WNT regulation of embryonic development likely involves pathways independent of nuclear CTNNB1

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Abstract

The bovine was used to examine the potential for WNT signaling to affect the preimplantation embryo. Expression of seven key genes involved in canonical WNT signaling declined to a nadir at the morula or blastocyst stage. Expression of 80 genes associated with WNT signaling in the morula and inner cell mass (ICM) and trophectoderm (TE) of the blastocyst was also evaluated. Many genes associated with WNT signaling were characterized by low transcript abundance. Seven genes were different between ICM and TE, and all of them were overexpressed in TE as compared to ICM, including *WNT6*, *FZD1*, *FZD7*, *LRP6*, *PORCN*, *APC* and *SFRP1*. Immunoreactive CTNNB1 was localized primarily to the plasma membrane at all stages examined from the 2-cell to blastocyst stages of development. Strikingly, neither CTNNB1 nor non-phospho (i.e., active) CTNNB1 was observed in the nucleus of blastomeres at any stage of development even after the addition of WNT activators to culture. In contrast, CTNNB1 associated with the plasma membrane was increased by activators of WNT signaling. The planar cell polarity pathway (PCP) could be activated in the embryo as indicated by an experiment demonstrating an increase in phospho-JNK in the nucleus of blastocysts treated with the non-canonical WNT11. Furthermore, WNT11 improved development to the blastocyst stage. In conclusion, canonical WNT signaling is attenuated in the preimplantation bovine embryo but WNT can activate the PCP component JNK. Thus, regulation of embryonic development by WNT is likely to involve activation of pathways independent of nuclear actions of CTNNB1.

Reproduction (2017) **153** 405–419

Introduction

WNT signaling is a complex signaling system regulated by 19 WNT ligands that interact with a variety of receptors including FZD, ROR, PTK7 and RYK (Cadigan & Nusse 1997, Logan & Nusse 2004). Among the downstream signaling cascades are the canonical pathway involving binding of WNT to FZD and recruitment of the co-receptor LRP5 or LRP6 (MacDonald *et al.* 2009), the planar cell polarity pathway (Veeman *et al.* 2003, Seifert & Mlodzik 2007) and calcium signaling pathway (Kühl *et al.* 2000, Kohn & Moon 2005). The specific phenotype induced by WNT signaling depends upon the ligands and receptors in play, as well as other specific characteristics of signaling molecules in the cell; thus, a specific WNT–receptor interaction can invoke different outcomes in different cell types (Amerongen *et al.* 2008).

Canonical WNT signaling is the most well-described pathway for WNT signaling and is crucial for a number of developmental processes through regulation of cell

proliferation (Logan & Nusse 2004), maintenance of pluripotency (Sato *et al.* 2004, Sokol 2011), differentiation (Liu *et al.* 2014) and migration (Morosan-Puopolo *et al.* 2014). The central effector of this signaling pathway is CTNNB1, a protein that not only modulates gene expression upon translocation to the nucleus after WNT activation but also serves as a constitutive protein for adherens junctions involved in cell–cell adhesion (Fleming *et al.* 2001). Nuclear accumulation of CTNNB1 is triggered by the inhibition of its degradation by the proteasome induced by a complex consisting of CKI, GSK3 and APC. Once in the nucleus, CTNNB1 displaces GROUCHO to interact with the transcription factors LEF1 and TCF7 to regulate the transcription of genes involved in cell proliferation and pluripotency such as *CCNDBP1* (Tetsu & McCormick 1999) and *MYC* (He *et al.* 1998) respectively.

WNTs are important regulators of mammalian development, but their role during the preimplantation period has not been resolved. In the mouse, inhibition of canonical WNT signaling does not impair blastocyst development (Huelsken *et al.* 2000, Kemler *et al.* 2004, Xie *et al.* 2008, Lyashenko *et al.* 2011) or affect identity, expansion or self-renewal of embryonic stem cells (ESCs) (Lyashenko *et al.* 2011, Wray *et al.* 2011). In other species, the role of canonical WNT signaling in the preimplantation embryo is less clear because of the experimental use of inhibitors or activators of canonical WNT signaling that could have effects on multiple signaling pathways. In the cow, for example, one inhibitor of GSK3B (which causes activation of the canonical pathway) increased the competence of embryos to develop to the blastocyst stage, whereas another inhibitor reduced development (Aparicio *et al.* 2000). A physiological antagonist of canonical WNT signaling, DKK1, enhanced the ability of porcine embryos to undergo hatching (Lim *et al.* 2013), increased trophectoderm (TE) differentiation in pig and cattle (Lim *et al.* 2013, Denicol *et al.* 2014) and increased competence of bovine embryos to establish pregnancy after transfer to females (Denicol *et al.* 2014). Although such results suggest that activation of canonical WNT signaling may inhibit TE differentiation, DKK1 can also regulate other signaling pathways independent of canonical WNT signaling (Caneparo *et al.* 2007, Tahinci *et al.* 2007).

In the human embryo, accumulation of CTNNB1 in the nucleus in response to inhibition of GSK3B depends upon the stage of development, with accumulation being attenuated after Day 3 of development and absent in blastocysts (Krivega *et al.* 2015). Such a result is consistent with findings in the mouse that canonical WNT signaling is not required for development, at least after Day 3, and that developmental changes in the embryo cause a dampening of canonical WNT signaling.

For the current study, the bovine embryo was used as a model to test the overall hypotheses that (1) canonical WNT signaling (i.e. WNT signaling mediated by nuclear localization of CTNNB1) is attenuated in the preimplantation embryo and (2) WNT can activate other signaling pathways in the embryo, as evaluated for activation of JNK. These hypotheses were evaluated in several experiments to characterize the developmental changes in expression of genes involved in WNT signaling, localization of CTNNB1 in blastomere nuclei and accumulation of phospho-JNK in the nucleus after WNT activation.

Materials and methods

Embryo production

Bovine embryos were produced *in vitro* from oocytes obtained from *Bos* (admixture of *B. taurus* and *B. indicus*) ovaries

collected at a local abattoir. Procedures for oocyte recovery and maturation were as described elsewhere (Dobbs *et al.* 2013). After oocyte maturation, oocytes were fertilized for 8–10 h in groups of up to 300 oocytes with sperm pooled from three randomly selected *B. taurus* and *B. indicus* bulls using procedures described elsewhere (Denicol *et al.* 2014). Groups of 25–30 presumptive zygotes were placed in 50 µL microdrops of SOF-BE2 (Kannampuzha-Francis *et al.* 2017) covered with mineral oil (Sigma-Aldrich) and cultured at 38.5°C in a humidified atmosphere of 5% O₂ and 5% CO₂ with the balance N₂. Treatments were applied to cultured embryos by removing 5 µL of culture medium and adding the treatment in a volume of 5 µL.

For immunofluorescence experiments, embryos were produced *in vitro* following procedures described above with a few modifications. Oocytes were harvested using BoviPRO oocyte washing medium (MOFA Global, Verona, WI, USA), and fertilization of matured oocytes was performed using IVF-TL (Parrish *et al.* 1986) (Caisson Laboratories, Logan, UT, USA) containing PHE (80 µL of 0.5 mM penicillamine, 0.25 mM hypotaurine and 25 µM epinephrine in 0.9% (w/v) NaCl) as described by Ortega and coworkers (Ortega *et al.* 2016).

Developmental changes in the expression of selected genes involved in WNT signaling (Experiment 1)

To prepare pools of matured oocytes, cumulus-oocyte complexes (COCs) were harvested at the end of oocyte maturation (20–22 h). Cumulus cells were removed by vortexing for 5 min in HEPES-SOF medium (Denicol *et al.* 2014) containing 1000 U/mL hyaluronidase. Denuded oocytes were washed three times in Dulbecco's phosphate-buffered saline (DPBS) containing 1% (w/v) polyvinylpyrrolidone (DPBS-PVP), incubated in 0.1% (w/v) proteinase solution (protease from *Streptomyces griseus*; Sigma-Aldrich) in DPBS to remove the zona pellucida, washed three times in DPBS-PVP and suspended in groups of 30 in 100 µL extraction buffer from the PicoPure RNA Isolation kit (Applied Biosystems). Samples were stored at –80°C.

Embryos were produced by *in vitro* fertilization in 19 replicates. Embryos were harvested from culture drops at the following stages: 2-cell (28–32 h after insemination (hpi)); 3–4 cell (44–48 hpi); 5–8 cell (50–54 hpi); 9–16 cell (72 hpi); morula (120 hpi) and blastocyst (168 hpi). Embryos were collected, processed as for denuded oocytes to remove the zona pellucida, suspended in groups of 30 in 100 µL extraction buffer from the PicoPure RNA Isolation kit (Applied Biosystems) and stored at –80°C. A separate pool of bulls was used for each replicate, resulting in a total of 19 different bulls.

Transcript abundance was examined for seven genes related to WNT signaling by quantitative real-time PCR (qPCR). Genes included two transcription factors (*LEF1* and *TCF7*), two transcription factor inhibitors (*AES* and *LOC505120* (GROUCHO-like)), two canonical WNT co-receptors (*LRP5* and *LRP6*) and a soluble inhibitor of canonical WNT signaling (*DKK1*) as well as three reference genes (*GAPDH*, *SDHA* and *YWHAZ*). The reference genes were chosen because expression is stable over preimplantation development (Goossens *et al.* 2005), and we verified that developmental

stage did not affect the expression of any of these three genes. Primers are listed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article). Primers for *DKK1*, *GAPDH*, *LRP6*, *SDHA* and *YWHAZ* were previously published ([Goossens et al. 2005](#), [Denicol et al. 2013](#)), whereas those for *AES*, *LEF1*, *LOC505120*, *LRP5* and *TCF7* were designed using software from Integrated DNA Technologies (Coraville, Iowa, USA). Primers were synthesized by Integrated DNA Technologies. All newly designed primer pairs were validated using cDNA from pools of Day 7 bovine blastocysts by generation of a standard curve (efficiency varied from 92.4 to 108.5%), evaluation of melt curves and sequencing of PCR amplicons. Sequences were mapped to the *B. taurus* genome using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information. All sequences aligned to the corresponding gene. RNA of pools of oocytes and embryos was extracted using the PicoPure RNA Isolation kit (Applied Biosystems) following the manufacturer's protocol. DNase treatment was performed using the QIAGEN DNase kit, and mRNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit of Applied Biosystems. The qPCR utilized SsoFast EvaGreen Supermix reagent (Bio-Rad) and was performed with a Bio-Rad CFX96-Real-Time system using conditions described previously ([Dobbs et al. 2013](#)). Two technical replicates were prepared for each sample and the mean cycle threshold (C_T) was calculated. Mean C_T values greater than 35 were considered non-detectable and were assigned a value of 35 for statistical analysis.

A total of five biological replicates containing 30 oocytes or embryos each were subjected to qPCR. Data analyzed were ΔC_T values, which were calculated by subtracting the geometric mean of the three reference genes from the mean C_T value of the sample. For graphical purposes, the relative transcript abundance was calculated as the $2^{\Delta C_T}$. Therefore, abundance of each mRNA type is expressed relative to expression of reference genes.

Data were analyzed by least-squares analysis of variance using the GLM procedure of SAS for Windows, version 9.4 (SAS Institute Inc, Cary, NC, USA). Assumptions of analysis of variance were tested using the univariate procedure of SAS. Results are reported as least-squares means $\pm$ S.E.M. The level of significance was $P < 0.05$.

Characteristics of the WNT signaling system in the morula and ICM and TE as revealed by RNA-Seq (Experiment 2)

Data sets of the transcriptome of three pools of *in vitro*-produced morulae collected at Day 6 after insemination, and three pools of ICM and TE purified from *in vitro*-produced blastocysts at Day 8 after insemination, were examined for stage and cell type effects on expression of 80 genes involved in WNT signaling. Reads were mapped to Btau_4 (<http://genome.ucsc.edu/>). Procedures and data for ICM and TE have been published previously ([Ozawa et al. 2012](#)), and raw data were deposited in the DDBJ Sequence Read Archive at <http://www.ddbj.nig.ac.jp/index-e.html> (Submission DRA000504). The samples of morulae (100 per pool) were produced using the same procedures and in the same replicates of

in vitro fertilization as for ICM and TE. Data were processed following the same bioinformatics methods as reported earlier ([Ozawa et al. 2012](#)). Details of the magnetic-activated cell sorting procedure used to separate ICM from TE were previously published ([Ozawa & Hansen 2011](#)).

RNA-seq data were obtained using a SOLiD v4 sequencer (Applied Biosystems). Data on a subset of genes involved in WNT signaling were evaluated for treatment effects by least-squares analysis of variance using the GLM procedure of SAS for Windows, version 9.4. The dependent variable was number of reads of the transcript, and the independent effect was cell type (morula, ICM and TE). The total transcript reads per sample was used as a covariate. Orthogonal contrasts were used to determine whether transcript abundance differed between morulae and blastocysts (morula vs ICM+TE) or between ICM and TE. Results are reported as least-squares means $\pm$ S.E.M. The level of significance was $P < 0.05$.

Localization of total and active CTNNB1 in bovine embryos as determined by immunofluorescence (Experiments 3 and 4)

Embryos produced *in vitro* were harvested at different stages of development using the same schedule as described earlier. Embryos were washed three times in cold DPBS-PVP, fixed in 4% (v/v) paraformaldehyde in DBPS/PVP for 15 min and washed in DPBS/PVP three times. Immediately thereafter, embryos were incubated in permeabilization solution (DPBS containing 0.5% (v/v) Triton X-100) for 30 min at room temperature, followed by incubation for 1 h in blocking buffer (DPBS containing 5% (w/v) bovine serum albumin (BSA)). Embryos were then incubated overnight at 4°C with 1 μ g/mL of primary antibody in antibody buffer (DPBS containing 0.1% (v/v) Tween 20 and 1% BSA (w/v)). For the detection of total CTNNB1 (Experiment 3), rabbit polyclonal anti-human CTNNB1 antibody (Abcam) was used. For detection of active CTNNB1 (Experiment 4), rabbit polyclonal anti-human non-phospho (active) CTNNB1 antibody (Ser33/37/Thr41; Cell Signaling Technology) was used, and for negative control, primary antibody was replaced with the same concentration of rabbit IgG. Details of all primary antibodies are in [Supplementary Table 2](#). After three washes in washing buffer (DPBS containing 0.1% (v/v) Tween 20 and 0.1% BSA (w/v)), oocytes and embryos were incubated with 1 μ g/mL goat anti-rabbit IgG conjugated with Alexa Fluor 555 (Life Technologies) for 1 h at room temperature in the darkness. Primary and secondary antibodies were diluted in antibody buffer. Nuclear labeling was achieved by incubation with 1 μ g/mL Hoechst 33342 (Sigma-Aldrich) for 15 min at room temperature. Embryos were finally rinsed in DPBS/PVP and placed on a slide containing SlowFade Gold anti-fade reagent (Life Technologies), covered with a coverslip and observed with a 40 $\times$ objective using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss) and Zeiss filter sets 02 (4,6-diamidino-2-phenylindole (DAPI)) and 04 (rhodamine).

Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera. For Experiment 3, evaluation of total CTNNB1 was replicated 7 times with a total of 450 embryos.

For Experiment 4, evaluation of active CTNNB1 was replicated 5 times with a total of 417 embryos.

Changes in immunoreactive active CTNNB1 in embryos after activation of canonical WNT signaling (Experiments 5–8)

For Experiment 5, *in vitro*-produced embryos were cultured in drops as described previously. Culture drops were randomly assigned to stage and treatments. Developmental stages included 3–4 cell (44–48 hpi), 5–8 cell (50–54 hpi), 9–16 cell (72 hpi) and compact morula (120 hpi). At the corresponding time for each developmental stage, 5 μ L of SOF-BE2 in the culture drop were replaced by either 5 μ L GSK3 inhibitor (CHIR-99021 HCL; Tocris Bioscience, Bristol, UK), 5 μ L of the nuclear export inhibitor leptomycin (Sigma), 5 μ L leptomycin + GSK3 inhibitor or 5 μ L vehicle (SOF-BE2 containing 0.04% (v/v) ethanol). Final concentrations were 10 μ M for the GSK3 inhibitor and 22 ng/mL for leptomycin. After 30 min of treatment while embryos were maintained in culture conditions (30 min was chosen because leptomycin B increases nuclear NF κ B p65 in HeLa cells at this time (Wolff *et al.* 1997)), embryos were fixed and labeling for immunofluorescence was carried out as described earlier using antibody against non-phospho (active) CTNNB1 or the corresponding rabbit IgG. A total of 191 embryos were evaluated.

For Experiment 6, canonical WNT signaling in 5–8-cell embryos and morulae was stimulated by addition of final concentrations of either 100 ng/mL human recombinant WNT1 (Sigma-Aldrich; 99% identical amino acid sequence to bovine WNT1), 0.7 μ M of the WNT agonist 2-amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl) pyrimidine (AMBMP, Sigma-Aldrich) or SOF-BE2 containing 0.1% (v/v) DMSO (vehicle). Embryos were harvested 1, 6 and 24 h after treatment and were analyzed by immunofluorescence for nuclear active CTNNB1 ($n=36$ embryos). For Experiment 7, compact morulae were treated with 0.7 μ M AMBMP or vehicle at Day 5 after insemination. Blastocysts were harvested 48 h later for the assessment of nuclear active CTNNB1 ($n=78$ embryos). For Experiment 8, embryos were treated with 10 μ M GSK3 inhibitor (CHIR-99021) on Day 6 after insemination or were used as a control group that received an equivalent amount of vehicle (SOF-BE2 containing 0.04% (v/v) ethanol). Blastocysts were harvested 24 h after treatment for the assessment of nuclear active CTNNB1 ($n=15$ embryos).

Localization of active CTNNB1 in mouse and bovine embryos as evaluated by confocal microscopy (Experiment 9 and 10)

For Experiment 9, cryopreserved 5–8-cell mouse embryos ($B_6C_3F_1 \times B_6D_3F_1$) were obtained from Embryotech Laboratories (Haverhill, MA, USA) and thawed following the supplier's instructions. Embryos were washed with HEPES-TALP and incubated in 50 μ L oil-covered microdrops of EmbryoMax KSOM medium with amino acids (EMD Millipore) for 3 h at 38.5°C in a humidified atmosphere of 5% CO₂ and 5% O₂. The 5–8-cell embryos were fixed in 10% (v/v) formalin for 30 min, permeabilized in 2.5%

(v/v) Tween 20 in DPBS for 20 min, blocked with DPBS containing 5% (w/v) BSA for 1 h and incubated overnight with 1 μ g/mL purified mouse monoclonal IgG against active CTNNB1 (anti-active-CTNNB1 (anti-ABC) clone 8E7; Millipore) at 4°C. After sequential washes with DPBS containing 0.1% (w/v) BSA and 0.1% (v/v) Tween 20, embryos were incubated in affinity-purified goat anti-mouse IgG coupled to fluorescein isothiocyanate (FITC; Abcam) for 1 h at room temperature, followed by 5-min incubation in 1 μ g/mL Hoechst 33342. Embryos were suspended in 10 μ L drops of ProLong Gold anti-fade mounting medium (Thermo Fisher Scientific) in chamber slides. Embryos ($n=5$) were observed using a spinning disk confocal scanner mounted to an Olympus DSU-IX81 inverted fluorescent microscope. Digital images were captured with a 60 $\times$ objective and DAPI and FITC filter sets, using an attached Hamamatsu C4742-12AG monochrome CCD camera.

For Experiment 10, bovine embryos were produced *in vitro* and harvested at the morula stage on Day 5 and blastocyst stage on Day 7 after insemination. Labeling of active CTNNB1 was performed following same procedure and antibody as for Experiment 4 except that embryos were suspended in 10 μ L drops of ProLong Gold anti-fade mounting medium (Thermo Fisher Scientific) in chamber slides. Embryos ($n=4$) were observed and images were captured using the spinning disk confocal scanner and camera mentioned previously, with 40 or 60 $\times$ objectives and DAPI and Texas Red filter sets.

Figure 1 Developmental changes in expression of selected genes involved in WNT signaling for bovine embryos produced *in vitro* (Experiment 1). Expression was assessed by qPCR. Expression of each gene was affected by stage of development ($P=0.004$ for *LRP5* and $P<0.0001$ for the other genes). Data are presented as least-squares means $\pm$ S.E.M. of results from 5 replicates. Blast, blastocyst (168 hpi).

Nuclear localization of CTNNB1 in bovine embryonic fibroblast cells after activation of canonical WNT signaling (Experiment 11)

Cells of the bovine embryonic fibroblast (BEF) cell line developed in our laboratory (Ozawa *et al.* 2012) were studied to verify nuclear labeling of non-phospho (active) CTNNB1

in non-embryonic cells. Cells were cultured with Dulbecco's modified Eagle's Medium (Gibco, Thermo Fisher Scientific) containing 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic (10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B; Sigma-Aldrich). Cells were initially cultured in cell culture flasks (Corning)

Table 1 Effect of stage of development and cell lineage (inner cell mass (ICM) vs trophectoderm (TE)) on expression of genes involved in WNT signaling for Day 6 morula and Day 8 bovine blastocysts produced *in vitro*.^a

Gene	Number of reads			P value	
	Morula	ICM	TE	Morula vs (ICM+TE)	ICM vs TE
WNTs ^b					
WNT2	18.49	15.19	17.66	0.778	0.784
WNT2B	22.00	21.00	50.67	0.079	0.952
WNT6	0.20	2.96	14.51	<0.0001	<0.0001
WNT8A	5.82	0.27	1.12	0.079	0.664
WNT10A	395.0	468.0	652.7	0.068	0.548
WNT11	101.0	91.33	119.7	0.376	0.745
WNT16	4.09	23.65	12.26	0.236	0.409
WNT processing					
PORCN	196.97	213.19	450.17	0.016	0.004
Frizzled receptors and LRP co-receptors ^c					
FZD1	145.00	82.33	140.00	0.159	0.016
FZD3	9.62	3.43	5.29	0.098	0.585
FZD6	62.29	108.59	132.79	0.032	0.367
FZD7	31.21	18.56	55.90	0.231	0.001
FZD8	91.00	50.49	58.17	0.385	0.877
FZD10	73.33	59.33	75.33	0.539	0.415
LRP6	302.90	264.38	412.72	0.390	0.024
Proteins involved in canonical signaling ^d					
APC	14.43	12.47	19.77	0.247	0.006
AXIN1	30.56	18.00	31.44	0.450	0.186
CNBP	11.79	9.83	9.38	0.469	0.901
CTNNB1	584.6	5897.4	6838.01	0.673	0.540
DVL2	9.89	4.84	6.60	0.315	0.717
DVL3	49.99	37.32	66.36	0.913	0.203
GSK3B	1144.6	549.73	720.33	0.060	0.539
Transcription factors ^e					
LEF1	61.55	1.64	3.14	0.096	0.968
TCF7L1	12.16	16.53	10.31	0.859	0.486
TCF7L2	556.98	211.86	116.16	0.003	0.339
Transcription factors inhibitors ^f					
AES	48.68	136.79	181.86	0.04	0.402
TLE3	17.67	89.67	122.00	0.154	0.187
R-spondin signaling ^g					
LGR4	166.23	60.65	102.46	0.032	0.289
LOC100337123 ^h	11.74	3.25	5.67	0.002	0.174
RSPO1	202.67	187.00	211.00	0.703	0.749
RSPO2	11.91	0.62	4.04	0.118	0.514
RSPO3	1925.6	643.08	301.03	0.019	0.540
Other soluble WNT regulatory proteins ⁱ					
DKK1	15.71	3.22	1.06	0.056	0.761
NDP	1498.1	1510.6	1779.3	0.670	0.530
SFRP1	124.75	48.81	143.11	0.145	0.006
SFRP2	61.00	16.00	25.67	0.700	0.236
SFRP3	63.47	23.03	16.83	0.236	0.882
SFRP4	31.67	25.67	38.67	0.127	0.393
WIF1	96.78	30.92	33.30	0.040	0.937
Other WNT signaling proteins ^j					
DACT2	49.56	41.19	43.25	0.367	0.830
KREMEN1	53.14	9.67	15.87	0.011	0.643
RYK	97.14	46.14	79.40	0.172	0.265

^aData are least-squares means of number of reads. ^bThe following genes had <5 reads: WNT1, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT7A, LOC100337066 (WNT7B paralog), WNT8B, WNT9A, WNT9B and WNT10B. ^cThe following genes had <5 reads: FZD2, FZD4, FZD5, FZD9 and LRP5. ^dThe following genes had <5 reads: AXIN2, DVL1. ^eThe following genes had <5 reads: TCF7. ^fThe following genes had <5 reads: LOC505120 (GROUCHO ortholog), TLE1, TLE2, TLE4 and TLE6. ^gThe following genes had <5 reads: LGR5, LGR6 and RSPO5. ^hRSPO3 like. ⁱThe following genes had <5 reads: DKK4 and SFRP5. ^jThe following genes had <5 reads: ANKRD6, DACT1, DACT3, FRAT1, KREMEN2, NKD2, VANGL1 and VANGL2.

Figure 2 Representative examples of localization of immunoreactive CTNNB1 at various stages of preimplantation development of bovine embryos produced *in vitro* (Experiment 3). Embryos were labeled with antibody to CTNNB1 (red) and DNA (blue). The total number of embryos evaluated was 450.

at 38.5°C in a humidified atmosphere of 5% CO₂. At 90% confluency, cells were trypsinized (0.25% (w/v) trypsin, Life Technologies), mixed with an equal volume of culture medium, centrifuged for 10 min at 1750g, resuspended in culture medium and counted (Automated Cell Counter, Bio-Rad). Cells were seeded in 8-chamber slides (Sigma-Aldrich) at a density of 10,000 cells/well and were allowed to adhere overnight in culture medium. Culture medium containing treatments were used to replace medium. Treatments included 10 µM GSK3 inhibitor (CHIR-99021), 0.7 µM AMBMP or vehicle (0.5% (v/v) DMSO). Concentrations were chosen based on the effect of these molecules in other studies (Denicol *et al.* 2013, Lappas 2014). Fixation and immunolabeling of cells were performed

24 h after treatment using 4% paraformaldehyde for 20 min, followed by permeabilization with DPBS containing 0.5% (v/v) Triton X-100 during 30 min and blocking for 1 h with DPBS containing 5% (w/v) BSA. Incubation with primary antibody (rabbit anti-human polyclonal non-phospho (active) CTNNB1 (Ser33/37/Thr41); Cell Signaling Technology) or rabbit IgG proceeded overnight at 4°C. Incubation with goat anti-rabbit IgG conjugated with Alexa Fluor 555 proceeded for 1 h, followed by DNA labeling using Hoechst 33342. Cells were observed and images were captured following methods described for mouse embryos; DAPI and Texas Red fluorescence filters were used. Image analyses consisted of quantification of proportion of cells depicting nuclear CTNNB1.

Figure 3 Representative examples of localization of immunoreactive non-phospho (active) CTNNB1 during preimplantation development of bovine embryos produced *in vitro* (Experiment 4). Embryos were labeled with antibody to non-phospho (active) CTNNB1 (red) and DNA (blue). A total of 417 embryos was evaluated.

Figure 4 Lack of localization of active CTNNB1 in the nucleus of *in vitro*-produced bovine embryos after activation of canonical WNT signaling with the GSK3 inhibitor (Experiment 5). Leptomycin was added to reduce nuclear export of CTNNB1. Shown are representative images of individual embryos labeled with antibody to non-phospho (active) CTNNB1 (red) and DNA (blue) treated at the 9–16-cell stage and harvested 24 h after stimulation. Similar results were seen for embryos treated at other stages of development (total number of embryos examined = 191).

Vehicle	+	+	+	+
Leptomycin	-	+	-	+
GSK3 inhibitor	-	-	+	+

A total of 41 images were analyzed. The proportion of cells showing nuclear localization of active CTNNB1 was analyzed by logistic regression using the LOGISTIC procedure of SAS for Windows, version 9.4 (SAS Institute Inc.) including treatment as a fixed effect. The PDIF means separation test was used to determine which treatments differed from control cells. The level of significance was $P < 0.05$.

Non-canonical WNT signaling mediated by phosphorylation of JNK (i.e. MAPK8) by WNT11 in bovine blastocysts (Experiments 12–14)

Experiment 12 was performed to determine whether WNTs could activate one component of the planar cell polarity pathway in bovine embryos. Embryos were produced *in vitro* as described. At Day 7 after insemination, culture drops were randomly assigned to treatments. In each culture drop, 5 μ L of SOF-BE2 were replaced by either 5 μ L human recombinant WNT11 (R&D Systems; 98% amino acid sequence identity with bovine WNT11) or 5 μ L vehicle (SOF-BE2 containing 0.01% (w/v) BSA (in addition to BSA included in SOF-BE2 formulation)). Final concentrations of WNT11 were 0.5, 1 and 2.5 μ g/mL. Blastocysts were harvested 6 h after treatment and fixed in 4% (v/v) paraformaldehyde. Immunolabeling was performed as described for CTNNB1 except that the primary antibody was 1 μ g/mL anti-phospho-JNK ((Thr183/Tyr185 and Thr221/Tyr223), Millipore) and 1 μ g/mL rabbit IgG served as a negative control. Note that the phosphorylation site is conserved among human, mouse and bovine and that the amino acid sequence identity between these species is 97–99%. Embryos were then incubated with 1 μ g/mL goat anti-rabbit IgG conjugated with Alexa Fluor 555 (Life Technologies), and nuclear labeling was performed using Hoechst 33342. Embryos were evaluated using a Zeiss Axioplan 2 epifluorescence microscope as described earlier. Quantification of intensity of labeling with

antibody was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA; version 1.70_02). Total immunoreactive phospho-JNK was measured as follows: after selection of the area encompassing the entire embryo, the mean intensity was obtained using the Measure Analysis feature and the background intensity was obtained from the area surrounding the embryo. The latter was subtracted from the embryo intensity before statistical analysis. A total of 49 embryos were analyzed. Data were analyzed by least-squares analysis of variance using the PROC GLM procedure of SAS for Windows, version 9.4 (SAS Institute Inc.) including WNT11 concentration as a fixed effect. Differences between individual concentrations of WNT11 and control embryos were assessed using the PDIF mean separation test of SAS.

Experiment 13 was performed to test whether WNT11 affected the competence of embryos to develop to the blastocyst stage and allocation of blastocyst blastomeres to TE and ICM. Treatments (vehicle and 2.5 μ g/mL WNT11) were applied at Day 5 after fertilization as described previously. Blastocyst development was assessed on Day 7 after fertilization, and blastocysts with clearly delineated blastocoels were harvested, fixed, permeabilized and blocked as described previously. Trophectoderm cells were identified by localization of a transcription factor crucial for differentiation of TE (caudal type homeobox 2 (CDX2); (Berg *et al.* 2011)). Labeling was achieved by sequential incubation with mouse anti-human polyclonal CDX2 antibody ready to use (Biogenex, Fremont, CA, USA) and 1 μ g/mL goat anti-mouse IgG conjugated with FITC (Abcam). Total number of cells was determined by counting DNA-labeled nuclei by labeling with 1 μ g/mL Hoechst 33342 after immunolabeling for CDX2. Number of ICM was calculated as the difference between total cells and TE. The experiment was performed in 5 replicates with a total of 484 COCs and semen from 13 different bulls. Blastocyst cell number was evaluated for 74 embryos, and data were analyzed for the effect of treatment using Proc Glimmix of SAS for Windows, version 9.4 (SAS

Figure 5 Consequences of treatment of bovine embryos with WNT agonists for immunolocalization of active CTNNB1. Shown in panel A (Experiment 6; $n=36$ embryos) are representative images of individual embryos produced *in vitro* that were treated at the 5–8 cell stage with vehicle, the WNT agonist AMBMP or recombinant WNT1, harvested 24 h later and labeled with non-phospho (active) CTNNB1 (red) and DNA (blue). Shown in panel B (Experiment 8; $n=15$ embryos) are representative images of individual embryos produced *in vitro* that were treated at Day 6 after insemination with vehicle or GSK3 inhibitor, harvested 24 h later and labeled with non-phospho (active) CTNNB1 (red) and DNA (blue).

Institute Inc.). Each embryo was considered an observation, and development (0=not developed to blastocyst; 1=developed to blastocyst) was considered a binary variable. Results are presented as least-squares means $\pm$ S.E.M.

Results

Developmental changes in expression of selected genes related to WNT signaling for embryos produced *in vitro* (Experiment 1)

Results on the expression of seven genes related to WNT signaling in embryos produced *in vitro* are shown in Fig. 1. Expression of each gene was affected by stage of development ($P=0.004$ for *LRP5* and $P<0.0001$ for the other genes). There were several distinct developmental patterns in gene expression. The first was a continuous decline in transcript abundance from the oocyte to the morula stage. Only *AES* showed this pattern and,

for this gene, there was a slight increase in transcript abundance by the blastocyst stage. A second pattern, shown for *DKK1*, was a continuous increase from the oocyte stage to the 9–16 cell stage followed by a decline in transcript abundance. For the other five genes, there was a slight increase in transcript abundance from the oocyte to the 2-cell or 4-cell stage followed by a decline in transcript abundance after the 2-cell, 4-cell or 5–8-cell stage. Transcripts for two genes, *DKK1* and *LEF1*, were not detectable at the blastocyst stage.

Characteristics of the WNT signaling system in the morula and ICM and TE of *in vitro*-produced embryos as revealed by RNA-Seq (Experiment 2)

A RNA-Seq database of the transcriptomes of *in vitro*-produced Day 6 morulae and isolated TE and ICM of Day 8 blastocysts (Ozawa *et al.* 2012) was assessed for the expression of 80 genes associated with

Figure 6 Localization of active CTNNB1 in mouse and bovine embryos by confocal microscopy. Shown in panel A (Experiment 10) are images of 2 individual 5-cell mouse embryos labeled with non-phospho (active) CTNNB1 (green) and Hoechst (blue). A total of 5 embryos were examined. Shown in panel B (Experiment 11) are representative confocal images of bovine embryos at the morula stage (top), at the blastocyst stage (middle), and at the blastocyst stage captured with higher magnification (bottom). A total of 4 embryos were examined.

WNT signaling. Genes were considered expressed if the average number of reads was ≥ 5 . Results are summarized in Table 1.

Only 7 of 19 WNT genes were expressed with the remaining 12 WNT genes having < 5 reads or being not detected. Of the 7 WNT that were expressed, only one, *WNT6*, varied in expression between groups. Expression was higher for TE than ICM or morula. A key gene involved in post-translational modification of WNTs, *PORCN*, was highly expressed, and transcript abundance was higher for TE than morula or ICM.

A total of 6 of 10 FZD receptor genes were expressed in the morula or blastocyst. Expression of *FZD1* and *FZD7* was lower for ICM than that for TE. In contrast, expression of *FZD6* was higher for both ICM and TE of the blastocyst than that for the morula. The FZD co-receptor gene, *LRP6*, was abundantly expressed but number of reads for *LRP5* was < 5 .

Among the genes involved in canonical WNT signaling that were expressed were *CTNNB1* and genes involved in the CTNNB1 destruction complex (*APC*, *AXIN1* and *GSK3B*). *DVL1*, which acts to bind FZD

proteins and transmit information about WNT binding, was not considered expressed (< 5 reads). However, two other disheveled genes (*DVL2* and *DVL3*) were expressed. The only gene involved in CTNNB1 metabolism that varied with stage of development was *APC*, which was expressed more in TE than ICM.

Expression of the two WNT-regulated transcription factor genes, *TCF7* and *LEF1*, was low. In addition, expression of *TCF7L1* was relatively low in both morula and blastocysts. In contrast, *TCF7L2* was highly expressed although expression declined from the morula to the blastocyst stage for both ICM and TE. In contrast, the transcription factor inhibitor, *AES*, increased in transcript abundance from the morula to blastocyst stage for both ICM and TE.

Expression was also examined for several genes that can promote or antagonize canonical WNT signaling. Among such genes that were affected by developmental stage, were the WNT stimulatory molecule, *RSPO3* and one of its receptors, *LGR4*. Expression of both genes was significantly lower for ICM and TE of the blastocyst than for morula. Two antagonists of canonical WNT signaling also declined from the morula to blastocyst stage, *DKK1*

and *WIF1*. *KREMEN1*, which can function as a DKK1 receptor, was also reduced in expression for ICM and TE of the blastocyst as compared to the morula.

Note that the pattern of gene expression was generally consistent with the earlier experiment (Fig. 1). In the first experiment, expression of six genes declined from the Day 5 morula to the Day 7 blastocyst. A similar decline occurred from the Day 6 morula to Day 8 blastocyst for *DKK1* and *LEF1*. *LRP6* did not change in expression, and *LOC505120* and *TCF7* were not detected by RNA-seq and *LRP5* was barely detectable. There was one

gene whose expression increased very slightly from the morula to blastocyst stage in the first experiment, *AES*, and a larger increase was observed when comparing the Day 6 morula to Day 8 blastocyst.

Localization of total and active CTNNB1 in bovine preimplantation embryos as determined by immunofluorescence (Experiments 3 and 4)

Expression of genes coding for molecules involved in WNT signaling was indicative that canonical WNT

Figure 7 Immunoreactive phospho-JNK in bovine blastocysts produced *in vitro* (Experiment 12). Shown in panel A are representative images of embryos labeled with phospho-JNK (red) and Hoechst (blue) that were treated at the blastocyst stage with 0, 0.5, 1 or 2.5 µg/mL of recombinant WNT11 and harvested 6 h later. Shown in panel B are average values of intensity of fluorescence of phospho-JNK in the whole area of the embryo (*n* = 49 embryos). ****P* < 0.0001 from 0 ng/mL.

signaling is partially silenced at the morula and blastocyst stages. To further explore this idea, two experiments were conducted to evaluate whether a key feature of canonical WNT signaling, nuclear localization of CTNNB1, occurs during preimplantation embryonic development.

For Experiment 3, an antibody recognizing both active (non-phosphorylated) and inactive (phosphorylated) CTNNB1 was used to determine the localization of total CTNNB1. Representative images are shown in Fig. 2. Regardless of stage of development, most immunoreactive CTNNB1 was localized to cell membranes; immunoreactive protein in the nucleus was absent in all but the occasional cell.

As nuclear CTNNB1 was not observed at any developmental stage, Experiment 4 was performed using an antibody specific for active CTNNB1 (i.e., non-phosphorylated CTNNB1) (Fig. 3). As for total CTNNB1, most active CTNNB1 was localized to plasma membranes and, except for the scattered nucleus, nuclear localization was absent at all developmental stages.

Failure of canonical WNT activators to induce localization of nuclear active CTNNB1 (Experiments 5–8)

One possible reason for the lack of nuclear CTNNB1 in embryos is the absence of stimulation by canonical WNTs. To test this hypothesis, three experiments were conducted to evaluate the localization of active CTNNB1 after stimulation of WNT signaling. In Experiment 5, embryos were treated with a GSK3 inhibitor at the 3–4-cell, 5–8-cell, 9–16-cell and morula stages of development. Inhibition of GSK3 leads to the accumulation of CTNNB1 and import into the nucleus (Yuan *et al.* 2005). Some control and GSK3 inhibitor-treated embryos were also treated with leptomycin to block nuclear exportins. This treatment was added to enhance nuclear localization of CTNNB1 in case nuclear CTNNB1 induced by GSK3 inhibition is rapidly exported from the nucleus. Representative images are shown in Fig. 4. Active CTNNB1 remained localized to non-nuclear regions

Table 2 Effect of 2.5 µg/mL WNT11 from Day 5 to Day 7 after insemination on the development of bovine embryos to the blastocyst stage at Day 7 after insemination.^a

Treatment	Percent blastocyst	Blastocyst cell number		
		Total	TE	ICM
Vehicle	17.8±2.5	127±6	89±5	38±3
WNT11	25.5±2.7	138±6	93±4	45±3
<i>P</i> value	0.042	0.210	0.529	0.137

^aData are the least-squares means±S.E.M. of results. Data on percent blastocyst represent the percent of inseminated oocytes that developed to the blastocyst stage. The total number of oocytes was 484. Blastocyst cell number was evaluated for 74 embryos.

of the cells and to the plasma membrane in particular. With the exception of the occasional cell, there was no accumulation of CTNNB1 in the nucleus at any stage of development.

For Experiment 6, embryos at the 5–8-cell or morulae stages of development were treated with either the WNT agonist AMBMP or human WNT1 and localization of active CTNNB1 was determined after 1, 6, 24 and 48 h of incubation. Although both treatments increased the intensity of labeling for active CTNNB1 in the plasma membrane, there was no accumulation of detectable CTNNB1 in the nucleus (Fig. 5A). For Experiments 7 (results not shown) and 8 (Fig. 5B), treatment of morulae with AMBMP at Day 5 or GSK3 inhibitor on Day 6 after insemination also failed to increase nuclear labeling with CTNNB1 in resultant blastocysts at Day 7. Both treatments did increase the immunoreactive active CTNNB1 localized in the plasma membrane (Fig. 5B).

Localization of active CTNNB1 in mouse and bovine embryos evaluated by confocal microscopy (Experiments 9 and 10)

As found for bovine embryos using epifluorescence microscopy, there was no observable active CTNNB1 in the nucleus of mouse 5–8-cell embryos (Fig. 6A). Similarly, no active CTNNB1 was observed in the nuclei of bovine embryos when embryos were examined by confocal microscopy (Fig. 6B).

Nuclear localization of CTNNB1 in bovine embryonic fibroblast cells after activation of canonical WNT signaling (Experiment 11)

To test whether the absence of nuclear localization of active CTNNB1 was a unique feature of preimplantation embryos, localization of the protein was also examined in BEF cells derived from bovine embryonic fibroblasts. In these cells, punctuate labeling of active CTNNB1 was observed in the nucleus of a fraction of cells (Supplementary Fig. 1). The proportion of cells depicting nuclear localization of active CTNNB1 was 310/763 (40.6%) for control cells vs 67/148 (45.3%) for cells treated with AMBMP (*P*=0.29 for difference from control) and 750/839 (89.4%) for cells treated with GSK3 inhibitor (*P*<0.0001 for difference from control).

Actions of WNT11 on phosphorylation of the non-canonical signaling protein JNK and development to the blastocyst stage (Experiments 12 and 13)

Immunoreactive phospho-JNK was localized in nuclei (Fig. 7A). Moreover, the pattern of nuclear labeling of phospho-JNK was punctuate. The degree of labeling varied between cells although labeling was not consistently elevated in TE or ICM. Treatment of

blastocysts with human recombinant WNT11 caused a significant increase in intensity of labeling of phospho-JNK at 2.5 µg/mL ($P < 0.0001$) but not at 0.5 or 1 µg/mL (Fig. 7B).

In Experiment 13, effect of WNT11 on development and blastocyst cell number was determined (Table 2). Treatment with WNT11 increased the proportion of oocytes that developed to the blastocyst stage ($P = 0.042$) but had no effect on number of ICM, TE or total cells in the resulting blastocysts.

Discussion

The mouse embryo does not require canonical WNT signaling for either development to the blastocyst stage or ESC identity, expansion or self-renewal (Huelsenken *et al.* 2000, Kemler *et al.* 2004, Xie *et al.* 2008, Lyashenko *et al.* 2011). The situation for other species is less clear. Here, we show that one of the characteristics of preimplantation development in the cow is a temporal decrease in the expression of key genes involved in WNT signaling along with a paucity of nuclear CTNNB1, even after stimulation of the embryo with molecules that activate canonical WNT signaling. These observations are consistent with the idea that, like the mouse, canonical WNT signaling is dispensable for blastocyst development in the cow. In contrast, non-canonical WNT signaling improved embryonic development because WNT11 increased the proportion of embryos becoming blastocysts while also increasing the phosphorylation of JNK, a central player in WNT/planar cell polarity (PCP) pathway (Zeke *et al.* 2016). Observed changes in gene expression also mean that, like for the human (Krivega *et al.* 2015), characteristics of WNT signaling are likely to change during development. By the blastocyst stage, WNT signaling may play different roles in the ICM and TE because of the differences in expression of several important genes in the WNT signaling system.

A key observation of the current series of experiments was, with rare exceptions, the absence of observable immunoreactive CTNNB1 in the nucleus of embryos at every stage examined. Failure to observe nuclear CTNNB1 was not because of failure of the antibodies used to recognize the molecule because immunoreactive total and active CTNNB1 could be localized to the plasma membrane. Lack of nuclear CTNNB1 was observed even after embryos were treated with molecules expected to activate canonical WNT signaling including a GSK3 inhibitor, the WNT agonist AMBMP (Liu *et al.* 2005) or the canonical WNT1 (Shimizu *et al.* 1997, Yuan *et al.* 2005). The lack of nuclear CTNNB1 was not due to rapid export from the nucleus because inhibition of nuclear exportins with leptomycin did not lead to the accumulation of CTNNB1 in the nucleus. Failure of the molecules to induce nuclear localization was not

because the molecules were inactive because all three WNT activators increased active CTNNB1 associated with the plasma membrane and because GSK3 inhibition increased the percent of cells with nuclear CTNNB1 in cells of the BEF cell line. Immunolabeling of nuclear active CTNNB1 in BEF cells was characterized by a punctuate pattern resembling that previously described in newly differentiated chondrocytes (Guo *et al.* 2004) and intrahepatic cholangiocarcinoma cells (Wang *et al.* 2015).

An absence of CTNNB1 in the nucleus of the preimplantation embryo may be a widespread phenomenon in the mammal, at least for certain stages of development. In the human embryo, GSK3B-induced accumulation of CTNNB1 in the nucleus depends upon stage of development, with accumulation being attenuated after Day 3 of development and absent in blastocysts (Krivega *et al.* 2015). In the pig, immunoreactive nuclear CTNNB1 was faint in expanded blastocysts and absent in hatching blastocysts (Lim *et al.* 2013). Moreover, accumulation in the nucleus was not induced by LiCl inhibition of GSK3 (Lim *et al.* 2013).

Results with respect to the mouse are contradictory. No nuclear CTNNB1 was detected in mouse blastocysts in one study (Kemler *et al.* 2004), whereas active CTNNB1 was observed in the nucleus of embryos at the 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst stages of development in another (Xie *et al.* 2008). Present results fail to replicate findings of nuclear CTNNB1 in mouse 5–8-cell embryos even though the antibody used in the present experiment was the same as used earlier (Xie *et al.* 2008).

The findings that CTNNB1 does not translocate to the nucleus in the bovine embryo after treatment with canonical WNT activators does not mean that WNTs are not involved in the regulation of embryonic development. In addition to canonical signaling, there are a variety of other signaling cascades activated by WNTs termed non-canonical pathways (Filmus *et al.* 2008, Chien *et al.* 2009, van Amerongen & Nusse 2009, Gao 2012). Some of these pathways use FZD as a receptor (PCP- and Ca^{++} -mediated signaling), whereas others use other receptor molecules such as ROR and RYK. Individual WNTs preferentially stimulate canonical or non-canonical signaling depending upon the ability to bind FZD and recruit LRP5/6 and other co-receptor molecules. Thus, some documented actions of WNTs on the preimplantation embryo, for example, promotion of TE development in human embryos by WNT3 (Krivega *et al.* 2015), could involve signaling through one or more pathways independent of accumulation of CTNNB1 in the nucleus. Here, we show that WNT11, which is considered to preferentially activate non-canonical pathways (Flaherty & Dawn 2008, Uysal-Onganer & Kypta 2012), can activate a key component of the PCP pathway in bovine blastocysts by phosphorylating the signaling kinase JNK in the nucleus. Activation of JNK

has been implicated in actions of WNT11 in other cells (Pandur *et al.* 2002, Cha *et al.* 2008, Chen *et al.* 2014, Geetha-Loganathan *et al.* 2014) although, under certain circumstances, WNT11 can inhibit JNK signaling (Railo *et al.* 2008). The observation that activated JNK was localized to the nucleus suggests that the protein translocates to the nucleus after activation, as has been described for other cells (Schreck *et al.* 2011, Coffey 2014). Furthermore, WNT11 participates in the regulation of preimplantation developmental processes because addition of exogenous WNT11 to the culture medium resulted in higher proportion of inseminated oocytes that developed to the blastocyst stage. Further investigations are needed to unravel the downstream effect of this WNT in the preimplantation bovine embryo, but the nuclear localization of phospho-JNK in response to WNT11 suggests an effect on gene expression (reviewed in Zeke *et al.* 2016).

In addition, although WNT activation did not cause accumulation of CTNNB1 in the nucleus in bovine embryos, it did increase CTNNB1 in the embryo, with the protein being localized primarily to the plasma membrane. Similar effects have been observed in human embryos (Krivega *et al.* 2015). Thus, certain actions of WNT on the embryo could involve signal transduction pathways using membrane-bound CTNNB1. In mouse embryonic stem cells, CTNNB1 bound to E-cadherin is required for the expression of *Klf4* and *Nanog* via STAT3 phosphorylation (Hawkins *et al.* 2012). It may also be possible that activation of WNT signaling does lead to some accumulation of CTNNB1 in the nucleus but at amounts too low to be detected by immunofluorescence. Further investigation is needed to understand the alternative WNT signaling pathways regulating developmental processes, as well as the role of each of these pathways in preimplantation embryo development.

Analysis of gene expression during development is consistent with a reduction in WNT signaling as the embryo develops. In Experiment 1, transcript abundance for all genes examined declined to a nadir at the morula or blastocyst stage of development. This was true for the WNT co-receptors, *LRP5* and *LRP6*, the canonical WNT antagonist *DKK1*, two WNT-dependent transcription factors, *LEF1* and *TCF7*, as well as two repressors of WNT-dependent transcription factors, *LOC505120* (encodes for GROUCHO-like protein) and *AES*. The decline in gene expression is not an artifact of *in vitro* fertilization or culture because similar developmental patterns of gene expression were seen for 6 of the 7 genes for embryos that developed *in vivo* (Supplementary Table 3 in Jiang *et al.* 2014). The only exception was for *AES*, which rose in transcript abundance at the blastocyst stage for *in vivo* embryos (Jiang *et al.* 2014) but remained low for *in vitro*-produced embryos. It is possible that the developmental decline in abundance of most transcripts examined is part of the large-scale destruction of maternally derived

mRNA in the oocyte after fertilization (Tadros & Lipshitz 2009, Graf *et al.* 2014).

More research is required, but analysis of differences in gene expression between the ICM and TE of the blastocyst are consistent with the idea that WNT signaling functions differently in the two cell types. Genes upregulated in the TE included three receptors or co-receptors (*FZD1*, *FZD7* and *LRP6*) and two genes involved in the inhibition of canonical WNT signaling (*APC* and *SFRP1*). Expression of *WNT6* was also upregulated in the TE. This WNT, which can promote the differentiation of primitive endoderm (Krawetz & Kelly 2008), functions as a canonical WNT when binding FZD 1/2/7 and as a non-canonical WNT when binding FZD 5/8 (Schmidt *et al.* 2007, Lhomond *et al.* 2012, Li *et al.* 2014). Perhaps WNT6 is secreted by TE cells to participate in the differentiation of cells of the ICM to primitive endoderm.

In conclusion, the accumulation of CTNNB1 in the nucleus in response to canonical WNT activators is blocked in the preimplantation bovine embryo. Moreover, there is a decline in expression of several genes important for canonical WNT signaling as the embryo advances in development. In contrast, at least one non-canonical signaling pathway involving JNK and the PCP pathway, can be activated in the bovine preimplantation embryo. Moreover, WNT11, which causes JNK activation, improves the competence of the embryo to develop to the blastocyst stage. Thus, some actions of WNTs on the preimplantation embryo are likely to involve signaling through mechanisms independent of nuclear CTNNB1. Differences in gene expression between the TE and ICM mean that, by the blastocyst stage, WNT signaling may play different roles in the ICM and TE.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0610>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Agriculture and Food Research Initiative Competitive Grant no. 2011-67015-30688 from the USDA National Institute of Food and Agriculture. Data were presented in part at 41st Annual Conference of the IETS, January 10–13, 2015, Versailles, France.

Acknowledgements

The authors thank William Rembert and Eddie Cummings, for ovary collection; owners and employees of Central

Beef Packing Co. (Center Hill, FL), for providing ovaries; Southeastern Semen Services (Wellborn, FL), for donation of semen; and Douglas Smith and the Cell and Tissue Analysis Core of the McKnight Brain Institute of the University of Florida for capturing confocal images. Authors also thank Amy Ralston, Michigan State University, for the advice on use of leptomycin and the anonymous reviewers, for careful scrutiny of the data.

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Received 4 October 2016

First decision 31 October 2016

Revised manuscript received 7 December 2016

Accepted 9 January 2017