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Consequences of endogenous and exogenous WNT signaling for development of the preimplantation bovine embryo†

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Abstract

The specific role of WNT signaling during preimplantation development remains unclear. Here, we evaluated consequences of activation and inhibition of β-catenin (CTNNB1)-dependent and -independent WNT signaling in the bovine preimplantation embryo. Activation of CTNNB1-mediated WNT signaling by the agonist 2-amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine (AMBMP) and a glycogen synthase kinase 3 inhibitor reduced development to the blastocyst stage. Moreover, the antagonist of WNT signaling, dickkopf-related protein 1 (DKK1), alleviated the negative effect of AMBMP on development via reduction of CTNNB1. Based on labeling for phospho c-Jun N-terminal kinase, there was no evidence that DKK1 activated the planar cell polarity (PCP) pathway. Inhibition of secretion of endogenous WNTs did not affect development but increased number of cells in the inner cell mass (ICM). In contrast, DKK1 did not affect number of ICM or trophectoderm (TE) cells, suggesting that embryo-derived WNTs regulate ICM proliferation through a mechanism independent of CTNNB1. In addition, DKK1 did not affect the number of cells positive for the transcription factor yes-associated protein 1 (YAP1) involved in TE formation. In fact, DKK1 decreased YAP1. In contrast, exposure of embryos to WNT family member 7A (WNT7A) improved blastocyst development, inhibited the PCP pathway, and did not affect amounts of CTNNB1. Results indicate that embryo-derived WNTs are dispensable for blastocyst formation but participate in regulation of ICM proliferation, likely through a mechanism independent of CTNNB1. The response to AMBMP and WNT7A leads to the hypothesis that maternally derived WNTs can play a positive or negative role in regulation of preimplantation development.

Summary Sentence

Endogenous WNTs are dispensable for blastocyst formation, but participate in the regulation of ICM proliferation, likely through a mechanism independent of β-catenin.

Key words: embryo development, preimplantation development, WNT, DKK1, WNT7A.

Introduction

The WNT family members (WNTs) are a family of 19 extracellular growth factors whose secretion depends upon palmitoylation mediated by acyltransferases [1, 2]. WNT signaling participates in a number of developmental processes including cellular proliferation [3] and differentiation [4], maintenance of pluripotency [5, 6], asymmetrical cell division [7], the epithelial-mesenchymal transition [8], and axis elongation [9, 10]. The outcome of WNT signaling depends upon the cellular context established by the availability of receptors, coreceptors, and molecules involved in different signaling pathways. WNTs may interact with any of 10 frizzled (FZD) receptors as well as alternative receptors including retonoic-acid receptor-related orphan receptor, tyrosine-protein kinase-like 7, and receptor-like tyrosine kinase to activate different downstream signaling cascades [3, 11]. The best described signaling pathway, the so-called canonical pathway, is mediated by nuclear β-catenin (CTNNB1). CTNNB1 has a dual function because it provides an indirect link between cadherin and the contractile cortical actin cytoskeleton [12] and can also localize into the nucleus where it interacts with transcription factors to regulate gene expression [13, 14]. Other downstream signaling pathways activated by WNTs include the planar cell polarity (PCP) pathway [15, 16], and Ca^{+} mediated cascade [17, 18]. More recently, non-nuclear CTNNB1 has also been associated with downstream signaling [19].

The role of embryo- and maternally derived WNTs in preimplantation development is unclear. Blastocyst-stage embryos express only a subset of WNT genes. Of the 19 WNT ligands, only 10, 8, and 6 are expressed in mouse, human, and bovine blastocysts, respectively [20–24]. There is also limited or no detectable nuclear accumulation of CTNNB1 in these three species even in the presence of WNT agonists [25, 26]. In mouse embryos, inhibition of secretion of endogenous WNT does not affect blastocyst formation [27] and neither does depletion of CTNNB1 [28]. Furthermore, inhibition of CTNNB1-mediated WNT signaling with dickkopf-related protein 1 (DKK1) does not impair blastocyst formation in mouse, cow, or pig embryos [24, 29–31]. Taken together, evidence suggests that endogenous WNT signaling mediated through nuclear accumulation of CTNNB1 is dispensable for blastocyst formation. A role for embryo-derived WNT acting through other pathways is possible however.

Maternally derived WNTs could also contribute to regulation of embryonic development although the consequences of maternal WNT for the embryo are unclear. There may also be a physiological role for the WNT antagonist DKK1 in preimplantation development. DKK1 is a product of the endometrium [32–35] and can have multiple functions in WNT signaling. For example, DKK1 blocks the canonical signaling pathway by interfering with formation of the WNT ligand/FZD receptor/low density lipoprotein-related protein 5 (LRP5) or LRP6 complex. In addition, DKK1 can stimulate the PCP pathway through activation of c-Jun N-terminal kinases (JNK) [36, 37]. Exposure of pig embryos to DKK1 increased the number of trophectoderm (TE) cells in the blastocyst without changing the number of inner cell mass cells (ICM) [31]. In cattle, DKK1 increased the proportion of cells in the blastocyst that were TE and improved competence of embryos to establish pregnancy after transfer into recipients [38]. In addition, DKK1 increased the proportion of pig blastocysts that hatched in vitro [31].

The overall objective of the series of experiments documented here was to determine consequences of activation and inhibition of CTNNB1-dependent and -independent WNT signaling on the development of bovine preimplantation embryos including allocation of cells of the blastocyst into ICM and TE lineages. Consequences of activation of WNT signaling mediated by CTNNB1 were tested by increasing accumulation of CTNNB1 through inhibition of glycogen synthase kinase 3 (GSK3) and by addition of the WNT agonist 2-amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine (AMBMP) [39, 40]. Furthermore, effects of inhibition of CTNNB1-mediated WNT signaling on development were examined using the WNT antagonist DKK1. Because DKK1 has previously been associated with embryonic competence to establish pregnancy [38], a set of experiments were designed to further explore effects of DKK1 on preimplantation bovine embryos. We examined consequences of exposure of embryos to DKK1 on blastocyst formation, cell lineage commitment at the blastocyst stage, and changes in the nuclear amounts of the transcription factors caudal type homeobox 2 (CDX2) and yes associated protein 1 (YAP1). CDX2 is important for continued development of the trophoblast in cattle [41], and Yap1 plays an important role in TE formation in the mouse by interacting with TEA domain transcription factor 4 (TEAD4) to induce transcription of Cdx2 [42, 43]. We also tested the hypothesis that DKK1 activates JNK to stimulate signaling through the PCP pathway. The role of endogenous WNT on blastocyst development was tested by blocking the acylation of WNT with a porcupine homolog (*Drosophila*) (PORCN) inhibitor to abolish WNT secretion [44]. Lastly, the WNT family member 7A (WNT7A) was used to test the hypothesis that maternally derived molecules regulate WNT signaling in the preimplantation embryo. This WNT was chosen because the gene is highly expressed in the bovine endometrium [44] but not in the bovine embryo [24, 45].

Taken together, results indicate a limited role of embryo-derived WNTs in blastocyst development and indicate that, depending on the type of ligand, maternally derived WNT can potentially either promote or inhibit competence of the embryo to become a blastocyst.

Materials and methods

Embryo production using non-sex-sorted sperm

Formulation of media used for production of bovine embryos in vitro is described elsewhere [46]. Cumulus-oocyte complexes (COC) were obtained from cattle ovaries (including *Bos taurus* and cattle that were of a genetic type composed of an admixture of *B. taurus* and *B. indicus* genetics) collected at a local abattoir by bisecting follicles 3–8 mm in diameter with a scalpel. Procedures for oocyte recovery and maturation, fertilization, and embryo culture were performed following procedures described elsewhere [47] with a few modifications, as follows. Oocytes were harvested using BoviPRO oocyte washing medium (MOFA Global, Verona, WI, USA) and matured for 20–22 h in groups of 10 in 50 μl drops of oocyte maturation

medium covered by mineral oil (Sigma-Aldrich, St. Louis, MO, USA). Groups of up to 300 matured oocytes were then fertilized for 8–10 h in 1700 μl in vitro fertilization Tyrode-lactate-pyruvate (IVF-TALP) solution (Caisson Laboratories, Logan, UT, USA) to which sperm (final concentration, 1×10^6 cells/ml) and 80 μl of a solution of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine) were added. Sperm used for each fertilization procedure consisted of a pool from three *B. taurus* or Brangus bulls that were randomly selected from available bulls. A different assortment of bulls was used for each procedure. Sperm from frozen-thawed straws were purified before fertilization using an Isolate gradient [(Irvine Scientific, Santa Ana, CA; 50% (v/v) and 90% (v/v) isolate] and diluted in IVF-Tyrode albumin lactate pyruvate solution (Caisson Laboratories). After removal of cumulus cells, groups of 25–30 presumptive zygotes were placed in 50 μl microdrops of synthetic oviduct fluid-bovine embryo 2 (SOF-BE2) covered with mineral oil (Sigma-Aldrich) and cultured at 38.5°C in a humidified atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2 with the balance N_2. Unless stated otherwise, treatments were administered on Day 5 of development [120 h postinsemination (hpi)]. The procedure consisted of removing 5 μl of culture medium and replacing it with 5 μl of culture medium containing treatment at 10× concentration.

Embryo production using sex-sorted sperm

Procedures were as described above except for semen preparation and fertilization. Commercially available X- and Y-sorted sperm from Angus sires were obtained from ABS Global (De Forest, WI, USA) and Genex Cooperative, Inc. (Shawano, WI, USA). Separated pools of X- and Y-sorted sperm from the same two bulls, randomly selected from those available, were used in each fertilization procedure. The total number of bulls used was six. Sperm were purified before fertilization using PureSperm 40/80 gradient column (Nidacon International AB, Mölndal, Sweden). Sperm was first centrifuged (2600× *g* for 5 min) in 2.0 ml microcentifuge tubes containing 250 μl sperm over two layers of 200 μl of Puresperm (top layer of Puresperm 40 and bottom layer of Puresperm 80). The pellet representing the bottom 100 μl was transferred to a new microcentrifuge tube, washed in 1000 μl of IVF-TL that had been pre-equilibrated at 38.5°C under 5% CO_2, and centrifuged at 600× *g* for 3 min. Fertilization of groups of 30 matured COC was performed in 60 μl oil-covered microdrops of IVF-TL medium containing 3.5 μl of PHE. Final concentration of sperm in the fertilization drop was 2×10^6 cells/ml. Fertilization was carried out for 18–20 h at 38.5°C and a humidified atmosphere of 5% (v/v) CO_2. Treatments were administered as described for embryos produced with non-sex-sorted semen.

Immunolabeling

Details of antibodies used are presented in Supplemental File S1. Procedures for labeling embryos against CTNNB1 and phosphorylated JNK (pJNK) were as follows. Embryos were fixed in 4% (v/v) paraformaldehyde, and permeabilized in Dulbecco phosphate-buffered saline (DPBS) containing 0.5% (v/v) Triton X-100. Blocking was performed using DPBS containing 5% (w/v) bovine serum albumin (BSA), and incubation with primary antibody diluted in antibody buffer [DPBS containing 0.1% (v/v) Tween 20 and 1% BSA (w/v)] was performed overnight at 4°C. Total immunoreactive CTNNB1 was detected using 1 μg/ml rabbit polyclonal antihuman CTNNB1 (Abcam, Cambridge, MA, USA). Immunoreactive pJNK was detected using 1 μg/ml rabbit polyclonal antiphospho-JNK [(Thr183/Tyr185, Thr221/Tyr223) (Millipore, Billerica, MA, USA)]. Note that the phosphorylation site is conserved among human and bovine JNK and that the amino acid sequence identity between these species is 99% (comparison of sequences using BLAST; https://blast.ncbi.nlm.nih.gov). Incubation with labeled secondary antibody [goat antirabbit IgG conjugated with Alexa Fluor 555 (Life Technologies, Carlsbad, CA, USA); 1 μg/ml diluted in antibody buffer] proceeded for 1 h at room temperature. Nuclear labeling was performed with 1 μg/ml Hoechst 33342 (Sigma-Aldrich) in antibody buffer. Slides were mounted using SlowFade Gold antifade reagent (Life Technologies), and observed with a 40× objective using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) and Zeiss filter sets 02 [4′,6′-diamidino-2-phenylindole], 03 (FITC filter), and 04 (rhodamine). Digital images of individual blastocysts were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera. For a negative control, IgG of the same species was used to replace primary antibody using the same concentration.

For dual immunolocalization of YAP1 and CDX2, embryos were processed as described above with few modifications. After overnight incubation with rabbit monoclonal antihuman YAP1 (Cell Signaling Technology, Beverly, MA, USA) at a concentration of 0.015 μg/ml, embryos were washed three times with washing buffer [DPBS containing 0.1% (v/v) Tween 20 and 0.1% (w/v) BSA] and incubated with secondary antibody [goat antirabbit IgG conjugated with Alexa Fluor 555 (Life Technologies); 1 μg/ml] for 1 h at room temperature. Embryos were washed again three times and incubated for 1 h with primary antibody against CDX2 (mouse antihuman polyclonal CDX2 antibody, ready to use; BioGenex, Fremont, CA, USA) and 1 h with 1 μg/ml goat antimouse IgG conjugated with fluorescein isothiocyanate (FITC; Abcam, Cambridge, MA, USA). Nuclear labeling, slide mounting, and image acquisition were performed as described above.

Quantification of intensity of labeling in either the entire embryo or in the nuclei was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). For labeling of the entire embryo, the area encompassing the entire embryo was selected and the mean intensity obtained using the Measure Analysis feature of ImageJ. Background intensity was obtained from the area surrounding the embryo using the same technique and the value subtracted from embryo intensity. Labeling in the nucleus was determined using a similar technique except that the software was used to isolate nuclear regions within each embryo based on labeling with Hoechst 33342.

Number of ICM and TE cells was determined for embryos labeled with anti-CDX2 as described above. Those cells with nuclei labeled with CDX2 were considered TE and the number of ICM cells was determined by subtracting number of TE cells from the total number of cells determined by counting number of nuclei labeled with Hoechst 33342.

Experiment 1: effect of activation of canonical WNT signaling by inhibition of glycogen synthase kinase 3 on development

Embryos were cultured in 50 μl microdrops of SOF-BE2. Treatments were either 10 μM of the GSK inhibitor CHIR99021 (Tocris Bioscience, Avonmouth, Bristol, UK; final concentration in the drop) or vehicle (SOF-BE2). The concentration of inhibitor was chosen because a similar concentration (5 μM) was effective in activating WNT/CTNNB1 signaling pathway in mouse embryonic stem cells

[39]. Blastocyst development was evaluated on Day 7 of development (168 hpi). The experiment was performed in 5 replicates using a total of 803 COC and conventional semen from 10 different bulls.

Experiment 2: effect of activation of canonical WNT signaling by the agonist 2-amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl) pyrimidine in the presence or absence of dickkopf-related protein 1 on development and β-catenin labeling

The source of AMBMP was Calbiochem (San Diego, CA, USA). Human recombinant DKK1 was purchased from R&D Systems (Minneapolis, MN, USA). Both reagents were reconstituted as previously described [24]. Embryos were cultured in 50 μl microdrops of SOF-BE2. Treatments were either vehicle [SOF-BE2 containing 0.1% (v/v) dimethyl sulfoximine (DMSO)], 0.7 μM AMBMP, 100 ng/ml DKK1, or 0.7 μM AMBMP plus 100 ng/ml DKK1 (final concentrations in the drop). Blastocyst development was evaluated on Day 7 of culture (168 hpi), and a fraction of blastocysts with a clearly delineable blastocoel were randomly selected for immunolabeling of CTNNB1. The experiment was performed in seven replicates using a total of 1006 COC and conventional semen from 10 different bulls. A total of 165 blastocysts were analyzed for immunolabeling of CTNNB1.

Experiment 3: effects of inhibition of endogenous WNT signaling with Wnt-C59 or dickkopf-related protein 1 on ability of embryos to develop to the blastocyst stage and blastocyst cell number

Wnt-C59 [2-(4-(2-methylpyridin-4-yl)phenyl)-N-(4-(pyridine-3-yl) phenyl)acetamide] is an inhibitor of PORCN [48], the enzyme that acylates WNTs to facilitate their secretion [44]. The inhibitor was purchased as a 10 mM stock in DMSO from Cellagen Technology (San Diego, CA, USA). The Wnt-C59 was serially diluted to 100 nM in SOF-BE2 containing 0.001% (v/v) DMSO so that the final concentration of Wnt-C59 in the culture drop was 10 nM. The concentration of inhibitor was chosen because it blocked WNT activation in HeLa cells [48]. Treatments were vehicle [SOF-BE2 containing 0.01% (w/v) BSA (in addition to the BSA included in SOF-BE2 formulation) and 0.0001% (v/v) DMSO]; 10 nM Wnt-C59; or 100 ng/ml DKK1 (final concentrations in the drop). Blastocyst development was assessed on Day 7 of development (168 hpi). A fraction of blastocysts with a clearly delineable blastocoel was randomly selected and subjected to immunolabeling to determine the numbers of TE and ICM cells. The experiment was performed in five replicates with a total of 905 COC and conventional semen from 13 different bulls. Immunolabeling was evaluated for 91 blastocysts.

Experiments 4 and 5: effects of dickkopf-related protein 1 on development and blastocyst cell number

In each experiment, treatments were 100 ng/ml DKK1 and vehicle [SOF-BE2 containing 0.01% (w/v) BSA (in addition to the BSA included in SOF-BE2 formulation)]. The concentration of DKK1 was chosen because it was effective at blocking actions of WNT signaling agonist on development of bovine embryos to the blastocyst stage [24].

Experiment 4 was performed in 10 replicates with a total of 1545 COC and conventional semen from seven bulls. Experiment 5 was replicated five times with a total of 1348 COC and X- and Y-sorted semen from six bulls. Blastocyst development was evaluated on Day 7 of development (168 hpi) and a fraction of blastocysts with a clearly delineated blastocoel (n = 89 for Experiment 4 and 204 for Experiment 5) were randomly selected for labeling with anti-CDX2 and Hoechst 33342 to determine number of TE and ICM.

Experiment 6: effects of dickkopf-related protein 1 on developmental changes in yes-associated protein 1 and caudal type homeobox 2 localization in morulae and blastocysts

Embryos were treated with either 100 ng/ml DKK1 or vehicle [SOF-BE2 containing 0.01% (w/v) BSA (in addition to the BSA included in SOF-BE2 formulation)]. Morulae on Day 5 (6 h after treatment), morulae on Day 6 (24 h after treatment), and blastocysts with a clearly delineated blastocoel on Day 7 (48 h after treatment) were harvested and fixed for immunolocalization of YAP1 and CDX2. Number of cells was counted based on DNA staining; number of $YAP1^+$ and $CDX2^+$ were also quantified. Intensity of immunofluorescence associated with nuclear YAP1 and nuclear CDX2 was obtained. Data are presented as absolute and relative number of $CDX2^+$ and $YAP1^+$ nuclei. The experiment was performed in five replicates with a total of 1510 COC and conventional semen from seven bulls. A total of 232 individual embryos were assessed for immunofluorescence.

Experiment 7: effects of dickkopf-related protein 1 on activation of c-Jun N-terminal kinases

Treatments, which were added at Day 5 of development, included vehicle [SOF-BE2 containing 0.01% (w/v) BSA (in addition to the BSA included in SOF-BE2 formulation)] and 100 ng/ml DKK1 (final concentration in the drop). Morulae were harvested 6 h after adding the treatment and immunolabeled for pJNK. The experiment was performed in four replicates with a total of 587 COC and conventional semen from 11 different bulls.

Experiments 8–11: embryo responses to WNT family member 7A

Actions of WNT7A on development were examined because the gene is highly expressed in the bovine endometrium [44] but not in the bovine embryo [24, 45]. WNT7A can alter cellular function through activation of CTNNB1-dependent [49] and -independent pathways [50, 51]. For these experiments, treatments were 66 ng/ml human recombinant WNT7A (eBioscience Inc., San Diego, CA, USA) or vehicle [SOF-BE2 containing 0.01 mM $NaPO_4$, 0.5 mM NaCl and 0.0005% (w/v) CHAPS in water]. The concentration of WNT7A was chosen because it is the upper limit of the range suggested for biological activity of the product by the manufacturer, as determined by inhibition of Wnt-3a-induced alkaline phosphatase production in MC3T3-E1 cells. Also, 50 ng/ml was effective at activating Akt/mTOR anabolic growth pathway in skeletal muscle [50]. Blastocyst rate was assessed on Day 7 of development (180 hpi) and a fraction of blastocysts with clearly delineated blastocoel was randomly selected for further analyses.

For experiment 8, embryos were produced in vitro as described above with few modifications. Oocytes were matured in BO-IVM (IVF Bioscience, Falmouth, Cornwall, UK) for 24 h, and fertilization proceeded for 12–14 h. Culture drops were randomly assigned to one of four treatments in a 2 × 2 arrangement with two treatments (WNT7A or vehicle) and treatment on either Day 1 (15 hpi) or Day 5 of development (115 hpi). This experiment was performed in six

Table 1. Effect of exposure of embryos to GSK3 inhibitor from Day 5 to 7 of development on the ability of embryos to develop to the blastocyst stage.[a]

	Treatment		
	Vehicle	GSK3 inhibitor	*P* value
Blastocysts/oocyte (%)	28.5 ± 2.3	21.9 ± 2.0	0.03
Blastocysts/cleaved embryo (%)	34.5 ± 2.7	27.4 ± 2.4	0.05

[a]Data are the least-squares means ± SEM of results from five replicates representing 803 COC.

replicates with a total of 1566 COC and conventional semen from eight bulls.

For experiments 9 and 10, embryos were produced in vitro. Treatments were added on Day 5 of development (120 hpi). Blastocysts were harvested to determine number of ICM and TE cells (experiment 9) and intensity of total CTNNB1 (experiments 10) by immunolabeling as described above. Experiment 9 was performed in seven replicates with a total of 1413 COC and conventional semen from seven bulls. A total of 54 blastocysts were assessed by immunofluorescence. Experiment 10 was performed in 10 replicates with a total of 1471 COC and conventional semen from 11 bulls. A total of 239 individual blastocysts were assessed by immunofluorescence to quantify total CTNNB1.

For experiment 11, embryos were produced following procedures for experiment 8. Treatments were added on Day 5 of development (120 hpi), and blastocysts were harvested on Day 7 of culture for immunolabeling of pJNK as described above. A total of 42 individual blastocysts were assessed by immunofluorescence to quantify total pJNK.

Statistical analysis

Effects of treatment on the percent of oocytes or cleaved embryos developing to the blastocyst stage were evaluated using Proc Glimmix of SAS for Windows, version 9.4 (SAS Institute Inc., Cary, NC, USA). Each embryo was considered an observation (0 = not developed to blastocyst; 1 = developed to blastocyst). The analysis was performed with the dependent variable considered as a binomial distribution, and treatments as fixed effects. Results are presented as least-squares means ± standard error of the mean. The level of significance was considered as $P < 0.05$.

Data on intensity of immunolabeling were analyzed by least-squares analysis of variance using the PROC MIXED procedure of SAS. Treatments were fixed effects and replicate was considered a random effect. Results are presented as least-squares means ± standard error of the mean. The level of significance was $P < 0.05$.

Results

Effect of activation of canonical WNT signaling on development (Experiments 1 and 2)

Two experiments were conducted to determine whether activation of canonical WNT signaling would affect development of embryos to the blastocyst stage. In Experiment 1, addition of the GSK3 inhibitor CHIR 99021 to cultured embryos from Day 5 to 7 of development decreased the proportion of oocytes and cleaved embryos becoming blastocysts ($P = 0.03$ and $P = 0.05$, respectively; Table 1).

In Experiment 2, canonical WNT signaling was activated by addition of the WNT agonist AMBMP. To test whether AMBMP acts by increasing CTNNB1 accumulation, embryos were treated with DKK1 which blocks coactivation of WNT receptors. Immunoreactive CTNNB1 was localized primarily to the plasma membrane and was never found in the nucleus even after addition of AMBMP (Figure 1A). Intensity of immunoreactive CTNNB1 was increased by AMBMP ($P < 0.0001$) and decreased by DKK1 ($P = 0.0001$). There was no interaction between AMBMP and DKK1 because AMBMP increased immunoreactive CTNNB1 even in the presence of DKK1 ($P < 0.0001$). Note, however, that the amount of CTNNB1 in embryos treated with AMBMP combined with DKK1 was similar to the amount of CTNNB1 in embryos treated with vehicle alone. Thus, while DKK1 did not prevent actions of AMBMP to increase CTNNB1, the total amount of CTNNB1 was not elevated as compared to controls (Figure 1B). AMBMP reduced development to the blastocyst stage and, while DKK1 alone did not alter development, the effect of AMBMP was blocked by DKK1 (DKK1 by AMBMP interaction: $P = 0.04$ for blastocysts/oocyte and $P = 0.03$ for blastocysts/cleaved; Table 2).

Results of these experiments indicate that exogenous molecules that induce accumulation of CTNNB1 are detrimental for blastocyst formation. In addition, DKK1 can reduce CTNNB1 accumulation without affecting development of preimplantation embryos to the blastocyst stage.

Effects of inhibition of endogenous WNT signaling with Wnt-C59 or dickkopf-related protein 1 on ability of embryos to develop to the blastocyst stage and blastocyst cell number (Experiment 3)

To evaluate the role of embryo-derived WNTs during preimplantation development, embryos were exposed to Wnt-C59, which blocks secretion of WNTs, or DKK1, which interferes with activation of the WNT-FZD-LRP5/6 receptor complex. Neither treatment altered the proportion of oocytes or cleaved embryos developing to the blastocyst stage (Table 3). However, blastocysts formed in the presence of Wnt-C59 had increased number of ICM cells ($P = 0.02$) and tended to have reduced TE:ICM ratio ($P = 0.06$) than embryos treated with vehicle. There was no effect of DKK1 on numbers of ICM or TE cells in the blastocyst (Table 3). Results indicate that endogenous WNTs are not required for blastocyst formation but do regulate ICM cell numbers. Because the number of ICM cells was increased when secretion of WNTs was prevented but not in embryos exposed to DKK1, it is likely that endogenous WNTs regulate ICM proliferation through a signaling pathway independent of CTNNB1.

Effects of dickkopf-related protein 1 on development and blastocyst cell number (Experiments 4 and 5)

These two experiments were designed to test whether DKK1 alters competence of embryos to become blastocysts and the number of TE and ICM cells in the blastocyst. An additional goal of experiment 5 was to determine whether effects of DKK1 varied with embryo sex. There was no effect of addition of DKK1 from Day 5 to 7 of development on the proportion of oocytes or cleaved embryos that became blastocysts or on the numbers of ICM or TE cells in the resulting blastocysts. This was true whether embryos were produced using conventional semen (Table 3) or whether male and female embryos were tested separately after fertilization (Table 4). There was a tendency for female blastocysts to have a smaller TE:ICM ratio than male blastocysts ($P = 0.10$). Taken together, actions of DKK1 do not affect development of embryos to the blastocyst stage or number of cells in the blastocyst.

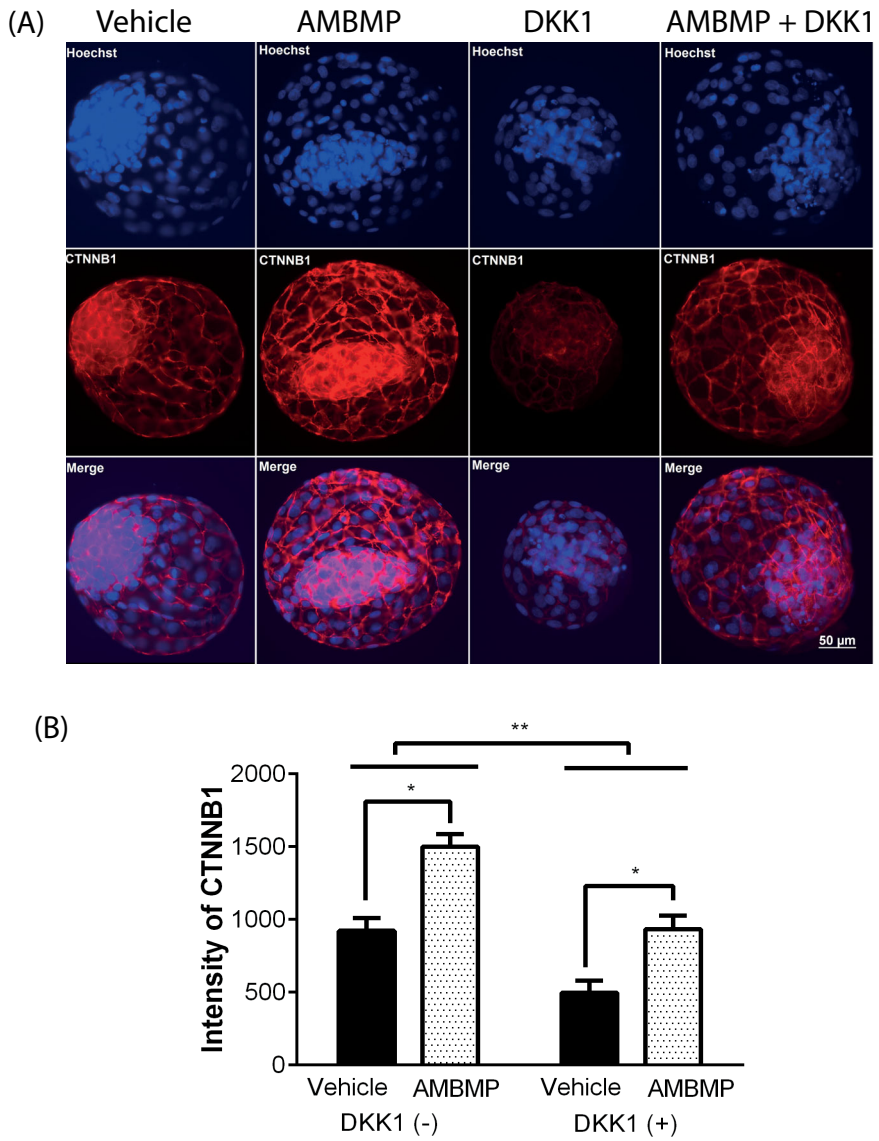

Figure 1 Treatment of embryos from Day 5 to 7 of development with 100 ng/ml DKK1 reduces amounts of immunoreactive CTNNB1 in the resulting blastocysts but does not prevent a WNT agonist (AMBMP) from increasing amounts of CTNNB1. (A) Representative images of blastocysts immunolabeled for CTNNB1 (red) and DNA (blue). (B) Quantification of intensity of CTNNB1. Immunoreactive CTNNB1 was affected by AMBMP (***$P < 0.0001$) and DKK1 (****$P = 0.0001$) but not by the AMBMP by DKK1 interaction ($P = 0.9$). Data are least-squares means ± SEM of results from seven replicates, with a total of 165 labeled blastocysts.

Effects of dickkopf-related protein 1 on developmental changes in immunoreactive yes-associated protein 1 and caudal type homeobox 2 in morulae and blastocysts (Experiment 6)

To further test the effect of DKK1 on TE differentiation, experiment 6 was conducted to test whether DKK1 modifies the developmental pattern of immunoreactive YAP1 and CDX2 from Day 5 to Day 7 of development (when the embryo transitions from the morula to the blastocyst stage). The transcription factor YAP1 plays an important role in TE formation in the mouse by interacting with the transcription factor TEAD4 to induce transcription of CDX2 [42, 43]. Signaling mediated by the small GTPases RHO-ROCK regulates YAP1 [52], and DKK1 activates noncanonical WNT signaling in other systems including the PCP pathway mediated by small GTPases [36].

Representative examples of labeling for YAP1 and CDX2 are shown in Figure 2A. Both YAP1 and CDX2 were localized exclusively to nuclei. For Day 5 morulae, nuclei positive for YAP1 and CDX2 were not frequent and labeling was faint. By Day 6, however, there were abundant numbers of $YAP1^+$ and $CDX2^+$ cells. By Day 7, both types of cells were confined to the TE. Quantification of nuclei that were positive for YAP1 and CDX2 indicated that the number of cells positive for both markers increased during development (Figure 2B and F). However, the proportion of total cells that were $YAP1^+$ declined after Day 5 (Figure 2C), while the proportion of total cells that were $CDX2^+$ increased from Day 5 to 7 (Figure 2G). Similarly, the percent of $CDX2^+$ cells that were also $YAP1^+$ declined over time (Figure 2H), while the percent of $YAP1^+$ cells that were also $CDX2^+$ increased (Figure 2D). Thus, as the embryo developed, an increasing number of $CDX2^+$ cells

Table 2. Effect of treatment of embryos from Day 5 to 7 of development with the WNT agonist AMBMP and the WNT regulatory molecule DKK1 on the ability of embryos to develop to the blastocyst stage.[a]

Treatment		Blastocysts/ oocyte (%)	Blastocysts/cleaved embryo (%)
AMBMP	DKK1		
–	–	24.3 ± 4.3	31.9 ± 5.1
+	–	18.7 ± 3.6	23.0 ± 4.2
–	+	18.4 ± 3.5	25.3 ± 4.5
+	+	21.7 ± 4.0	28.3 ± 4.8
Statistical significance (*P*)			
AMBMP		0.62	0.29
DKK1		0.72	0.86
Interaction		0.04	0.03

[a]Data are the least-squares means ± SEM of results from seven replicates representing 1006 COC.

lost expression of YAP1. Intensity of labeling for YAP1 and CDX2 in nuclei positive for the marker increased during development (Figure 2E and I).

There was no effect of DKK1 on total number of blastomeres or on the number or proportion of blastomeres that were YAP1$^+$ or CDX2$^+$. Similarly, DKK1 did not affect intensity of CDX2$^+$ cells. In contrast, the intensity of YAP1 labeling in YAP1$^+$ cells was reduced by DKK1 ($P = 0.04$; Figure 2E). Results confirm findings of experiments 4 and 5 that DKK1 does not affect TE cell numbers but indicate that DKK1 can affect accumulation of nuclear YAP1.

Dickkopf-related protein 1 does not activate planar cell polarity mediated by phosphorylated c-Jun N-terminal kinases (Experiment 7)

DKK1 can activate the PCP pathway mediated by small GTPases [36]. Accordingly, experiment 7 was conducted to test the hypothesis that DKK1 could activate WNT/PCP pathway mediated by JNK signaling. Embryos were exposed to DKK1 at Day 5 of development. There was, however, no effect of DKK1 on accumulation of immunoreactive pJNK in morulae at Day 5 of development (Figure 3).

Regulation of WNT signaling by WNT family member 7A (Experiments 8–11)

WNT7A was used to test the hypothesis that exogenous WNTs can regulate WNT signaling in preimplantation embryos. This WNT was chosen because *WNT7A* is highly expressed in the female reproductive tract [44] but is not expressed in the morula or blastocyst [24, 45].

Effects of WNT7A on development to the blastocyst stage were evaluated in Experiment 8 (Table 5). Treatment of embryos with WNT7A beginning at either Day 1 (20 hpi) or Day 5 (115 hpi) increased the proportion of oocytes ($P = 0.0005$) and cleaved embryos ($P = 0.02$) that became a blastocyst at Day 7 of development. There was no effect of day of treatment or interaction of day with treatment. Actions of WNT7A on allocation of blastomeres into ICM and TE were evaluated in Experiment 9 in which WNT7A was added on Day 5 of development. WNT7A again increased the proportion of oocytes ($P = 0.02$) and cleaved embryos ($P = 0.04$) that became a blastocyst at Day 7 of development, but there was no effect of WNT7A on blastocyst cell number (Table 6).

Table 3. Effects of inhibition of endogenous WNT signaling from Day 5 to 7 of development with either Wnt-C59 or DKK1 on ability of embryos to develop to the blastocyst stage, and cell number of Day 7 blastocysts.

		Development		Blastocyst cell number			
Experiment	Treatment	Blastocysts/ oocyte (%)	Blastocysts/cleaved embryo (%)	Total	TE	ICM	TE:ICM ratio
3[a]	Vehicle	16.6 ± 2.3	23.9 ± 3.9	133.6 ± 9.1	88.2 ± 6.1	43.3 ± 4.4	2.2 ± 0.1
	Wnt-C59	19.2 ± 2.2	26.4 ± 3.7	141.7 ± 8.1	89.1 ± 5.2	52.7 ± 4.1[b]	1.7 ± 0.1[c]
	DKK1	18.1 ± 2.1	26.1 ± 3.3	127.8 ± 9.0	81.2 ± 6.0	45.2 ± 4.4	1.8 ± 0.1
4[d]	Vehicle	25.1 ± 1.6	34.2 ± 2.0	129.3 ± 11.2	83.1 ± 7.6	46.6 ± 4.0	2.0 ± 0.1
	DKK1	23.2 ± 1.5	31.8 ± 1.9	134.6 ± 11.6	85.9 ± 7.8	48.7 ± 4.2	1.8 ± 0.1

[a]Data are the least-squares means ± SEM of results from five replicates representing 905 COC.
[b]Differs from control ($P = 0.02$).
[c]Differs from control ($P = 0.06$).
[d]Data are the least-squares means ± SEM of results from 10 replicates representing 1545 COC. There were no effects of treatment ($P > 0.10$).

Table 4. Effect of treatment of embryos with DKK1 from Day 5 to 7 of development on the ability of male and female embryos to develop to the blastocyst stage and cell number of Day 7 blastocysts.[a]

		Development		Blastocyst cell number			
Treatment	Sex	Blastocysts/ oocyte (%)	Blastocysts/cleaved embryo (%)	Total	TE	ICM	TE:ICM ratio[b]
Vehicle	Female	20.6 ± 2.1	23.6 ± 2.5	148.2 ± 7.1	88.9 ± 4.7	59.5 ± 4.1	1.7 ± 0.1
DKK1	Female	17.1 ± 2.0	20.1 ± 2.4	135.6 ± 6.8	93.1 ± 5.0	51.6 ± 4.0	1.9 ± 0.1
Vehicle	Male	14.3 ± 1.9	17.1 ± 2.2	144.0 ± 7.4	83.7 ± 4.9	60.3 ± 4.2	1.5 ± 0.1
DKK1	Male	15.9 ± 2.0	19.3 ± 2.4	153.1 ± 7.6	83.6 ± 4.4	60.1 ± 4.3	1.6 ± 0.1

[a]Data are the least-squares means ± SEM of results from five replicates representing 1348 COC.
[b]Sex effect, $P = 0.10$.

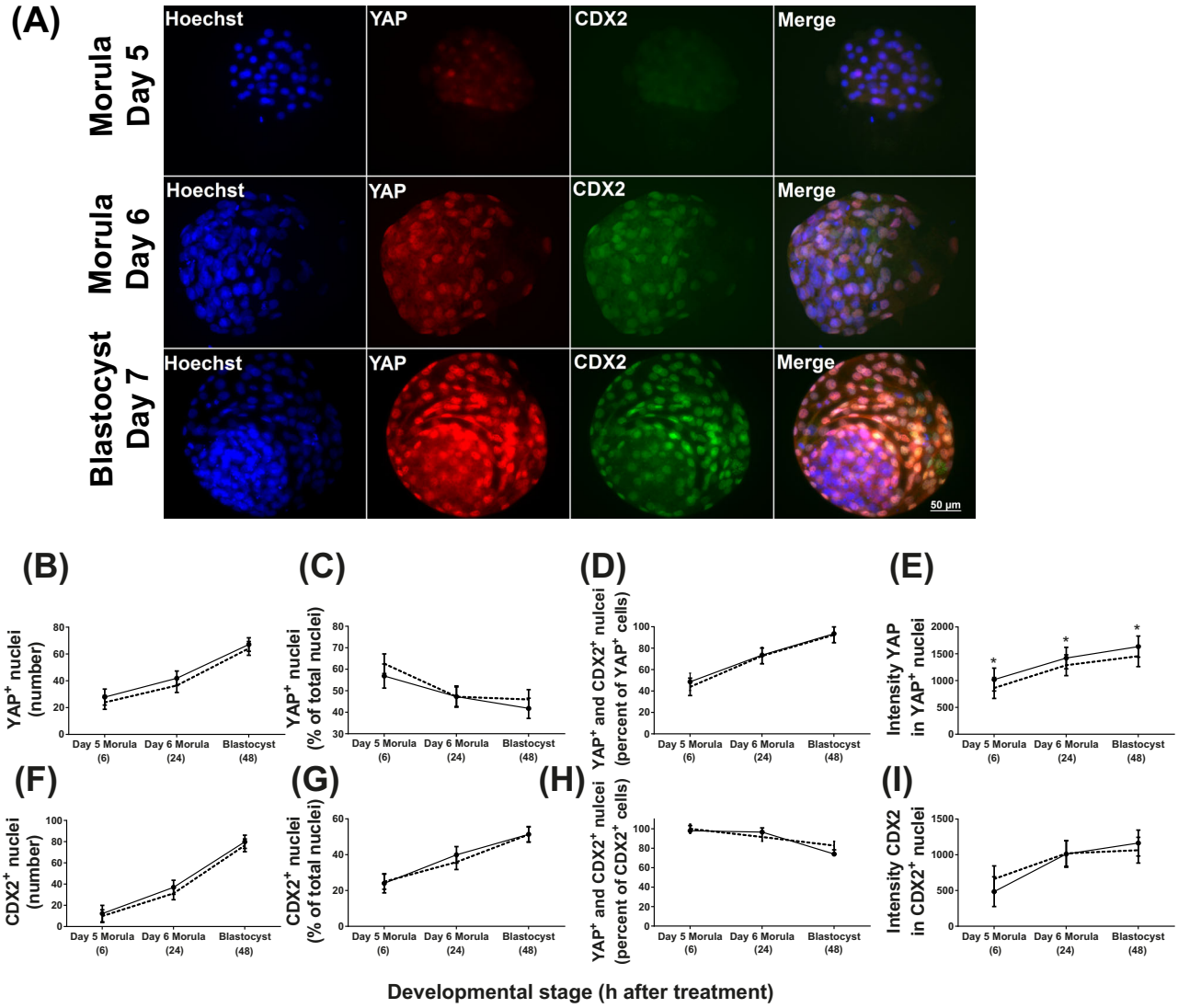

Figure 2 Immunolocalization of the transcription factors YAP1 and CDX2 in morulae and blastocysts. (A) Shown are representative images of individual embryos immunolabeled for YAP1 (red), CDX2 (green), and DNA (blue) at Days 5 (morulae), 6 (morulae), and 7 (blastocyst) of development. (B–I) Effect of treatment of embryos with DKK1 at Day 5 of development on immunoreactive YAP1 and CDX2 at Days 5 (morulae), 6 (morulae) and 7 (blastocysts) of development. Data represent the absolute number (B) and percent of total cells (C) positive for YAP1 and absolute number (F) and percent of total cells (G) positive for CDX2. Panels D and H show quantification of dual labeling for YAP1 and CDX2 expressed relative to number of $YAP1^+$ (D) and number of $CDX2^+$ nuclei (H). Panels E and I show intensity of labeling of nuclei for YAP1 (E) and CDX2 (I). Data are least-squares means ± SEM of results from five replicates. Embryos treated with vehicle are represented by closed circles and solid lines while embryos treated with DKK1 are represented by broken lines and open circles. Data are least-squares means ± SEM of results from five replicates, with a total of 232 labeled embryos. Asterisks indicate effect of DKK1 on intensity of YAP1 immunofluorescence ($P = 0.04$).

To investigate the signaling pathways involved, effects of WNT7A on accumulation of CTNNB1 and pJNK were evaluated in Experiments 10 and 11, respectively. There was no effect of WNT7A on total immunoreactive CTNNB1 on Day 7 (Figure 4B). Moreover, there was no nuclear localization of CTNNB1 regardless of treatment (Figure 4A and B). Treatment with WNT7A actually reduced JNK signaling as indicated by a reduction in immunoreactive pJNK measured in the total area of the embryo ($P < 0.0001$) or in nuclei ($P < 0.0001$; Figure 4C–E).

Taken together, results indicate that WNT7A increases proportion of embryos developing to the blastocyst stage through a mechanism independent of CTNNB1 that may involve reduction of pJNK.

Discussion

Collectively, data suggest that embryo-derived WNTs are dispensable for blastocyst formation in bovine embryos but participate in regulation of ICM proliferation, likely through a mechanism independent of CTNNB1. In contrast, exogenous WNTs can regulate competence of the embryo to develop to the blastocyst stage, with WNT agonists that increase intracellular CTNNB1 inhibiting development and WNTs like WNT7A that do not regulate intracellular CTNNB1 improving competence of the embryo to develop to the blastocyst stage.

There are two lines of evidence that endogenous WNT are not required for development to the blastocyst stage. The percent of

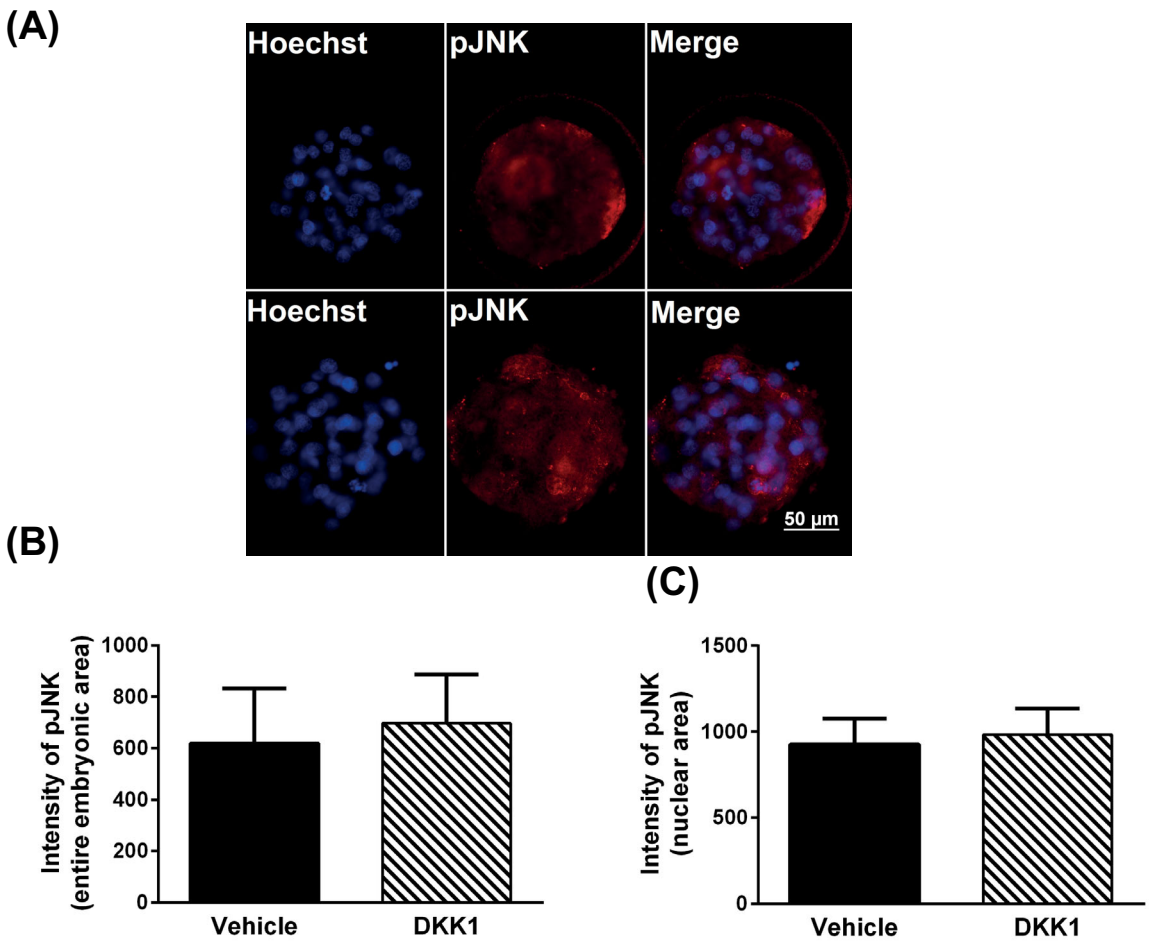

Figure 3 Effect of treatment of embryos with DKK1 on Day 5 of development on accumulation of pJNK in the resultant morulae. (A) Representative images of individual morula immunolabeled for pJNK (red) and DNA (blue). (B) Quantification of intensity of pJNK in whole embryonic area. (C) Quantification of intensity of pJNK in nuclear area. Data are least-squares means ± SEM of results from four replicates with a total of 91 labeled blastocysts.

Table 5. Effect of treatment of embryos with WNT7A from Day 1 to 7 or from Day 5 to 7 of development on the ability of embryos to develop to the blastocyst stage.[a]

		Development	
Timing of treatment	Treatment	Blastocysts/ oocyte (%)	Blastocysts/cleaved embryo (%)
Day 1 to 7	Vehicle	24.4 ± 2.2	37.5 ± 3.6
Day 1 to 7	WNT7A	31.0 ± 2.4	44.2 ± 3.6
Day 5 to 7	Vehicle	20.1 ± 2.0	31.7 ± 3.6
Day 5 to 7	WNT7A	29.8 ± 2.3	42.8 ± 3.6
Statistical significance (*P*)			
WNT7A		0.0005	0.02
Day		0.26	0.33
Interaction		0.51	0.54

[a]Data are the least-squares means ± SEM of results from six replicates representing 1566 COC.

embryos developing to the blastocyst stage was not reduced by inhibition of secretion of endogenous WNTs through a Wnt-C59-mediated block of PORCN, the enzyme that acylates WNTs for secretion [44]. Moreover, inhibition of CTNNB1-mediated WNT signaling with DKK1 did not alter the proportion of oocytes or cleaved embryos becoming a blastocyst. In earlier studies as well, there was no effect of DKK1 on development [24,30].

Similar to our findings, endogenous WNTs do not play a role in development to the blastocyst in mouse embryos. Blastocyst formation was not impaired in either *Ctnnb1* or *Porcn*-deficient mice [27, 28]. Also, there was no effect of Dkk1 on development to the blastocyst stage [30].

Although the cow parallels the mouse with respect to the dispensability of WNT signaling for development to the blastocyst stage, there may be divergence between species in role of embryo-derived WNT in formation of the ICM. As shown here, inhibition of PORCN increased the number of cells in the ICM of the bovine blastocyst, indicating that endogenous WNTs limit number of these cells. In contrast, numbers of ICM and TE cells were unperturbed in *Porcn*-mutant mouse blastocysts [27]. The mechanism by which WNTs regulate number of cells in the ICM could potentially be mediated by the proportion of blastomeres in the morula that remain pluripotent after differentiation of the TE or by the degree of proliferation and apoptosis of cells in the ICM. Specific WNTs can promote differentiation [26] and apoptosis [53] and decrease proliferation [54]. The importance of the number of ICM and TE cells at the

Table 6. Effect of treatment of embryos with WNT7A from Day 5 to 7 of development on the ability of embryos to develop to the blastocyst stage and cell number of Day 7 blastocysts.[a]

	Development		Blastocyst cell number			
Treatment	Blastocysts/ oocyte (%)	Blastocysts/cleaved embryo (%)	Total	TE	ICM	TE:ICM ratio
Vehicle	24.5 ± 1.7	32.1 ± 2.2	125.8 ± 11.2	76.9 ± 8.9	48.4 ± 5.9	1.7 ± 0.2
WNT7A	30.7 ± 1.7	39.2 ± 2.2	129.4 ± 11.6	74.1 ± 8.1	56.9 ± 4.6	1.5 ± 0.2
P value	0.02	0.04	0.75	0.70	0.24	0.36

[a]Data are the least-squares means ± SEM of results from seven replicates representing 1471 COC.

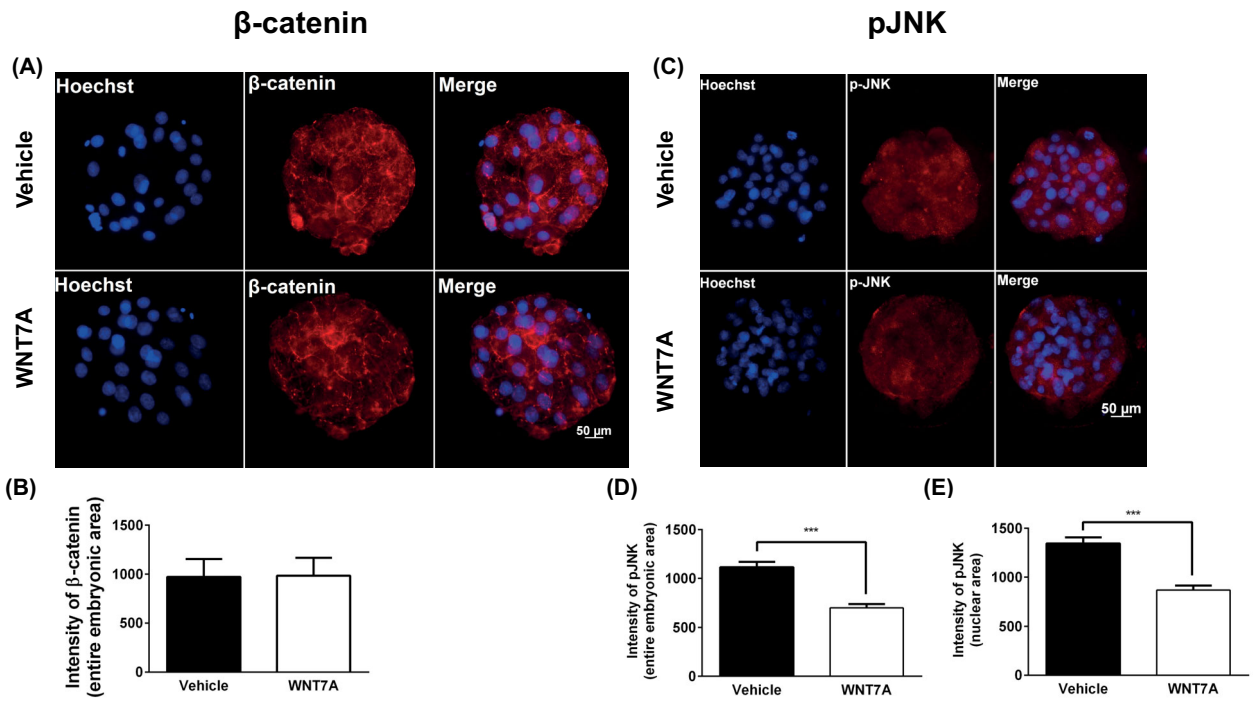

Figure 4 Effect of treatment of embryos with WNT7A from Day 5 to 7 of development on accumulation of CTNNB1 and pJNK. (A) Representative images of individual blastocysts immunolabeled for CTNNB1 (red) and DNA (blue). (B) Quantification of intensity of CTNNB1 in whole embryonic area. Data are least-squares means ± SEM of results from 10 replicates with 239 labeled embryos. (C) Representative images of individual blastocysts immunolabeled for pJNK (red) and DNA (blue). (D) Quantification of intensity of pJNK in whole embryonic area. (E) Quantification of intensity of pJNK in nuclear area. Data are least-squares means ± SEM of results from one replicate and 42 labeled blastocysts. ***$P < 0.0001$.

blastocyst stage for competence of the embryo to establish pregnancy has not been well defined in cattle. In the cow, about 20–30% of embryos produced in vitro fail to develop an embryonic disk after transfer to recipients [55, 56], and it is possible that failure of the disk to form could be the result of inadequate development of the ICM. In the human, characteristics of the ICM appear to be a less important determinant of potential for development after transfer than are properties of the TE [57–60]. Treatment of embryos with DKK1, which was shown to reduce CTNNB1 in the embryo, did not have an effect on numbers of ICM or TE cells. Thus, it is likely that the endogenous WNTs regulate number of ICM cells through a mechanism independent of CTNNB1. There was also no effect of DKK1 on the number of TE cells in the blastocyst.

Additional evidence against a role for DKK1 in differentiation of the bovine blastocyst was the finding that DKK1 reduced accumulation of YAP1, a transcription factor important for TE formation in mouse [61] and did not affect amounts of the transcription factor CDX2 that is responsible for TE differentiation [62]. These findings stand in contrast to earlier studies in cattle [38] and pigs [31] that DKK1 increases the number of TE cells in the blastocyst. The reason for the discrepancy between current findings and earlier ones with respect to actions of DKK1 on TE numbers is not known.

Embryo sex was one possible cause of variation in response of the embryo to DKK1 that was examined. Indeed, changes in gene expression in the bovine morula induced by DKK1 were different in some cases for male embryos than for female embryos [63]. Sex has an even larger effect on response of the bovine embryo to colony stimulating factor 2 [64, 65]. Despite these observations, the lack of effect of DKK1 on TE numbers was seen for both male and female embryos.

Although embryo-derived WNTs have little effect on the competence of an embryo to become a blastocyst, activation of WNT signaling, either by treatment with AMBMP or WNT7A, can modify the proportion of embryos developing to the blastocyst stage. The uterine endometrium expresses a wide number of WNT ligands including WNT family member 1 (*WNT1*), WNT family member

5A (*WNT5A)*, WNT family member 6 (*WNT6)*, *WNT7A*, WNT family member 8A (*WNT8A),* WNT family member 9A (*WNT9A)*, and WNT family member 9B (*WNT9B)* [44,66], and it is likely that maternally derived WNTs participate in embryonic development. Consequences of maternal WNT signaling are likely to depend on a complex array of factors including the abundance of specific WNT ligand, receptor and coreceptor availability, and presence of WNT regulatory molecules such as DKK1 and soluble FZD receptors, which are also expressed in the endometrium [44]. The fact that fertility in cows [34] and heifers [67] is associated with endometrial expression of DKK1 could reflect the importance of optimal WNT signaling for successful pregnancy.

Present results indicate that WNTs that increase CTNNB1 decrease developmental competence because two treatments that increase cellular CTNNB1, GSK3 inhibitor, and the WNT mimetic AMBMP decreased the proportion of embryos that developed to the blastocyst stage. In an earlier study, AMBMP decreased development of bovine embryos [24]. Effects of AMBMP were decreased by DKK1, which also decreased the amount of CTNNB1 in the embryo. This result is an indication that actions of AMBMP involve accumulation of CTNNB1 and that maternally derived molecules such as DKK1 can modify responses of the embryo to those WNTs that increase cellular CTNNB1. As reported earlier [45], CTNNB1 was not localized in the nucleus of the embryo after treatment with GSK inhibitor or AMBMP, and thus it is likely that CTNNB1 acts independent of nuclear site of action.

Not all maternally derived WNT are likely to inhibit development. Present results indicate that WNT7A, which does not affect amounts of CTNNB1 in the embryo but does decrease phosphorylation of JNK, increased the proportion of embryos that developed to the blastocyst stage. WNT7A is not expressed in the bovine preimplantation embryo [24, 45] but is highly expressed in bovine endometrium [44]. Although it has been described that WNT signaling mediated by JNK is required for cavity formation in mouse embryos [30, 68], the role of WNT signaling mediated by pJNK remains unknown in the cow.

One of the objectives of the series of experiments documented here was to identify downstream pathways affected by DKK1. This molecule, which can block canonical WNT signaling by interfering with recruitment of the LRP5/6 coreceptor to the WNT-FZD ligand receptor [69,70], may be an important determinant of fertility in the cow. Bovine embryos treated with DKK1 were more likely to establish and maintain pregnancy after transfer to recipient cows than embryos not treated with DKK1 [38]. Also, expression of *DKK1* in endometrium was lower for heifers diagnosed as infertile compared to heifers considered fertile [67] and was lower for endometrium of lactating cows than nonlactating cows [34].

Actions of DKK1 are complex because, in addition to interfering with WNT-FZD signaling, DKK1 can act as a WNT agonist to activate noncanonical signaling pathways such as the PCP pathway [36]. In the present work, the ability of DKK1 to reduce accumulation of CTNNB1 was documented, both because it reduced amounts of immunoreactive CTNNB1 and because it blocked actions of AMBMP. Treatment with DKK1 did not prevent AMBMP from increasing CTNNB1 but amounts of the protein were lower in embryos treated with both AMBMP and DKK1 than embryos treated with AMBMP alone. Thus, AMBMP increases CTNNB1 in the cell by acting downstream from formation of the WNT receptor–coreceptor complex. Even though DKK1 did not prevent AMBMP from increasing CTNNB1 accumulation, it blocked actions of AMBMP on development probably because of the AMBMP-independent actions of DKK1 to decrease accumulation of CTNNB1.

In contrast to regulation of CTNNB1 in the embryo, DKK1 had no effect on JNK phosphorylation even though DKK1 can activate JNK signaling in other cellular systems [37,71]. The lack of effect of DKK1 on activation of JNK in the bovine embryo may reflect insufficient amounts of one or more molecules involved in the pathway by which DKK1 activates JNK signaling.

To our knowledge, data presented here are first description of localization of YAP1 in the bovine embryo. This transcription factor plays an important role in TE formation in the mouse by interacting with TEAD4 to induce transcription of CDX2 [42,43]. The pattern of expression of YAP1 from the morula stage at Day 5 to the blastocyst stage at Day 7 indicates that YAP1 accumulation in the nucleus precedes that of CDX2, as revealed by higher number of $YAP1^+$ than $CDX2^+$ nuclei at Day 5 morulae, and presence of nuclear YAP1 in every nucleus that was $CDX2^+$ at this developmental stage. As embryos developed, however, the proportion of blastomeres that were $YAP1^+$ declined and fewer nuclei were $YAP1^+$ than were $CDX2^+$. By the blastocyst stage, both YAP1 and CDX2 were localized in the TE. This observation, as well as the observation that over 90% of $YAP1^+$ nuclei in the blastocyst were also $CDX2^+$, is consistent with a role of YAP1 in CDX2 expression and TE differentiation. Functional studies are needed to determine whether or not YAP1 is required for CDX2 expression in bovine embryos. Recently, it was shown that the binding partner for YAP1, TEAD4, is not required for *CDX2* expression or blastocyst formation [72].

Taken together, these data suggest that embryo-derived WNTs are dispensable for blastocyst formation in bovine embryos but do participate in the formation of the ICM. In contrast, exogenous WNTs can affect embryonic development in a positive or negative manner depending upon the nature of the WNT ligand and the downstream outcome. Such a result implies that maternally derived WNT could play important roles in development of the preimplantation embryo.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental File S1. Information on antibodies used.

Acknowledgments

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