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Canonical WNT signaling regulates development of bovine embryos to the blastocyst stage

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Objectives were to evaluate the role of canonical WNT signaling in development of the preimplantation embryo. Signaling was activated with 2-Amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine (AMBMP) and inhibited with Dickkopf-related protein 1 (DKK1). Treatment of bovine embryos with AMBMP at day 5 after insemination decreased development to the blastocyst stage at day 7 and reduced numbers of trophectoderm and inner cell mass cells. At high concentrations, AMBMP caused disorganization of the inner cell mass. DKK1 blocked actions of AMBMP but did not affect development in the absence of AMBMP. Examination of gene expression in day 6 morulae by microarray revealed expression of 16 *WNT* genes and other genes involved in WNT signaling; differences in relative expression were confirmed by PCR for 7 genes. In conclusion, the preimplantation embryo possesses a functional WNT signaling system and activation of the canonical pathway can inhibit embryonic development.

The WNT signaling system plays important roles in directing developmental processes including maintenance of pluripotency[1,2], cell migration during gastrulation and neurulation[3], and axis formation[4,5]. WNT proteins regulate cell function through a variety of cell signaling systems. The canonical pathway involves binding of WNT proteins to a membrane receptor, Frizzled (FZD), and co-receptor, LDL receptor-related protein (LRP) 5 and 6. This signal activates the downstream messenger Dishevelled that blocks action of the β-catenin destruction complex, resulting in accumulation of β-catenin in the cytoplasm and translocation to the nucleus, where it activates transcription factors such as T-cell factor and lymphocyte enhancer factor[6–8]. Non-canonical signaling pathways are activated by binding of WNTs to various receptors including FZD, ROR2, and RYK[9,10].

The canonical pathway has been associated with regulation of cell fate while non-canonical pathways have been implicated in regulation of polarity, asymmetric cell divisions, and cell movements during gastrulation[11]. While individual WNT proteins are often classified as to whether they activate canonical or non-canonical pathways, activation of a specific pathway is dependent not only on the ligand but also receptor availability, cell type, specific stage of embryonic development and agonistic or antagonistic effects of other WNT proteins[8,10,11].

Little is known about the role of WNT signaling in embryonic development during the preimplantation period. Evidence from the mouse indicates that the Wnt system is present and activated as early as the two-cell stage[12–15]. However, inhibition of Wnt signaling does not compromise development to the blastocyst stage[14] and, therefore, activation of this signaling system may not be a requirement for preimplantation development. Activation of WNT signaling in bovine embryos by inhibitors of glycogen synthase kinase 3 (GSK3) had inconsistent effects on development to the blastocyst stage. One inhibitor, $LiCl_2$, blocked development to the blastocyst stage while another, CT99021, increased the percent of zygotes that developed to the blastocyst stage[16].

Here we tested the hypothesis that activation of WNT signaling during early embryonic development inhibits development of embryos to the blastocyst stage. The basis for the hypothesis was the observation that colony stimulating factor-2 (CSF2), which alters gene expression in a way that would inhibit WNT signaling[17], can enhance the competence of embryos to develop to the blastocyst stage and to establish pregnancy when transferred into females[18]. WNT signaling was activated by administration of 2-Amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine (AMBMP), a WNT agonist that activates canonical signaling[19,20]. The timing of agonist administration, day 5 after insemination, represents a period in development after the

embryo has undergone major genome activation[21] and is at the morula stage of development. Moreover, it is identical to the time of development when CSF2 acts to enhance embryonic development. To determine whether actions of AMBMP were mediated by a canonical WNT pathway, it was tested whether effects of AMBMP could be blocked by Dickkopf-related protein 1 (DKK1), which interferes with binding of WNTs to the co-receptor LRP 5/6[22,23]. Administration of DKK1 was also used to assess the effects of signaling of endogenous WNTs through LRP 5/6 on early embryonic development. Finally, the capacity of the preimplantation embryo to express genes involved in WNT signaling was evaluated by querying a microarray database generated using mRNA from the bovine morula for expression of genes involved in WNT signaling.

Results

Effect of AMBMP on development to the blastocyst stage. The first experiment was designed to test if activation of canonical WNT signaling blocked development of embryos to the blastocyst stage. Addition of AMBMP to culture medium at day 5 after insemination caused a concentration-dependent decrease ($P < 0.001$) in the proportion of oocytes that reached the blastocyst stage at day 7 after insemination (Table 1). The minimum concentration necessary to obtain a significant inhibitory effect was 0.7 μM AMBMP.

AMBMP reduced significantly total cell number at concentrations of 0.7 μM and higher (Table 1). Similarly, AMBMP reduced significantly numbers of trophectoderm (TE) cells at 1.4 μM and higher and numbers of ICM cells at 0.7 and 2.8 μM (Table 1). The ratio of ICM/TE cells was not significantly affected ($P = 0.33$) by AMBMP but the ratios were numerically highest at the two highest concentrations of AMBMP. At the highest concentrations of AMBMP, the ICM appeared disorganized with several cells not labeled with CDX2 (i.e. ICM) being located outside the cluster of cells characterized by the ICM (Figure 1).

Inhibition of actions of AMBMP by CSF2. An experiment was conducted to determine whether AMBMP would inhibit embryonic development when embryos were exposed to CSF2, which has been reported to alter genes involved in WNT signaling. Embryos cultured with CSF2 (10 ng/ml) beginning at Day 5 were exposed to AMBMP (0.7 μM) at Day 6. Despite the preesence of CSF2, AMBMP decreased ($P = 0.04$) blastocyst development at day 7 (percent of embryos becoming blastocysts = 41.5 ± 3.7% vs 29.9% ± 3.7%, for embryos treated with vehicle and AMBMP, respectively).

Inhibition of actions of AMBMP by the WNT antagonist DKK1. A third experiment was designed to test whether AMBMP blocked development by acting through the WNT signaling pathway. The approach was to determine whether DKK1, which prevents binding of the co-receptor LRP 5/6 to the agonist-receptor complex, blocks effects of AMBMP on development. As shown in Figure 2, AMBMP decreased ($P = 0.02$) the proportion of embryos that developed to the blastocyst stage at day 7. Moreover, blastocyst development was affected by the interaction between AMBMP and DKK1 at day 7 ($P = 0.01$). This interaction occurred as a result of blockage of the negative effect of AMBMP on blastocyst development by DKK1 (Figure 2); combination of AMBMP and DKK1 resulted in 22.2 ± 2.1% of embryos developing to blastocysts at day 7 compared to 15.9 ± 2.1% for AMBMP alone. In the absence of AMBMP, however, DKK1 tended to reduce blastocyst development at day 7 (29.9 versus 21.1 ± 2.1% for control and 100 ng/ml of DKK1, respectively).

Effect of DKK1 on blastocyst development. Because there was a tendency of DKK1 to decrease development, an additional experiment was performed to determine whether DKK1 itself has deleterious effects on embryo competence to develop to the blastocyst stage. Embryos were cultured with concentrations of DKK1 ranging from 0 to 400 ng/ml, beginning at day 5 after insemination. As shown in Figure 3, addition of DKK1 at concentrations of 50, 100, 200 and 400 ng/ml did not change ($P = 0.47$) the proportion of embryos reaching the blastocyst stage at day 7 of development as compared to embryos cultured without DKK1.

Expression of genes involved in WNT signaling. Examination of gene expression in the day 6 embryo using microarray analysis (Table 2) revealed that the embryo expresses at least 16 *WNT* genes at this stage, with greatest hybridization signal for *WNT1, WNT2B, WNT11, WNT8A* and *WNT10B*. Moreover, genes for WNT receptors were expressed including 11 *FZD* genes, the co-receptors *LRP5* and *LRP6*, and non-canonical receptors *RYK* and *ROR2*. Genes encoding signal transduction molecules for both canonical and non-canonical pathways were expressed, such as *CTNNBL1* (gene encoding β-catenin), as were a variety of genes encoding for proteins that modulate WNT signaling (such as *DKK1*).

Analysis of a separate set of embryos at day 6 of development by qPCR confirmed results of the microarray. A total of 7 genes were analyzed and all but one (*WNT3*) were detectable by qPCR (Figure 4A). Moreover, the amount of mRNA, expressed as the fold-change relative to *WNT11* (the least-abundant detectable gene) was highly correlated ($r = 0.83$, $P = 0.02$) with amount of mRNA detected by microarray analysis (Figure 4B).

Discussion

Using the bovine embryo as a model, we demonstrate here that WNT signaling affects embryonic development to the blastocyst stage. Activation of canonical WNT signaling at day 5 of development decreased the number of embryos reaching the blastocyst stage. This conclusion is based on the observation that AMBMP decreased the proportion of embryos that developed to the blastocyst stage in a concentration-dependent manner. Likewise, this action of AMBMP was blocked by administration of DKK1 to interfere with FZD-LRP 5/6 receptor signaling.

The mechanism by which AMBMP inhibits development is not known. One possibility is that AMBMP blocked proliferation. Such an explanation is consistent with the reduction in blastocyst cell

Table 1 | Effects of addition of the WNT agonist AMBMP at day 5 after insemination on the proportion of oocytes that developed to the blastocyst stage at day 7 after insemination[a]

		Day 7 blastocyst cell numbers			
[AMBP] (μM)	Percent oocytes becoming blastocysts at day 7	Total cells	ICM	TE	ICM:TE ratio
0	20.1 ± 1.7	132.6 ± 8.0	69.9 ± 4.3	63.6 ± 6.0	1.02
0.35	19.7 ± 1.7	111.9 ± 8.0	58.5 ± 4.3	55.0 ± 6.0	1.03
0.7	12.3 ± 1.7**	103.0 ± 8.0*	55.2 ± 4.3*	47.8 ± 6.0	1.07
1.4	7.3 ± 1.7***	101.5 ± 10.2*	62.1 ± 5.6	39.2 ± 7.6*	1.46
2.8	2.9 ± 1.7***	79.9 ± 12.0**	48.5 ± 6.5*	31.0 ± 9.0**	1.53

[a]Columns with asterisks differ from 0 μM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

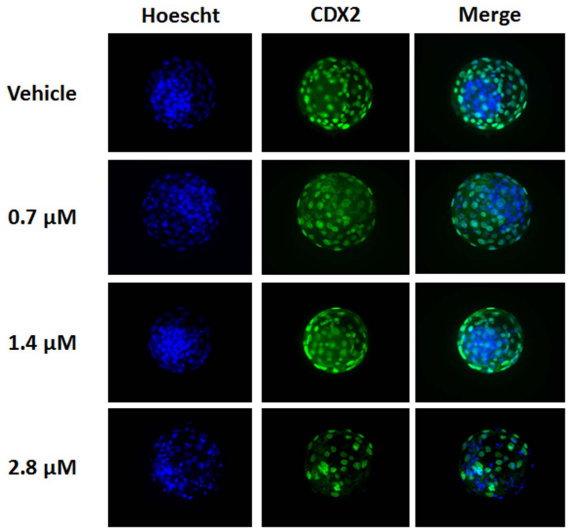

Figure 1 | Representative images of labeling of inner cell mass and trophectoderm cells. Blastocysts were harvested at day 7 and subjected to differential immunofluorescence. Blue: Hoescht 33342 staining (all nuclei); green: $CDX2^+$ cells (labeled with mouse anti-CDX2 and FITC-conjugated anti mouse IgG). Trophectoderm cells were labeled with Hoescht and anti CDX2 while inner cell mass cells were labeled with Hoescht only.

number caused by AMBMP. While canonical WNT signaling is often associated with an increase in cell proliferation[24–26], inhibition of canonical WNT signaling by DKK1 can induce cell proliferation in some cells such as endothelial colony-forming cells[27].

In addition to its role as the intra-cellular effector of canonical WNT signaling, β-catenin is also a main component of adherens junctions[28], linking the adhesive glycoprotein E-cadherin to actin filaments[29]. Knockout of E-cadherin is lethal to mice embryos, as it impairs formation of the blastocoele and subsequent hatching of the embryo[30]. Perhaps, over-activation of WNT signaling through AMBMP results in recruitment of adherens junctions associated

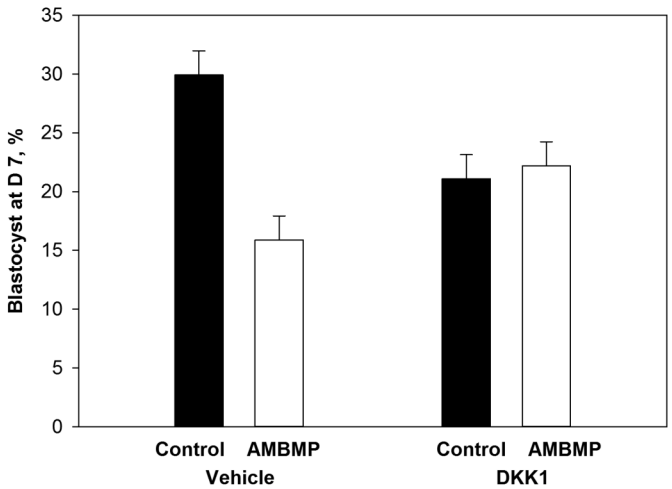

Figure 2 | Co-treatment of bovine embryos at day 5 after insemination with 100 ng/ml Dickkopf-related protein 1 (DKK1) reduces the inhibitory effect of a WNT agonist (AMBMP) on development to the blastocyst stage. Data are least-squares means ± SEM of results from 5 replicates. The percent of oocytes becoming blastocysts at day 7 was affected by agonist (P = 0.02) and the agonist by DKK1 interaction (P = 0.01).

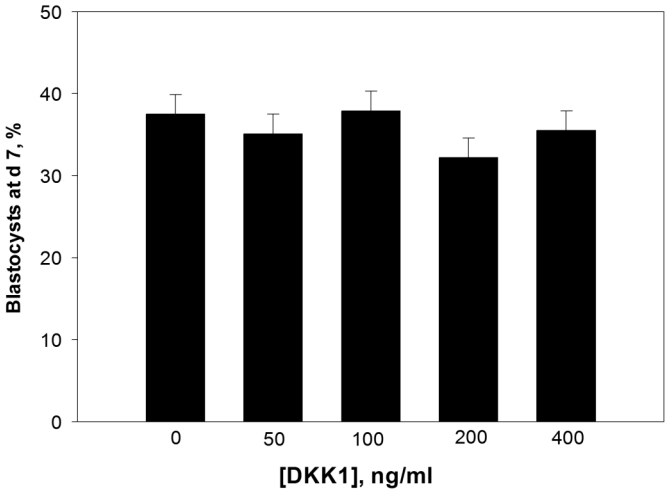

Figure 3 | Effect of administration of different concentrations of Dickkopf related protein 1 (DKK1) at day 5 after insemination on blastocyst development at day 7. Data are least-squares means ± SEM of results from 7 replicates. No difference in the percent of ooyctes that became blastocysts at day 7 after insemination was observed following treatment with DKK1 at concentrations between 50 and 400 ng/ml (P = 0.47).

β-catenin to the nucleus, decreasing E-cadherin mediated cell-cell adhesion and disrupting formation of the blastocoele.

It is also possible that the decreased blastocyst formation caused by AMBMP is the result of maintenance of pluripotency and inhibition of blastomere differentiation. There is abundant evidence that canonical WNT signaling can promote pluripotency[2,31–34]. Consistent with this idea is that there was a tendency for WNT to affect numbers of TE cells to a greater extent than numbers of ICM cells.

Given the inhibitory effect of activation of the canonical pathway achieved by AMBMP and the expression of canonical WNTs by the day 6 embryo, it was surprising that inhibition of endogenously produced canonical WNTs by addition of DKK1 did not increase the percent of embryos that developed to the blastocyst stage. DKK1 inhibits the canonical WNT pathway through binding and inactivation of the LRP 5/6 WNT co-receptors[22,35,36]. However, DKK1 has more recently been associated with inhibition of non-canonical pathways in human cancer cell lines[37] and with inhibition of both canonical and non-canonical pathways altering axis formation in the *Xenopus* embryo[38]. Thus, DKK1 could conceivably affect activation of canonical and non-canonical pathways activated by endogenously produced WNTs. Also, actions of one WNT can depend on the presence of another[8,39] so global inhibition of WNT signaling might cause actions different than actions of individual WNTs. In the mouse, inhibition of endogenous canonical WNT signaling with either recombinant DKK1 or PKF115-584 and CGP049090, two small chemical molecules that inhibit the β-catenin/TCF complex, had no effect on development to the blastocyst stage[14]. Inhibition did, however, compromise development through implantation. Conducting experiments to determine long-term consequences of regulation of WNT signaling during the preimplantation period in cattle will provide additional insight into the role of WNTs in the preimplantation period.

Present results demonstrate that the machinery for WNT signaling is present in the preimplantation embryo. The microarray data demonstrate that genes for canonical and non-canonical WNTs, non-WNT ligands such as Norrie disease protein and R-spondin, FZD and alternative WNT receptors such as RYK and ROR2, and a great variety of molecules involved in WNT signaling regulation are present in Day 6 bovine morulae. This finding was confirmed by qPCR using a subset of genes and different pools of day 6 morulae.

Table 2 | Expression of genes involved in WNT signaling in Day 6 embryos[a]

WNTs		Frizzled receptors and LRP co-receptors		Canonical signaling proteins		Alternative WNT receptors and signaling proteins	
Gene	Intensity	Gene	Intensity	Gene	Intensity	Gene	Intensity
WNT1	234	*FZD1*	909	*DVL1*	2139	*RYK*	102
WNT2B	91	*FZD2*	34	*DVL2*	8723	*ROR2*	63
WNT3	20	*FZD3*	21769	*DVL3*	44	*SRC*	83
WNT3A	37	*FZD4*	4583	*AXIN1*	42	*CAMK2D*	1620
WNT4	17	*FZD5*	21	*AXIN2*	772	*NDP*	16
WNT5A	25	*FZD6*	93	*APC*	459	*RSPO3*	3439
WNT5B	3	*FZD7*	7504	*GSK3B*	2885	*RSPO2*	422
WNT7B	5	*FZD8*	122	*CSNK1A1*	15405	*RSPO1*	99
WNT8A	72	*FZD9*	9	*CTNNBL1*	9092	*RSPO4*	4
WNT8B	13	*FZD10*	34			WNT regulatory proteins	
WNT9A	7	*FRZB*	44	Housekeeping genes		*DKK1*	139
WNT9B	48	*LRP5*	1748	*GAPDH*	14788	*DKK2*	2
WNT10A	11	*LRP6*	830	*H2A*	3062	*DKK3*	450
WNT10B	72			*LMNB1*	1032	*DKKL1*	1247
WNT11	80	WNT processing		*ACTB*	26571	*CCDC88*	52
WNT16	14	*CHURC1*	288			*SFRP1*	143
						SFRP2	18
						SFRP4	3
						SFRP5	103
						WIF1	25

[a]Data are average fluorescent intensities from four pools of embryos.

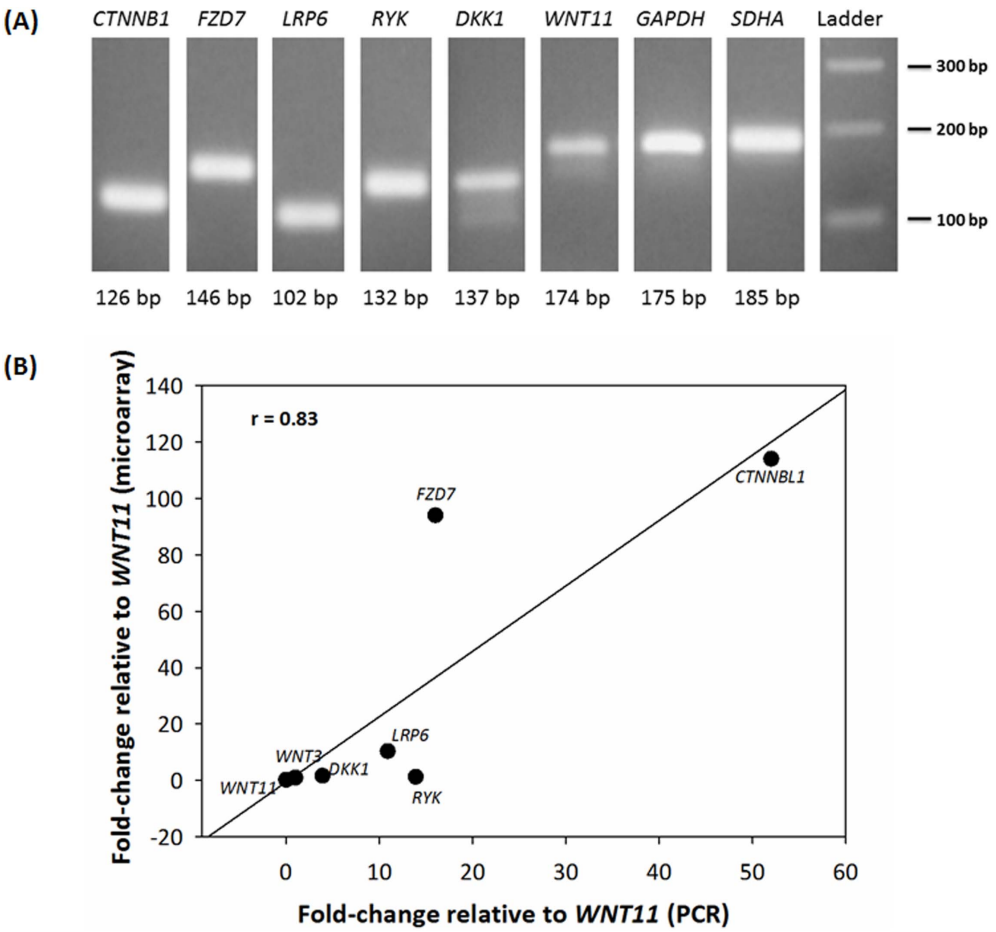

Figure 4 | Genes related to WNT signaling are expressed in day 6 morulae. (A) Agarose gel electrophoretogram of reverse transcriptase PCR products for *CTNNBL1*, *DKK1*, *FZD7*, *LRP6*, *RYK*, *WNT11*, *SDHA* and *GAPDH*. Note that another gene, *WNT3*, was not detectable. (B) Correlation between expression results for day 6 morulae obtained from microarray analysis and from real-time PCR. *WNT3* was undetectable and the amounts of other mRNA were expressed as a fold-change relative to amount of *WNT11*.

Table 3 | Primer sequences used for real time PCR

Gene name	Gene ID	Reference Sequence	Primers	Product size (bp)
CTNNBL1	282421	NM_174637.4	F: 5′-AATCCGAGCTGGACCTCAATGACA-3′ R: 5′-TATCATGTCCAAGCAGCCCGAGAA-3′	126
RYK	781353	XM_002685100.2	F: 5′-TAGTGATGTGTGGGCCTTTGGAGT-3′ R: 5′-TTGATTGGCTGGGCTATCCGGTAA-3′	132
FZD7	524255	NM_001144091.1	F: 5′-TTGTTTCTGGAACCTCCTTCCGCT-3′ R: 5′-GCATCATCCTGCAAGTCTTTGCCA-3′	146
LRP6	536328	XM_002687783.2	F: 5′-AGTGCCCTGGAACATGTGGTAGAA-3′ R: 5′-ATTGGTTCCTGTGTCTGCCCAGTA-3′	102
DKK1	504445	NM_001205544.1	F: 5′-GACTGGTGGAGGCGCTCGGA-3′ R: 5′-GCTGTGCCCAGAGCCGTCAT-3′	137
WNT3	540753	NM_001206024.1	F: 5′-TCTGTGGCTGCGACTCACATCATA-3′ R: 5′- CAGCCTCGTTGTTGTGCTTGTTCA -3′	—
WNT11	613288	NM_001082456.2	F: 5′-ATCAGGGAAGGAAAGCCAGGACAT-3′ R: 5′-AAGCTGGTGTCTCTGGCTACGAAA-3′	174
GAPDH	281181	XM_618013	F: 5′-TTCAACGGCACAGTCAAGG-3′ R: 5′-ACATACTCAGCACCAGCATCAC-3′	175
SDHA	281480	NM_174178	F: 5′-GCAGAACCTGATGCTTTGTG-3′ R: 5′-CGTAGGAGAGCGTGTGCTT-3′	185

Norrie disease protein, also called Norrin[40], and R-spondin have been identified as alternative FZD ligands that are capable of activating canonical WNT signaling[41]. Likewise, ROR and RYK are transmembrane tyrosine kinases that have been recently identified as alternative (non-FZD) WNT receptors[41]. There is additional evidence in the literature indicating the presence of WNT signaling systems in the early embryo. Genes involved in WNT signaling pathways are expressed in both bovine oocytes and blastocysts[42]. Aparicio and coworkers[16] demonstrated the presence of GSK3 protein, a key component of the β-catenin destruction complex, in bovine embryos from the 2-cell to the blastocyst stage. A variety of Wnts are expressed in murine blastocysts, with *Wnt3a, Wnt6, Wnt 7b, Wnt 9a*, and *Wnt 10b* showing the highest expression levels[13] and *Wnts* are expressed as early as the 2-cell stage[12]. Kemp and collaborators[13] also reported the expression of Wnt antagonists such as *Sfrp*s and *Dkk*s, with greatest detection of *Dkk1*, in murine blastocysts.

It is likely that maternal regulation of preimplantation embryonic development involves regulation of embryonic WNT signaling. One such maternal signal might be CSF2, which improves ability of bovine embryos to develop to the blastocyst stage and to establish pregnancy after transfer into recipients[18]. One action of CSF2, when administered at the morula stage, is alteration of gene expression in a manner that was interpreted as causing inhibition of WNT signaling[17]. For example, CSF2 decreased the non-canonical *WNT16* and increased expression of genes such as secreted frizzled-related protein 4 (*SFRP4*) that inhibit canonical WNT signals[17]. Our present findings indicate, however, that AMBMP inhibits development in the presence of CSF2. This result is suggestive that the positive action of CSF2 on embryonic survival is not mediated by inhibition of canonical WNT signaling.

In conclusion, our findings demonstrate that WNT signaling is present in pre-implantation embryos and plays a role in the regulation of development to the blastocyst stage. Activation of canonical signaling decreases development and blastocyst cell numbers; however, inhibition of WNT signaling by DKK1 is not beneficial to blastocyst development. The exact role for regulation of preimplantation development by WNTs is not yet clear. It has been postulated that the roles of different WNTs are variable according to developmental stage and factors such as cell environment, presence and abundance of different receptors and effector molecules[8]. It is very likely that regulation of embryonic development by WNTs involves a plethora of endogenous WNTs, receptors and signaling molecules, as well as a fine regulation of canonical and non-canonical pathways to balance maintenance of pluripotency, self-renewal and cell-fate commitment.

Methods

Reagents. Calbiochem (San Diego, CA, USA) was the source of AMBMP. The molecule was diluted in dimethylsulfoxide (DMSO) to a concentration of 2.8 mM. Aliquots of 100 µl-volume were stored at −20°C and protected from light until used. Human recombinant DKK1 was from R&D Systems (Minneapolis, MN). It was reconstituted using Dulbecco's phosphate buffered saline (DPBS) with 0.1% (w/v) bovine serum albumin (BSA) to a concentration of 100 µg/ml. Aliquots of 5 µl were stored at −20°C until use. Recombinant bovine CSF2 was donated by Novartis (Basel, Switzerland). Mouse monoclonal antibody (ascites) against CDX2 was purchased from Biogenex (Fremont, CA, USA) and affinity-purified goat anti-mouse IgG coupled to fluorescein (FITC) was from Abcam (Cambridge, MA, USA).

Production of embryos. Bovine embryos were produced by in vitro fertilization from oocytes obtained from abattoir-collected ovaries. All procedures for embryo production followed techniques previously described[43].

Effect of AMBMP on development to the blastocyst stage. Following fertilization, putative zygotes (a mix of fertilized and unfertilized oocytes) were randomly placed in groups of 30 in 45 µl-microdrops of culture medium (SOF-BE1)[43] covered with mineral oil. On day 5 after fertilization, AMBMP was added to each drop in a volume of 5 µl to achieve a final concentration of 0, 0.35, 0.7, 1.4 or 2.8 µM AMBMP and 0.1% (v/v) DMSO. Development to blastocyst stage was evaluated at day 7, and blastocysts were harvested and processed for determination of total cell number and number of ICM and TE cells. The experiment was conducted in 6 replicates using a total of 1,102 embryos.

Blastocyst cell numbers were determined as follows. All steps were conducted at room temperature unless otherwise described. Blastocysts were harvested from culture drops, washed three times in DPBS with 0.2% (w/v) polyvinylpyrrolidone (DPBS-PVP) and fixed in 4% (w/v) paraformaldehyde in DPBS-PVP for 15 min. Embryos were washed again three times in DPBS–PVP and permeabilized with 0.25% (v/v) Triton X-100 in DPBS for 20 min. After washing 3 times and incubation with a blocking buffer of 5% (w/v) BSA in DPBS (w/v) for 1 h, embryos were incubated with anti-CDX2 at 4°C overnight (used at the working concentration provided by the manufacturer). Embryos were washed with antibody buffer [10 mM Tris, pH 7.5 containing 0.15 M NaCl, 0.1% bovine serum albumin and 0.1% (v/v) Tween-20] and incubated with 1 µg/ml FITC-labeled goat anti-mouse IgG diluted in antibody buffer for 1 h at room temperature in the dark. Embryos were then incubated with 5 µg/ml Hoechst 33342 in DPBS-PVP for 15 min in the dark. Embryos were washed three times with DPBS–PVP, placed on clean microscope slides with Prolong® anti-fade solution (Invitrogen, Carlsbad, CA, USA), covered with a cover slip and examined for fluorescence using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Gottingen, Germany) with a 40× objective and using Zeiss filter set 02 (DAPI filter) and Zeiss filter set 03 (FITC filter). Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera. Total cell number was determined by counting nuclei staining with Hoescht 33342, number of TE cells determined by counting nuclei positive for CDX2 (FITC) and number of ICM cells determined by subtracting number of TE cells from total cell number.

Inhibition of actions of AMBMP by CSF2. Following fertilization, putative zygotes were randomly allocated in groups of 30 in 40 µl-culture drops. At Day 5 after fertilization, embryos received 5 µl of CSF2 in culture medium to a final concentration of 10 ng/ml. At day 6, an additional 5 µl of medium was added containing either vehicle or AMBMP (final concentration = 0.7 µM). Blastocyst development was evaluated and recorded at Day 7 after insemination. This experiment was performed in five replicates using a total of 394 embryos.

Inhibition of actions of AMBMP by the WNT antagonist DKK1. Following fertilization, putative zygotes were randomly allocated in groups of 30 in 40 µl-culture drops. At day 5 after fertilization, AMBMP (diluted as in the previous experiment) or vehicle was added in a volume of 5 µl. In addition, DKK1, diluted in SOF-BE1 was added in a volume of 5 µl. The final concentration of DKK1 was 100 ng/ml. Drops of embryos were treated with vehicle only [0.1% (v/v) DMSO in SOF – control group], 0.70 µM of AMBMP, 100 ng/ml of DKK1, or 0.70 µM of AMBMP plus 100 ng/ml DKK1 (final concentrations). Development to the blastocyst stage was evaluated at day 7 after insemination. The experiment was performed in 5 replicates using a total of 1,487 embryos.

Effect of DKK1 on blastocyst development. Following fertilization, putative zygotes were randomly allocated in groups of 30 in 45 µl-culture drops. Dilution of DKK1 was performed as described in the previous experiment. At day 5 after fertilization, drops were treated with vehicle only [SOF-BE1 – control group] or 50, 100, 200 or 400 ng/ml of DKK1 (final concentrations). Development to the blastocyst stage was evaluated at day 7. The experiment was replicated 7 times using a total of 1,467 embryos.

Expression of genes involved in WNT signaling. A previously reported microarray database obtained by analysis of four separate pools of mRNA from bovine embryos at Day 6 of development with the *Bos taurus* Two Color Microarray Chip (Agilent Technologies, Santa Clara, CA, USA)[17] was examined for expression of *WNT* genes as well as genes involved in WNT signaling. The relative abundance of each gene was determined by the intensity of the hybridization signal. Expression of four genes used as housekeeping genes in the bovine embryo (*GAPDH, H2A, LMNB1,* and *ACTB*)[45] was also assessed to compare intensity of hybridization with that of genes involved in WNT signaling.

Real-time quantitative polymerase chain reaction (qPCR) of WNT related genes. Of the genes shown by microarray to be expressed in day 6 embryos, seven that encode main components of the WNT pathway were chosen for qPCR: *WNT3, WNT11, FZD7, DKK1, LRP6, RYK* and *CTNNBL1*. *GAPDH* and *SDHA* were used as housekeeping genes.

Primers were designed and obtained from Integrated DNA Technologies (Coralville, Iowa, USA), except for the housekeeping genes *SDHA*[44] and *GAPDH*[45], which were previously published. Primer sequences are listed in Table 3. A standard curve was performed for each primer using bovine day 7 blastocysts. PCR amplicons were sequenced and the obtained products were mapped to the *Bos taurus* genome using the Basic Local Alignment Search Tool of NCBI. All amplicon sequences aligned to the genes for which primers were designed with identities between 93 and 99%. For qPCR of day 6 morulae, 2 pools of 40 embryos were used. RNA was isolated using PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. RNA concentration and quality were assessed using NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA). DNase treatment was performed using Qiagen DNase reagents (Qiagen, Valencia, CA) and reverse transcription using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qPCR reaction was performed using SsoFast EvaGreen Supermix reagent (Bio-Rad, Hercules, CA, USA). The Bio-Rad thermal cycler CFX96-Real-Time system (Bio-Rad, Hercules, CA, USA) was used to quantify abundance of gene transcripts. The following PCR protocol was used: 30 seconds at 95°C followed by 40 cycles each of 5 seconds at 95°C and 1 minute at 60°C. All samples were amplified in duplicate, and mean cycle threshold (Ct value) was used to compare transcript fold change. Fold changes were calculated relative to the Ct value for *WNT11*.

Statistical analysis. Analyses were performed with SAS for Windows, version 9.2 (SAS Institute Inc., Cary, NC). Data were tested for normality of distribution using the Univariate procedure. The General Linear Model procedure was used to perform least-squares analysis of variance to determine treatment effects and interactions. Replicate was considered random and other main effects were considered fixed. Tests of significance were made using the appropriate error terms after calculating expected means squares. The pdiff procedure of SAS was used as a means-separation test for multiple degree-degree-of-freedom effects. Results are presented as least-squares means ± standard error of the mean. The level of significance for all analyses was 0.05.

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Author contributions

All authors were involved in experimental design, data analysis and interpretation, and in writing the paper. AD, KD, KM and BL conducted the experiments.

Additional information

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