IWP2 impairs the development of porcine somatic cell nuclear transfer embryos via Wnt signaling pathway inactivation

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Abstract. Wnt signaling is critical in embryonic development and post-embryonic tissue homeostasis. The aim of the present study was to evaluate the expression levels of canonical Wnt signaling genes in porcine somatic cell nuclear transfer (SCNT) embryos. Quantitative polymerase chain reaction analysis was performed in porcine SCNT embryos, and the results indicated that the temporal expression patterns of canonical signaling genes were similar between in vivo and SCNT embryos from the 2-cell to the blastocyst stage. In addition, aberrant expression in a small number of Wnt signaling genes in SCNT embryos was identified. IWP2, an inhibitor of Wnt processing, was applied to the culture of SCNT embryos. The Wnt signaling pathway in the SCNT blastocysts may be inactivated via IWP2 treatment, reflecting the low expression levels of c-Myc and peroxisome proliferator-activated receptor δ. Furthermore, blastocyst hatching was damaged by IWP2 treatment. These findings indicate that the canonical Wnt signaling pathway is important for SCNT embryo development.

Introduction

Somatic cell nuclear transfer (SCNT) is an effective method of reprogramming differentiated nuclei and has many potential applications (1). Owing to incomplete or faulty reprogramming, however, the cloning efficiency of SCNT embryos remains low (2,3). Aberrant reprogramming leads to changes in the expression levels of certain genes important for embryonic development. Studies on the expression of development-associated genes may be valuable for improving the developmental competence of SCNT embryos.

Wnt signaling is a critical regulator of many cellular and physiological processes. Wnt signaling regulates cell proliferation and differentiation, controls migration and patterning during embryonic development, and maintains tissue homeostasis in adults (4,5). Depending on the involvement of β-catenin, these signaling pathways have been classified as canonical (β-catenin-dependent) or non-canonical (β-catenin-independent) (6). In the canonical Wnt signaling pathway, which has been better examined (7), β-catenin accumulates in the cytoplasm and eventually translocates to the nucleus where it acts as a transcriptional co-activator of transcription factors belonging to the T-cell factor/lymphoid enhancer factor-1(TCF/LEF) family. β-catenin that accumulates in the cytoplasm is degraded, however, by a protein complex that includes axin, glycogen synthase kinase-3 (GSK-3), protein phosphatase 2A (PP2A), casein kinase 1 (CK1) and adenomatous polyposis coli (APC) (6,8). This degradation complex becomes disrupted as soon as the Wnt protein binds to receptors of frizzled (Fz) and low-density lipoprotein receptor-related protein (LRP5/6), which activates multiple signaling cascades.

Dysfunctions in Wnt signaling result in severe complications of embryonic development. For example, abnormalities were observed in the size and shape of mouse embryos lacking intact maternal/zygotic β-catenin during pre- and post-implantation development (5). By contrast, transient expression of Wnt2 enhances cellular reprogramming efficiency by elevating β-catenin nuclear accumulation (9).

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SCNT cloning was associated with altered expression levels of specific Wnt-associated genes in extra-embryonic tissue in a time- and tissue-specific manner in cattle (10). Impaired expression of E-cadherin and β-catenin proteins, along with defective β-catenin signaling, contributes to insufficient placentation in bovine SCNT-derived fetuses (11). IWP2 is a potential inhibitor of Wnt processing and secretion; however, studies evaluating the effect of IWP2 on embryonic development are lacking. Therefore, the aim of the present study was to determine whether the low cloning efficiency of SCNT embryos was correlated with irregular coordination between Wnt signaling and embryonic development by applying IWP2 treatment.

Materials and methods

Somatic cell nuclear transfer. Oocyte collection, *in vitro* maturation and SCNT were performed in accordance with a previously described protocol (12) with slight modifications. Porcine ovaries were obtained from a local slaughterhouse (HuaZheng Agriculture Development Co. Ltd.) and transported to our laboratory (Jilin Provincial Key Laboratory of Animal Embryo Engineering) within 3 h in sterile saline solution containing penicillin (12 mg/l) and streptomycin (20 mg/l) at 30-35˚C. Cumulus-oocyte complexes (COCs) were harvested by aspirating ovarian follicles (diameter, 3-6 mm) with an 18-gauge needle attached to a 10-ml disposable syringe, and COCs with at least three uniform layers of cumulus cells were selected. The selected COCs were rinsed using PVA-TL HEPES stock solution and cultured in TCM-199 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 0.1% polyvinyl alcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 10 ng/ml epidermal growth factor (Sigma-Aldrich; Merck KGaA), 0.57 mM cysteine, 0.5 μg/ml follicle-stimulating hormone (Sigma-Aldrich; Merck KGaA), 0.5 μg/ml luteinizing hormone (Sigma-Aldrich; Merck KGaA), 75 μg/ml penicillin and 50 μg/ml streptomycin for 42-44 h at 38.5˚C in humidified air containing 5% CO_2.

The cumulus cells of the COCs were denuded by repeated blowing and suction in 0.1% hyaluronidase with a 100-μl pipette. Denuded oocytes with an extruded first polar body, round shape and intact cytoplasm were selected as SCNT recipients. Oocytes were enucleated with a beveled glass pipette by aspirating the first polar body and part of the surrounding cytoplasm. A single donor cell was injected into the perivitelline space of the enucleated oocyte cytoplast with the same glass pipette. SCNT was conducted in manipulation medium (Sigma-Aldrich; Merck KGaA) supplemented with 7.5 μg/ml cytochalasin B (Sigma-Aldrich; Merck KGaA). The oocyte-cell complexes were fused and activated in medium consisting of 0.3 M mannitol, 1.0 mM $MgCl_2 \cdot 6H_2O$, 1.0 mM $CaCl_2 \cdot 2H_2O$, and 0.5 mM HEPES using a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA). Reconstructed embryos were then cultured in PZM-3 (12) at 38.5˚C in a humidified atmosphere containing 5% CO_2. Embryos at the 2-cell, 4-cell, morula, early blastocyst, and hatching blastocyst stages were collected at 24, 36, 96, 132 and 156 h after activation, respectively.

Collection of in vivo embryos. All animal experiments were conducted according to the guidelines on animal care and use established by the Animal Care and Welfare Committee of Jilin University. All experimental protocols were approved by the Ethics Committee of Jilin University. Gilts for embryo collection were artificially inseminated at estrus and kept in Changchun HuiChang Livestock Co. Ltd. (Changchun, China). Subsequently, 2-cell, 4-cell, morula, and blastocyst embryos were collected by flushing the oviduct or uterus with 10% fetal bovine serum-phosphate-buffered saline (v/v; Wuhan Boster Biological Technology, Ltd., Wuhan, China) ~24, 36, 96, or 132 h post coitum, respectively. Embryo collection surgery was performed under anesthesia.

Synthesis of single-embryo cDNAs and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Briefly, RNA was extracted from 4-5 SCNT or *in vivo* embryos at the 2-cell, 4-cell, morula, and blastocyst stages. The zona pellucida of each embryo was removed by treatment with Acidic Tyrode's Solution (Sigma-Aldrich; Merck KGaA) and rinsed in bovine serum albumin (Sigma-Aldrich; Merck KGaA)-Dulbecco's phosphate-buffered saline (BSA-DPBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Each zona-free embryo was immediately selected and transferred into lysis buffer by mouth pipetting. The samples were incubated at 70˚C for 90 sec, followed by 1 min at 4˚C. Following release of mRNA, SuperScript III reverse transcriptase (Invitrogen; Thermo Fisher Scientfic, Inc.) was used for RT. Exonuclease I (New England BioLabs, Inc., Ipswich, MA, USA) was then used to remove the free primers. A poly (A) tail was added to the 3'-end of each first-strand cDNA using terminal deoxynucleotidyl transferase (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA amplification was performed with 20 cycles of PCR using TaKaRa Ex Taq™ HS DNA Polymerase (Takara Bio, Shiga, Japan). Purification of PCR products was conducted with a NucleoSpin® Extract II kit (Macherey-Nagel Co., Düren, Germany).

qPCR was conducted using the iQ5™ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantification was performed in triplicate using a BioEasy SYBR-Green I Real Time PCR kit (BIOER, Hangzhou, China) according to the following procedure: Denaturation at 94˚C for 2 min, followed by 40 cycles of 10 sec at 94˚C, 30 sec at the annealing temperature [with primers as previously described (13)], and 30 sec at 72˚C for extension. The fold change in gene expression was calculated from three replicates with the formula $2^{-\Delta\Delta Cq}$ (14) folloowing normalization against β-actin expression.

Inactivation of Wnt signaling. The small-molecule inhibitor IWP2 (Stemgent, Inc., Cambridge, MA, USA) was used to inactivate Wnt signaling in the SCNT embryos. Following electrical activation, embryos were transferred into PZM-3 with 5.0 μM IWP2. Control SCNT embryos were maintained in PZM-3 without any treatment.

Indirect immunofluorescence staining. The zona pellucida of each embryo was removed by treatment with Acidic Tyrode's Solution and rinsed in BSA-DPBS. Zona-free embryos were fixed in 4% paraformaldehyde (v/v) for 30 min at room
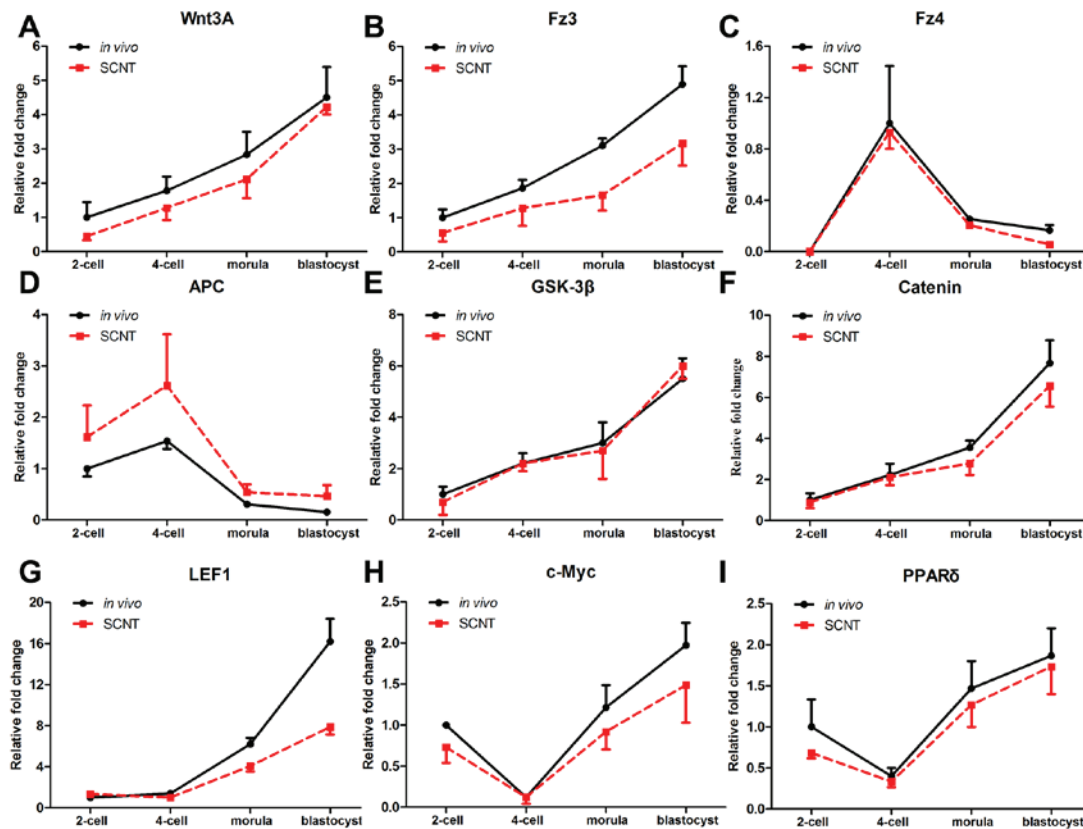
Figure 1. Temporal expression of canonical Wnt signaling components in *in vivo* and SCNT embryos. The relative fold-change of each gene was calculated following normalization to the expression level of β-actin in SCNT embryos at the 2-cell stage (Fz4 at the 4-cell stage). SCNT, somatic cell nuclear transfer; Fz4, frizzled homolog 4; Fz3, frizzled homolog 3; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; LEF1, lymphoid enhancer binding factor 1; PPAR δ, peroxisome proliferator activated receptor δ.

temperature and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich; Merck KGaA) for 30 min. Embryos were blocked in 5% goat serum (Wuhan Boster Biological Technology, Ltd.) in PBS for 30 min and incubated overnight with primary antibodies at 4˚C. Primary antibodies used in the analysis were anti-β-catenin mouse monoclonal antibody (1:200; sc-65480; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-active-β-catenin mouse monoclonal antibody (1:500; 05-655; Upstate Biotechnology, Inc., Lake Placid, NY, USA). Subsequent to removing the primary antibodies, embryos were rinsed three times with 0.2% Tween-20 in PBS. Embryos were incubated with Alexa Fluor 594 goat anti mouse IgG (1:2,000; R37121; Invitrogen; Thermo Fisher Scientific, Inc.) at 4˚C overnight. Embryos were stained with Hoechst 33342 and observed under an epifluorescent microscope (Nikon Corporation, Tokyo, Japan).

Results

Temporal expression patterns of Wnt signaling genes in porcine pre-implantation embryos. To determine the roles of canonical Wnt signaling pathway genes in porcine SCNT embryos, their temporal expression patterns were examined. The expression of various canonical Wnt signaling genes, including Wnt3A, frizzled receptor transcripts (Fz3 and Fz4), adenomatous polyposis coli (APC), glycogen synthase kinase (GSK)3β, β-catenin, lymphoid enhancer binding factor 1 (LEF1), c-Myc and peroxisome proliferator activated receptor

(PPAR)δ, was analyzed in individual porcine embryos by RT-qPCR. As shown in Fig. 1, changes in the expression patterns of Wnt signaling genes were similar between in vivo and SCNT embryos from the 2-cell to the blastocyst stage. The expression level of Wnt3A, Fz3, GSK3β, β-catenin and LEF1 steadily increased from the 2-cell to the blastocyst stage (Fig. 1A,B,E-G). For Fz4 and APC, there was a peak in expression at the 4-cell stage, and expression then gradually declined to the blastocyst stage (Fig. 1C and D). By contrast, the expression levels of Wnt target genes, c-Myc and PPARδ decreased from the 2-cell to the 4-cell stage, and subsequently increased, reaching a peak at the blastocyst stage (Fig. 1H and I). Thus, the temporal expression patterns of canonical Wnt signaling genes in SCNT embryos were similar with those of *in vivo* embryos.

Development of SCNT embryos was compromised with Wnt signaling inactivation. The results revealed that various genes were expressed abnormally in pre-implantation embryos, although the actual contribution of the Wnt signaling pathway to the development competence of SCNT embryos requires further elucidation. Our previous study revealed that parthenogenetic blastocyst hatching may be affected by activation or inactivation of Wnt signaling. Therefore, the effects of Wnt signaling blockade were accessed in SCNT embryos. As presented in Table I, no significant differences were identified between control and IWP2-treated embryos in blastocyst-formation rates or cell numbers in the early

Table I. Effects of IWP2 on the *in vitro* blastocyst development of somatic cell nuclear transfer embryos.

Treatment	Total oocytes	Early blastocysts (% ± SD)	Hatching blastocysts [% ± SD]	Cell numbers in early blastocysts (mean ± SD)
Control	948	248 (28.3±9.1)[a]	153 (64.3±11.1)[a]	46±10.2[a]
IWP2	575	160 (27.4±8.8)[a]	25 (15.4±2.2)[b]	47±8.1[a]

[a,b]Values with different superscripts within the same column denote significant difference (P<0.05). The blastocyst-formation rate was calculated by dividing the number of early blastocysts to the number of oocytes. The blastocyst hatching rate was based on the number of hatching blastocysts divided by the number of early blastocysts.
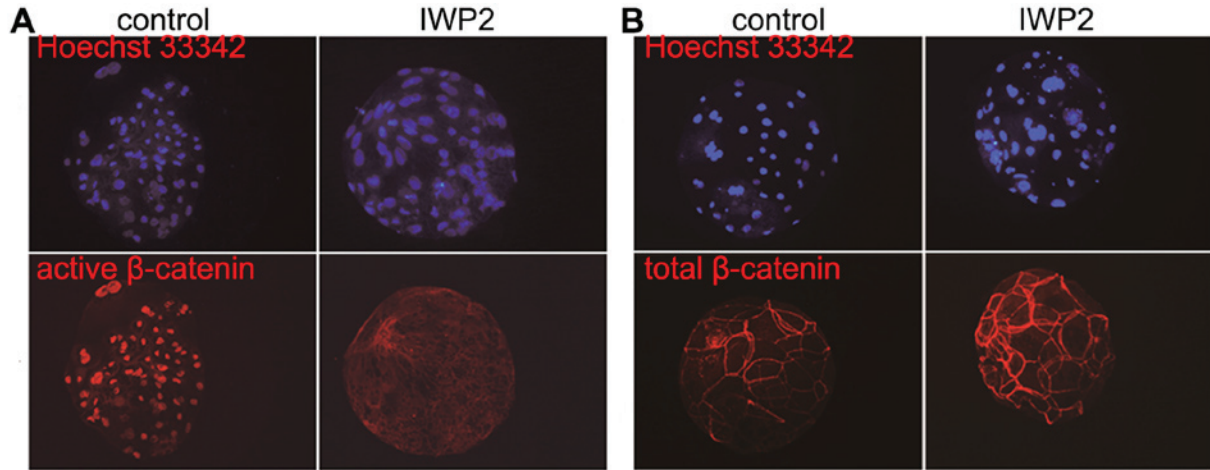

Figure 2. Expression of (A) active and (B) total β-catenin in porcine somatic cell nuclear transfer blastocysts treated with or without IWP-2 as determined by immunofluorescence staining. Magnification, x300.
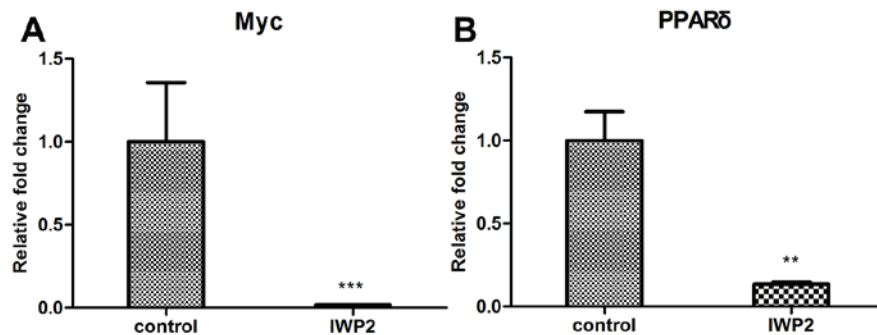

Figure 3. Gene expression in porcine SCNT blastocysts treated with or without IWP2. The relative fold change of each gene was calculated following normalization to its level in blastocysts without IWP2 treatment. The data are presented as means ± standard deviation, n=3. [**]P<0.01 vs. control, and [***]P<0.001 vs. control. SCNT, somatic cell nuclear transfer.

blastocyst. However, the hatching rate of embryos at 156 h was significantly reduced following IWP2 treatment.

To confirm the inhibition of Wnt signaling following IWP2 exposure, β-catenin immunofluorescence was observed in SCNT blastocysts as cytosolic β-catenin is transported into the nucleus when Wnt signaling is active. As shown in Fig. 2, no significant differences in total β-catenin abundance between control and IWP2-treated blastocysts were observed, although expression of active β-catenin was nearly undetectable following IWP2 treatment. In addition, expression levels of the Wnt target genes, c-Myc and PPARδ were analyzed. As presented in Fig. 3A and B, expression levels of c-Myc and PPARδ were downregulated in SCNT blastocyst following IWP2 treatment. These results confirm that the Wnt signaling pathway is inactivated by IWP2 treatment.

Discussion

The Wnt signaling pathway is important for embryonic development, tissue regeneration and homeostasis (15,16). In the present study, certain Wnt signaling genes were observed to be aberrantly expressed in SCNT embryo, such as the expression of Wnt3A, FZ3, LEF1 and APC. In our previous study, it

was primarily Wnt genes that exhibited different expression patterns in parthenogenetic and *in vivo* embryos (13). This may be one of the reasons for differences in developmental competence between parthenogenetic and SCNT embryos. While parthenogenetic embryos do not develop to term, a small number of SCNT embryos go through gestation and form cloned piglets.

Furthermore, the present study identified that blastocyst hatching was impaired following IWP2 treatment to inactivate the Wnt signaling pathway. Furthermore, β-catenin has been shown to be aberrantly expressed in bovine SCNT placentas (11). These findings indicate that Wnt signaling is markedly more important for trophectoderm development, affecting SCNT embryonic development by disturbing placentation. Notably, one component of Wnt signaling, LEF1, which was downregulated in SCNT embryos at the morula and blastocyst stages in the present study, is critical in the development of the placenta (17). Such aberrant gene expression patterns may contribute to the failure of embryonic development or to defects in the SCNT piglets. However, the present study did not identify the most critical Wnt gene for embryonic development. Further research should be focused on regulating the expression of Wnt signaling genes to improve SCNT reprogramming efficiency and embryonic development.

In summary, the present study demonstrated that only a small proportion of Wnt signaling genes were abnormally expressed during pre-implantation development, and this pathway serves important roles in the development of the trophectoderm in porcine SCNT blastocysts.

Acknowledgements

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