfered with implantation of the blastocyst (16). In the present study, *LEF1* mRNA was observed primarily in endometrial epithelia during early pregnancy. Thus, WNT7A may act in an autocrine manner to regulate endometrial function, as well as in a paracrine manner to regulate periimplantation conceptus morphogenesis. Moreover, it is likely that the other WNTs identified in the endometrial stroma and/or conceptus (WNT2, WNT2B, and WNT4) regulate endometrial function and conceptus growth and differentiation as proposed in the mouse (42) and human (18). In fact, *Wnt2* null mice have placentation defects, resulting in lower birth weights and perinatal lethality (43). The precise role of *Wnt4* in placental development is not known, because *Wnt4* homozygous null mice have a defect in differentiation of the fetal reproductive tract and suffer from perinatal lethality (44).

In the present study, the induction of *WNT7A* in the endometrial LE between d 14 and 16 of pregnancy was temporally correlated with elongation of the conceptus, production of IFNT, and the onset of trophoblast giant BNC differentiation. Indeed, our previous study determined that *WNT7A* was a novel IFNT-induced gene in the uterine LE (19). CTNNB1 and TCF7L2 proteins were particularly abundant in nuclei of trophoblast giant BNCs, suggesting that the canonical WNT signaling pathway is active in those cells of the conceptus. Indeed,

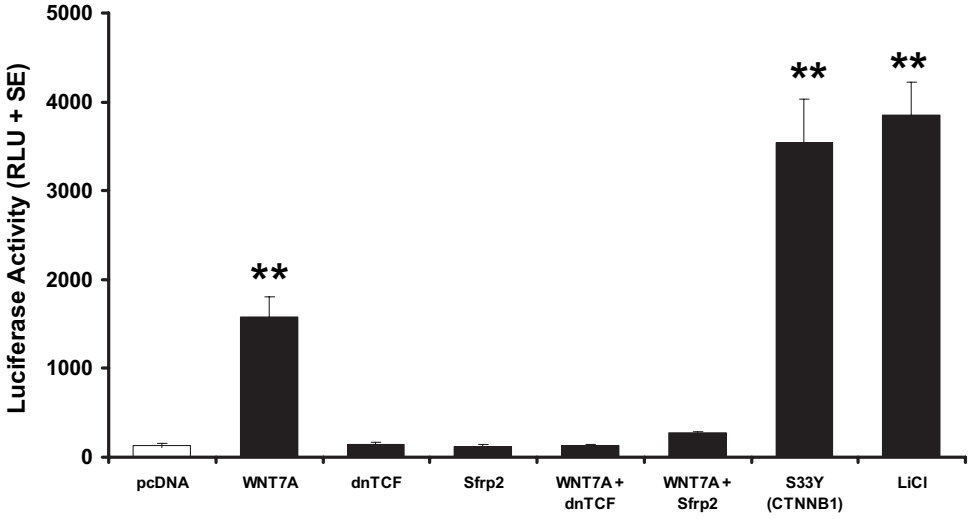

FIG. 4. WNT7A activation of the canonical signaling pathway in oTr cells. A trophectoderm cell line was established from a d 15 ovine conceptus and then transfected with a TCF-luciferase reporter (TOP-FLASH). Cells were cotransfected with pcDNA3.1 empty vector as a control or with *WNT7A*, *dnTCF*, *Sfrp2*, or *S33Y CTNNB1* expression constructs. As a positive control, cells were treated with LiCl, a GSK3B inhibitor, for 24 h. Data are presented as relative luciferase units (RLU) with SE. These experiments were repeated at least three times. **, $P < 0.0001$.

transfection of WNT7A into mononuclear oTr cells stimulated the canonical WNT signaling pathway and cell proliferation and increased abundance of *MSX2* and *MYC* mRNAs, which are genes up-regulated by the canonical WNT signaling pathway in other cell types (45–48). *MSX2*, a homeobox transcription factor, is also expressed in the epithelial-mesenchymal cell layers of developing chorionic villi of human placentae (49). *MYC*, a transcription factor that stimulates genes required for proliferation, is also expressed in the human placenta in which it regulates cell proliferation (50). Homozygous null mice lacking *Myc* perish before 10.5 d of gestation and also exhibit reduced fertility in heterozygous female mice (51). Collectively, available evidence supports the idea that IFNT from the elongating conceptus acts in a paracrine manner to induce *WNT7A* in the endometrial LE, and, in turn, WNT7A acts in a paracrine manner on the conceptus to promote proliferation of the mononuclear cells and perhaps differentiation into trophoblast giant BNCs. Indeed, the canonical WNT signaling pathway promotes invasive human trophoblast differentiation *in vitro* (18), and CTNNB1/TCF has been implicated in trophoblast giant BNC differentiation in cattle (52). Although activation of the WNT signaling pathway was not demonstrated in that study, nuclear accumulation of CTNNB1 was observed during differentiation of bovine trophoblast cells into BNCs by endoreduplication (52). Thus, results from studies of human, cow, and sheep support the hypothesis that WNT/TCF signaling is involved in differentiation of multinucleated cells of the placenta, *e.g.* trophoblast giant BNCs or syncytiotrophoblast depending on the species.

In addition to WNT7A, parallels between human and sheep endometria were also apparent in the expression of *DKK1*, an inhibitor of WNT signaling. DKK1 interacts with LRP5/6 to inhibit WNT signaling by disrupting LRP5/6 binding to the WNT/FZD complex (53, 54). In the present study, *DKK1* mRNA was detected specifically in the stratum compactum stroma of the endometrium beginning on d 16 and increased to d 20. In humans, *DKK1* is regulated by progesterone in uterine endometrial stromal cells (55), induced in the secretory phase of the cycle (17), and increased during the window of implantation (56). Furthermore, Pollheimer *et al.* (18) found that DKK1 reduced migration, invasion, and proliferation of cytotrophoblasts from human placentae. Thus, the induction of *DKK1* in the endometrial stroma of the ovine uterus is hypothesized to inhibit WNT signaling pathways in the trophoblast giant BNCs, and hence endometrial stroma, thereby preventing their migration into

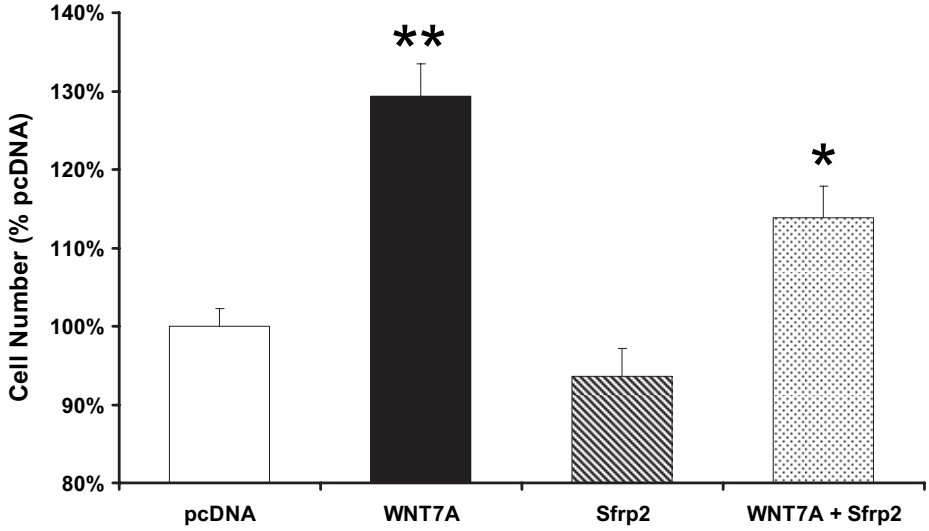

FIG. 5. WNT7A stimulates proliferation of oTr cells. A trophectoderm cell line was established from a d 15 ovine conceptus and then transfected with pcDNA3.1 empty vector as a control, *WNT7A*, *Sfrp2*, or their combination. Cell numbers were determined after 48 h, and data are expressed relative to pcDNA3.1 (100%). *, $P < 0.05$; **, $P < 0.01$.

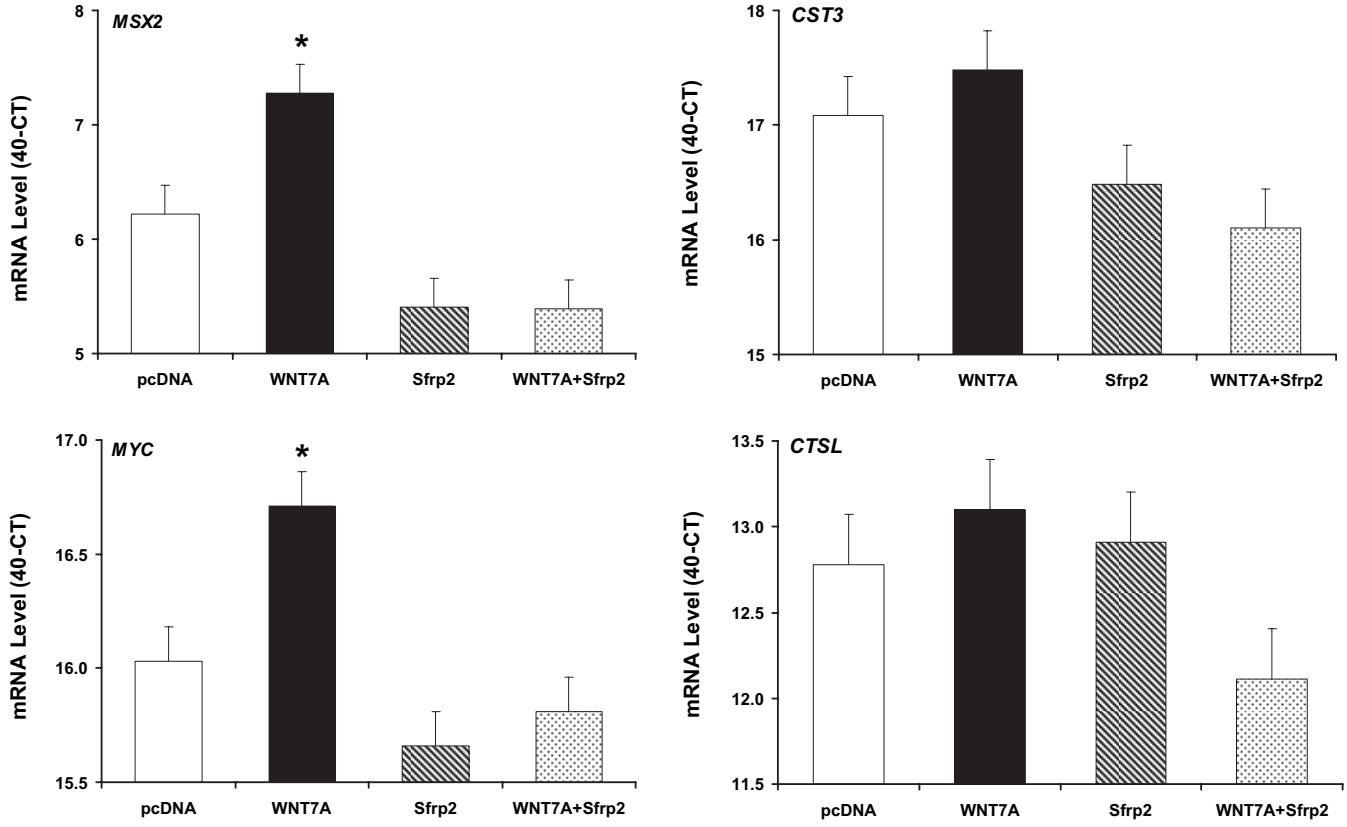

FIG. 6. WNT7A increases expression of *MSX2* and *MYC* but not *CST3* and *CTSL* in oTr cells. A trophectoderm cell line was established from a d 15 ovine conceptus and then transfected with pcDNA3.1 empty vector as a control, *WNT7A*, *Sfrp2*, or their combination. Target mRNA was measured by real-time PCR. Data are expressed as normalized expression ($40 - C_T$). *, $P < 0.05$.

the stroma. The trophoblast giant BNCs arise from mononuclear trophectoderm cells that exit from the mitotic cycle, undergo subsequent endoreduplication, and then migrate and fuse with the endometrial LE and subsequently each other to form multinucleated syncytial plaques that line the chorionic villi of the cotyledonary portion of the placentomes (23). A hallmark of synepitheliochorial placentation in sheep is the lack of invasion of trophoblast giant BNCs and multinucleated syncytial plaques into the endometrial stroma of both caruncular and intercaruncular areas of the uterus (23, 24). Indeed, neither the BNCs nor multinucleated syncytia proliferate after their differentiation.

In addition to being required for canonical WNT signaling, CTNNB1 is a critical component of the cadherin cell adhesion complex that comprises a major cell adhesion system in epithelia (57). In the present study, *CDH1* mRNA was abundant in the endometrial epithelia as well as conceptus trophectoderm, and CTNNB1 protein were localized both to the nucleus and at the periphery of endometrial epithelial cells and trophectoderm cells in d 18 and 20 conceptuses. Dynamic changes in the distribution of CDH1 and CTNNB1 in bovine conceptuses are hypothesized to have important roles in trophoblast giant BNC differentiation, including the rearrangement of cadherin-mediated cell adhesions during cell migration and the onset of endoreduplication, probably via nuclear transfer of CTNNB1 (52).

Results of the present studies also support the hypothesis that the noncanonical WNT or the planar cell polarity pathway also regulates conceptus development. *WNT5A* and *WNT5B* were expressed throughout early pregnancy in the endometrial stratum compactum stroma and at lower abundance in LE. *WNT11* mRNA was also detected throughout early pregnancy but primarily in endometrial glands. Those WNTs appear to primarily activate the noncanonical or planar cell polarity pathway involved in cell movement and proliferation via activation of Rho-ROCK and JNK-JUN pathways (9). In mice, *Wnt5a* and *Wnt11* were expressed after the morula-to-blastocyst transition, suggesting a candidate pathway for embryo-maternal signaling at implantation (58). However, null mutants of *Wnt5a* exhibit perinatal lethality (59), and *Wnt11* mutants exhibit extensive embryonic lethality and perinatal lethality if they survive to term (60). In the present study, Wnt5a increased migration of oTr cells, which was inhibited by an inhibitor of ROCK but not an inhibitor of JNK. Thus, endometrial-derived WNT5A and WNT5B, as well as perhaps WNT11, are proposed to act in a paracrine manner on conceptus trophectoderm to stimulate movement and migration via the Rho-ROCK pathway. The Rho-ROCK pathway stimulates cell movement and migration by regulating the organization of actin filaments required to maintain cell adhesion during movement (61). In contrast to humans and rodents, blastocysts of domestic ruminants must elongate before implantation (21, 22). Trophoblast elongation involves cell migration and proliferation and is required for

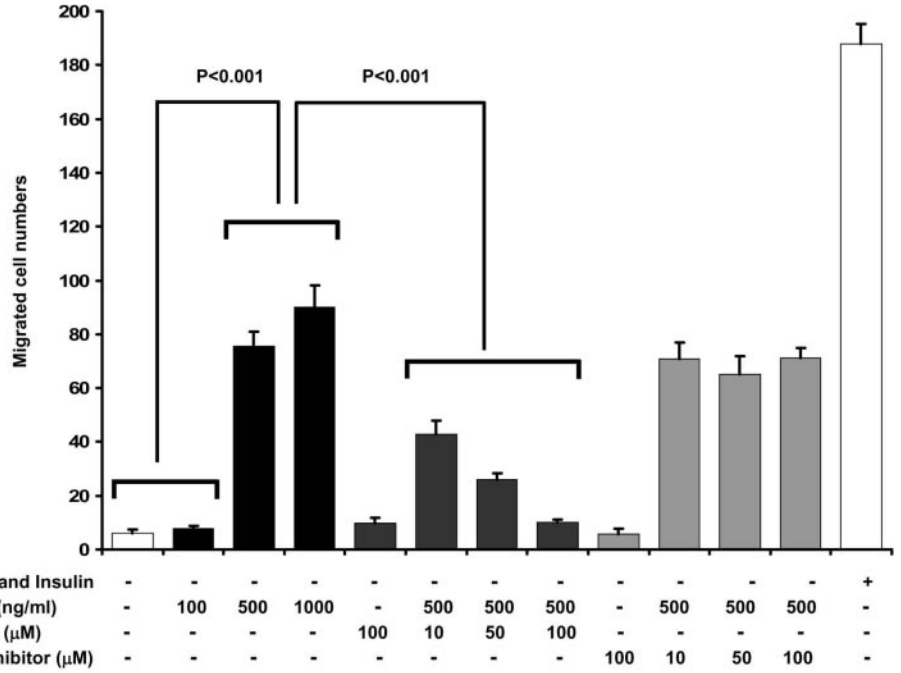

FIG. 7. Wnt5A stimulates migration of oTr cells. The trophectoderm cell line established from a d 15 ovine conceptus was cultured in a transwell plate (n = three wells per treatment) and treated with recombinant mouse Wnt5a, Y27632 (ROCK inhibitor), JNK inhibitor, or their combination. Cells grown in serum- and insulin-containing DMEM served as a positive control. Cell migration was determined after 8 h of treatment and expressed as least-square means ± SE. Statistically significant ($P < 0.001$) differences in cell migration attributable to transfection with Wnt5A and inhibition of cell migration by Y27632 are noted.

formation of the conceptus and developmentally regulated production of IFNT for pregnancy recognition (21, 62). In fact, blastocyst elongation is compromised in the uterine gland knockout ewe model, which lacks endometrial glands and has a reduced amount of LE and stroma (63). It is tempting to speculate that WNT11 may also be involved in conceptus elongation, because *WNT11* mRNA was most abundant in endometrial glands in the present study. Recent evidence indicates that WNT5A and WNT11 regulate gastrulation in zebrafish by activation of Rho kinase (64). The process of gastrulation is very similar to blastocyst elongation in ruminants because both involve massive cell rearrangements, including convergence and extension movements that involve narrowing and lengthening of embryonic tissues as well as cell differentiation (21, 65). In sheep, gastrulation begins after d 9 and is completed by d 16 (65).

In summary, these results support the ideas that canonical and noncanonical WNT signaling pathways are conserved regulators of conceptus-endometrial interactions in mammals and regulate periimplantation ovine conceptus development. Collectively, available results support the following working hypotheses: 1) as blastocysts elongate to a filamentous form, *WNT7A* is induced in the endometrial LE between d 14 and 16 by IFNT from conceptus trophectoderm, and, in turn, endometrial WNT7A acts in a paracrine manner to stimulate proliferation, expression of TCF target genes such as *MSX2* and *MYC*, and differentiation of mononuclear trophectoderm cells into trophoblast giant BNCs from d 14 to 16 of pregnancy; and 2) *WNT5A*, and perhaps *WNT5B* and *WNT11*, from the endometrium act in a paracrine manner to promote conceptus elongation via activation of the planar cell polarity pathway.

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Address all correspondence and requests for reprints to: Thomas E. Spencer, Center for Animal Biotechnology and Genomics, 442 Kleberg Center, 2471 TAMU, Texas A & M University, College Station, Texas 77843-2471. E-mail: tspencer@tamu.edu.

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