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—Original Article—

Effects of Downregulating Oct-4 Transcript by RNA Interference on Early Development of Porcine Embryos

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Abstract. The objective of this study was to investigate the role of the POU family transcription factor, Oct-4, in the early development of porcine embryos. We attempted Oct-4 downregulation of porcine early embryos by RNA interference, and evaluated Oct-4 suppression of developmental competencies and gene transcripts in porcine embryos. Injection of specific siRNA resulted in a distinct decrease in *Oct-4* mRNA and protein expression in porcine embryos until at least the morula stage. Although the porcine embryos injected with Oct-4 siRNA were able to develop to the morula stage, these embryos failed to form blastocysts. Gene transcripts of caudal-like transcription factor (*Cdx2*) and fibroblast growth factor 4 (*Fgf4*), which were involved in segregation of the trophectderm and functionalization of the inner cell mass, were unchanged by Oct-4 siRNA injection. Our results indicated that Oct-4 is an important factor for porcine embryos and, in particular, for the regulation of porcine blastocyst formation.

Key words: Early development, Gene expression, Oct-4, Porcine embryo, RNA interference

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Pigs have attracted increasing attention as suitable sources for xenotransplantation, production of specific proteins by transgenesis, and biomedical models for studying human physiology and pathology. Successful piglet production from *in vitro* produced embryos, such as those produced by *in vitro* fertilization (IVF) or somatic cell nuclear transfer [1, 2], has accelerated progress in these areas. However, *in vitro* production (IVP) of porcine embryos is still inefficient compared with that of other mammals, such as mice and cattle. This is attributed to low rates of development to the blastocyst stage, and to the production of poor quality blastocysts [3]. One of the reasons for the decreased development of the porcine IVP system is limited knowledge concerning the molecular mechanisms involved in early embryonic development. Therefore, to improve the IVP system for porcine embryos, it is important to focus on the molecular mechanisms underlying the regulation of early embryonic development.

In mammalian embryonic development, the first visible cell lineage segregation occurs during the transition from the morula to the blastocyst stage. The cells of the inner part of the blastocyst, called the inner cell mass (ICM), are pluripotent and eventually give rise to the fetus and additional extraembryonic tissues. By contrast, the cells of the outer layer differentiate into an epithelium, called the trophectoderm (TE), which subsequently develops to into the placenta. The segregation of the ICM and TE is regulated by the mutual interaction of various genes. In murine embryos, differences between the ICM and TE cellular populations have been proposed to be regulated by the POU family transcription factor Oct-4 (encoded by Pou5f1) [4–6] and the caudal-like transcription factor (Cdx2) [7, 8]. Slight differences are observed between the levels of Oct-4 and Cdx2 proteins expressed in the inner and outer cells. These protein levels are amplified through reciprocal repression to give a mutually exclusive pattern of Cdx2 on the outside and Oct-4 on the inside [9]. Eventually, these proteins display reciprocal expression with Cdx2 localized exclusively in the TE and Oct-4 localized in the ICM at the late blastocyst stage [8, 10]. On the other hand, fibroblast growth factor-4 (*Fgf4*) is a known target of Oct-4 [11]. *Fgf4* has been demonstrated to respond to Oct-4 in a Sox2-dependent manner [12, 13] and is coexpressed with Oct-4 in the ICM and epiblast [14, 15].

Previously, we demonstrated differences in the expression levels of several genes including *Oct-4* and *Cdx2* between ICM and TE lineage cells from bovine and porcine preimplantation embryos [16, 17]. In both bovine and porcine blastocyst embryos, the levels of *Oct-4* mRNA in the ICM were significantly higher than those in the TE [16, 17]. Thus, similar to the situation in murine embryos, Oct-4 may have an important role in the segregation of the ICM lineage in bovine and porcine embryos. On the other hand, several investigators reported that the *Oct-4* transcript and protein did not appear to be restricted to the ICM, and were detected in the TE lineage in bovine and porcine embryos at the blastocyst to elongated stages [16–21]. These findings raise the possibility that the role of Oct-4 in ICM segregation in bovine and porcine embryos is different from that in murine embryos. However, limited information is available concerning the molecular mechanism of ICM/TE segregation in domestic animals, and the functions of Oct-4 during embryonic development are largely unknown compared with those of murine embryos.

Small interfering RNAs (siRNAs) are identified molecules shown to silence specific genes via a targeted mRNA degradation and are widely used in molecular and cellular research [22]. Most recently, RNA interference (RNAi) has been proven to be functional in oocytes and preimplantation embryos of the pig [23, 24]. Knockdown of

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Table 1. Primers and siRNA sequences

Name	Nucleotide sequences (5'-3')	Annealing temperature (C)	Fragment size (bp)	GenBank accession no.
Oct-4	F- GTTCTCTTTGGGAAGGTGTT	56	313	NM_001113060
	R- ACACGCGGACCACATCCTTC			
Cdx2	F- CAGGCCCTCTGAGAAGTGTC	60	212	XM_003130908
	R- GGGGTCTTTCCTGAGGATTC			
Fgf4	F- GCGATGAGTGCAAGTTCAAA	60	155	XM_003122418
	R- GAGGAAGTGGGTGACCTTCA			
Gapdh	F- TCGGAGTGAACGGATTTG	52	219	AF017079
	R- CCTGGAAGATGGTGATGG			
siRNA-1	S- GAAAGCGGACAAGUAUCGAGA	N/A	N/A	N/A
	AS- UCGAUACUUGUCCGCUUUCUC	N/A	N/A	N/A
siRNA-2	S- GGGAAGGUGUUCAGCCAAATT	N/A	N/A	N/A
	AS- UUUGGCUGAACACCUUCCCTT	N/A	N/A	N/A

F, forward; R, reverse; S, sense strand; AS, antisense strand.

Oct-4 expression in porcine embryos may be achieved using siRNA. As mentioned above, it is necessary to clarify the role of Oct-4 in early embryo development in order to understand the molecular mechanisms responsible for the segregation of the ICM and TE lineages in porcine embryos. Therefore, the objective of this study was to investigate the role of Oct-4 during the early development of porcine embryos using siRNAs targeted to *Oct-4*.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Oocyte collection and in vitro maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and were maintained at 37 C during transport to the laboratory. Cumulus-oocyte complexes (COCs) were obtained from follicles 2–6 mm in diameter in TCM-199 medium supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 20 mM Hepes, 0.68 mM L-glutamine, 100 IU/ml penicillin G potassium (Meiji Seika, Tokyo, Japan), and 0.1 mg/ml streptomycin sulfate (Meiji Seika). Approximately 50 COCs with uniform ooplasm and a cumulus cell mass were cultured separately in four-well dishes (Thermo Fisher Scientific, Kanagawa, Japan) for 20 h in 500 µl of maturation medium, which was composed of a modified North Carolina State University (NCSU)-37 (mNCSU-37) [25] solution containing 10% porcine follicular fluid, 0.6 mM cysteine, 0.05 mM β-mercaptoethanol, 1 mM dibutyryl cAMP (dbcAMP), 10 IU/ml pregnant mare serum gonadotropin (PMS 1000, ZENOAQ, Koriyama, Japan), and 10 IU/ml human chorionic gonadotropin (hCG; Puberogen 1500, Novartis, Tokyo, Japan). The developing COCs were subsequently cultured in maturation medium without dbcAMP and hormones for 24 h. The maturation culture was performed at 39 C in a humidified atmosphere containing 5% CO_2, 5% O_2, and 90% N_2.

In vitro fertilization and in vitro culture

After *in vitro* maturation, COCs were washed with modified Pig-FM (mPig-FM) [1], and 15–20 COCs were placed into a 90- µl drop of mPig-FM. Cryopreserved semen was thawed, and spermatozoa were washed twice by centrifugation (at 1800 rpm for 3 min) in sperm washing medium [TCM 199 medium supplemented with 20 mM Hepes, 0.68 mM L-glutamine, 100 U/ml penicillin G potassium, 0.1 mg/ml streptomycin sulfate, 0.91 mM sodium pyruvate, 4.12 mM calcium lactate, 3.0 mM glucose, and 10% (v/v) FBS] adjusted to pH 7.8 [26]. The spermatozoa were resuspended in the sperm washing medium, and 10 µl of this suspension was added to 90 µl of mPig-FM containing matured COCs. The final concentration was adjusted to 1.0×10^6 /ml. COCs and sperm were incubated for 6 h at 39 C under a 5% CO_2, 5% O_2, and 90% N_2 atmosphere. Following microinjections of siRNA, the embryos were washed and cultured in PZM-5 [27] at 39 C under a 5% CO_2, 5% O_2, and 90% N_2 atmosphere until day 6 (IVF = day 0). Rates of embryo development were assessed on day 2 (2-cell ≤), day 3 (8-cell ≤), day 4 (16-cell and morula), day 5 and day 6 (blastocyst).

Design of siRNA and microinjection into embryos

The target sights of the Oct-4 transcript were selected from porcine sequences (GenBank accession number: NM_001113060). Two types of specific siRNA (siRNA-1 and siRNA-2) were respectively designed using siRNA design software, Enhanced siDirect (http://design.RNAi.jp/) and BLOCK-iT RNAi Designer (http://rnaidesigner.invitrogen.com/rnaiexpress/). Both sense and antisense RNA sequences for siRNA were commercially synthesized (Table 1). After insemination, cumulus cells and excess spermatozoa were removed from presumptive zygotes by pipetting. These embryos were subsequently transferred to a 20-µl drop of modified TALP (mTALP) medium [28], containing 1 mg/ml BSA (fraction V) for microinjection. Approximately 10 pl of 50 µM specific siRNA duplexes were injected into the cytoplasm of each embryo using a Transjector 5246 (Eppendorf, Hamburg, Germany). Approximately 10 pl of 20 µM nonsilencing siRNA (AllStars Negative Control siRNA, Qiagen, Tokyo, Japan) was injected as control siRNA by the same

method. The embryos were washed three times immediately after microinjection, and cultured as described above.

Determination of the relative abundance of gene transcripts in porcine embryos

On day 4, morula stage embryos were treated with 0.1% protease in 1% PVP-PBS for 5 min, and washed seven times in 1% PVP-PBS. Pools of four or five embryos were added to 5 µl lysis buffer [0.8% Igepal (ICN Biomedicals, Aurora, OH, USA), 5 mM DTT (Invitrogen) and 1 U/µl of RNasin (Promega, Madison, WI, USA)], snap-frozen in liquid nitrogen and stored at –80 C. RNA samples were heated to 80 C for 5 min and treated for reverse transcription (RT) using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The reaction mixture was diluted with DEPC-treated water to obtain a final volume of 21 µl. Real-time PCRs were performed using a StepOne™ system (Applied Biosystems, Tokyo, Japan), and products were detected with SYBR Green included in the QuantiTect SYBR Green PCR master mix (Qiagen). A 2-µl aliquot of the RT product was used for each quantification. The amplification program was as follows: preincubation at 95 C for 15 min to activate HotStarTaq DNA Polymerase (Qiagen), followed by 45 cycles of denaturation at 94 C for 15 sec, annealing of primers at different temperatures (Table 1) for 30 sec, and elongation at 72 C for 30 sec. After the end of the last cycle, a melting curve was generated by starting fluorescence acquisition at 60 C and recording measurements at 0.3 C increments up to 95 C.

A standard curve was generated for each amplicon by amplifying serial dilutions of a known quantity. PCR products for each gene were purified using a QIAquick PCR Purification Kit (Qiagen), quantified by measuring absorbance at 260 nm using NanoDrop (ND-1000; Thermo Fisher Scientific), and diluted as described. Serial 10-fold dilutions for creating the standard curve were amplified in every real-time PCR run. The standards and cDNA samples were then co-amplified in the same reaction prepared from a master mix. Fluorescence was acquired at each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence rose above the background for each sample. Final quantification was performed using StepOne™ quantification software. Expression of the target gene in each run was normalized to the internal standard glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Immunofluorescent staining

On day 1 (2-cell stage), day 3 (4-cell and 8-cell stages), day 4 (16-cell and morula stages), and day 5 (blastocyst stage), embryos at the appropriate developmental stage were fixed in 4% paraformaldehyde for 20 min at room temperature, and then washed twice in PBS containing 0.1% Triton X-100 (TXPBS) for 10 min each time. Samples were subsequently permeabilized in 0.2% Triton X-100 in PBS for 30 min and then incubated in Image-iT FX Signal Enhancer (Invitrogen) for 30 min, followed by washing twice for 10 min in TXPBS. Blocking was performed by incubation in 7% normal goat serum (Invitrogen) in TXPBS for 1.5 h, followed by washing in TXPBS for 5 min. Rabbit polyclonal Anti-OCT-4 primary antibody (SC-9081, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a concentration of 1:50 at 4 C overnight in PBS containing 0.5% BSA and 0.05% Triton X-100. Embryos were washed four times in TXPBS for 15 min each time. Embryos were then incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (A11034, Invitrogen) at a concentration of 1:400 at room temperature for 1 h in PBS containing 0.5% BSA and 0.05% Triton X-100. Washing in TXPBS was done four times in TXPBS for 20 min each time. Samples were then mounted on slides in a drop of VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Fluorescent images were obtained using an AxioCam MRc and AxioVision image analyzer system (Carl Zeiss, Oberkochen, Germany).

Assessment of cell number

On day 4, morula stage embryos obtained from siRNA-2 or control siRNA injection were used for assessment of total cell numbers. Some morula stage embryos were cultured until day 5, and then total cell numbers were counted. Embryos were fixed in 4% paraformaldehyde for 20 min at room temperature, and then were washed 7 times in PBS containing 0.1% PVA. Samples were then mounted on slides in a drop of VECTASHIELD mounting medium with DAPI. Fluorescent images were obtained using an AxioCam MRc and AxioVision image analyzer system, and total cell numbers were counted.

Statistical analysis

The percentage data for embryo development were subjected to arcsine transformation. The transformed values and *Oct-4* mRNA expression levels were analyzed by one-way analysis of variance (ANOVA) followed by multiple pairwise comparisons using the Tukey-Kramer method. Expression levels of *Cdx2* and *Fgf4* mRNA were analyzed by the Kruskal-Wallis test. The total cell numbers were analyzed by the F-test followed by the Student's *t* test. A P value less than 0.05 denoted a statistically significant difference.

Results

Effect of siRNA injection on Oct-4 expression in porcine embryos

The expression levels of *Oct-4* mRNA in morula stage embryos that were uninjected, injected with control siRNA, or injected with either of the two specific siRNAs were evaluated (Fig. 1). The relative abundance of *Oct-4* in embryos injected with siRNA-2 was significantly ($P<0.05$) lower than that in uninjected and control siRNA-injected embryos. Although the *Oct-4* mRNA level in siRNA-1-injected embryos was lower than that in the uninjected and control siRNA-injected embryos, the difference between these values was not significant (Fig. 1). Thus, siRNA-2 was selected as the specific siRNA for subsequent experiments.

Expression of the Oct-4 protein was evaluated by immunofluorescent staining. The Oct-4 signals in embryos at the 2-cell to blastocyst stage were compared (Fig. 2). As shown in Fig. 2A, a diffuse Oct-4 signal in the control siRNA-injected embryos was observed throughout the cytoplasm until the 8-cell stage. A strong nuclear signal was detected in the control siRNA-injected embryos at the 16-cell stage (Fig. 2A). The Oct-4 signal in the siRNA-2-injected embryos was weaker than that observed in the control siRNA-injected embryos, and it was difficult to detect a nuclear signal at the 16-cell

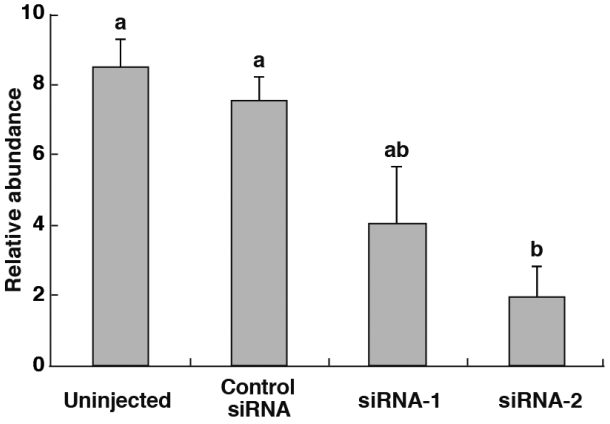
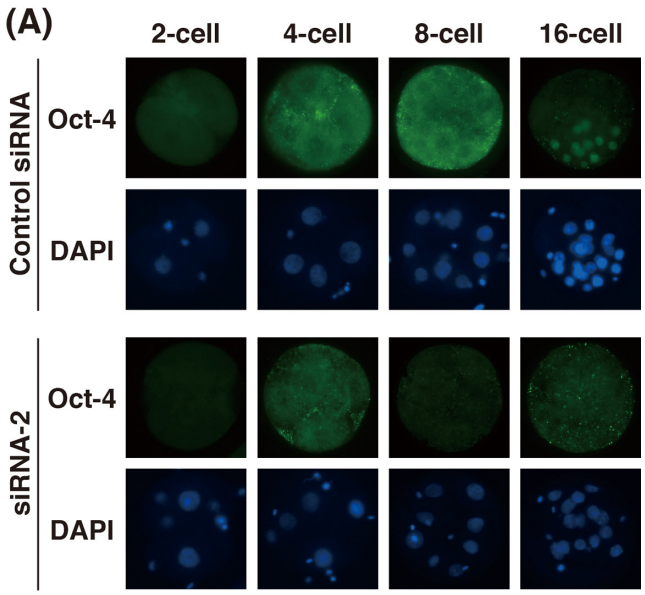
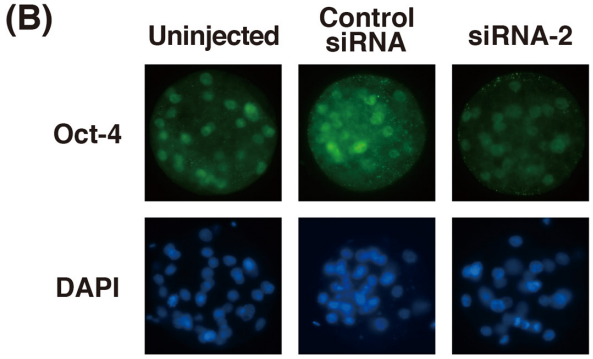


Fig. 1. Relative abundance (mean ± SEM) of *Oct-4* transcripts in uninjected porcine morula stage embryos (n=6) or porcine morula stage embryos treated with control siRNA (n=6), siRNA-1 (n=7) or siRNA-2 (n=6). [a, b] Different superscripts indicate a significant difference (P<0.05).





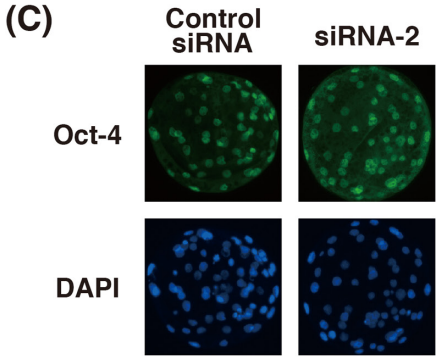


Fig. 2. Representative photographs of Oct-4 protein expression in porcine embryos. The embryos labeled for Oct-4 (green) and DAPI (blue). (A) Oct-4 signals in control siRNA and siRNA-2-injected embryos at each developmental stage. (B) Oct-4 signals in morula stage embryos obtained without injection (uninjected) or with the control siRNA or the siRNA-2 injection. (C) Oct-4 signals in blastocyst stage embryos obtained with the control siRNA or the siRNA-2 injection.

stage (Fig. 2A). At the morula stage, although the Oct-4 signal was also observed in nuclei of siRNA-2-injected embryos, the signal intensity was weaker than that observed in uninjected and control siRNA injected embryos (Fig. 2B). Thus, knockdown of Oct-4 by siRNA-2 was effective until at least the morula stage on day 4. At the blastocyst stage, Oct-4 signals were detected in both siRNA-2-injected and control siRNA-injected embryos (Fig. 2C).

Effect of Oct-4 downregulation on the development of porcine embryos

In vitro developmental competence of siRNA-2-injected embryos was evaluated (Table 2). No difference in developmental rates for the 2-cell to 16-cell stages was observed between siRNA-2-injected and control (uninjected and control siRNA injected) embryos. The siRNA-2-injected embryos developed to the morula stage on day 4, and there was no significant difference between the experimental groups (18.6–19.3%). However, on both day 5 and day 6, the blastocyst developmental rates of siRNA-2-injected embryos (4.6 and 7.2%, respectively) were significantly (P<0.05) lower than those of the uninjected (24.6 and 23.4%, respectively) and control siRNA-injected embryos (19.0 and 17.7%, respectively).

On day 4, total cell numbers of morula stage embryos obtained from control siRNA-injected embryos and siRNA-2-injected embryos were 23.3 ± 2.1 (n=15) and 21.5 ± 1.8 (n=15), respectively. These values were not significantly different. In contrast, the total cell number in siRNA-2-injected embryos on day 5 (21.9 ± 3.9, n=15) was significantly (P<0.01) lower than that in control siRNA-injected embryos (54.8 ± 3.4, n=18).

Representative photographs of embryos morphology are shown in Fig. 3. In the uninjected and control siRNA groups, the morula embryos developed to the blastocyst stage on day 5, and these embryos were expanded on day 6. However, the siRNA-2-injected embryos showed developmental arrest at the morula stage, with only a few embryos reaching the blastocyst stage. Moreover, almost all embryos injected with siRNA-2 were collapsed structures at day 6 (Fig. 3).

Table 2. Effect of Oct-4 siRNA injection on *in vitro* development of porcine embryos*

Treatment	Number of embryos cultured	No. (%)† of embryos developed to					
		Day 2	Day 3	Day 4		Day 5	Day 6
		2-cell ≤	8-cell ≤	16-cell	Morula	Blastocyst	Blastocyst
Uninjected	244	130 (53.3)	88 (36.1)	38 (15.6)	47 (19.3)	60 (24.6)[a]	57 (23.4)[a]
Control siRNA	237	130 (54.9)	83 (35.0)	22 (9.3)	44 (18.6)	45 (19.0)[a]	42 (17.7)[a]
siRNA-2	237	120 (56.0)	95 (40.1)	29 (12.2)	44 (18.6)	11 (4.6)[b]	17 (7.2)[b]

* Experiments were replicated five times. † Percentages of the number of embryos cultured. [a, b] Values with different superscripts within each column differ significantly (P<0.05).

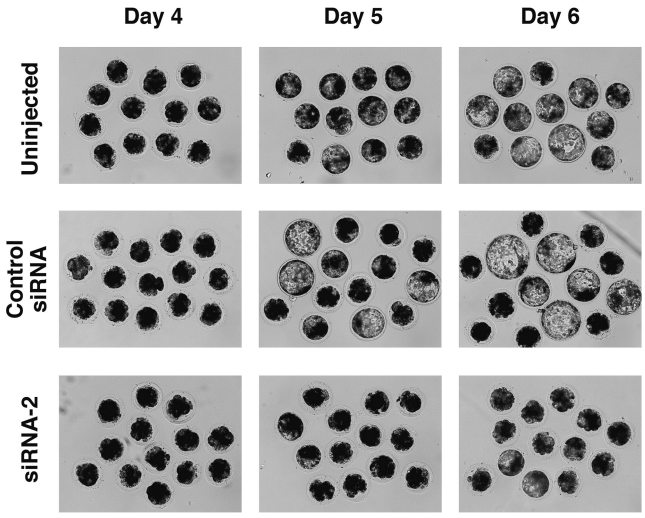


Fig. 3. Representative photographs showing the developmental morphology in late culture periods of porcine embryos obtained without injection (uninjected) or the control siRNA or the siRNA-2 injection. The morula stage embryos obtained from each treatment were selected on day 4, and these embryos were cultured continuously until day 6.

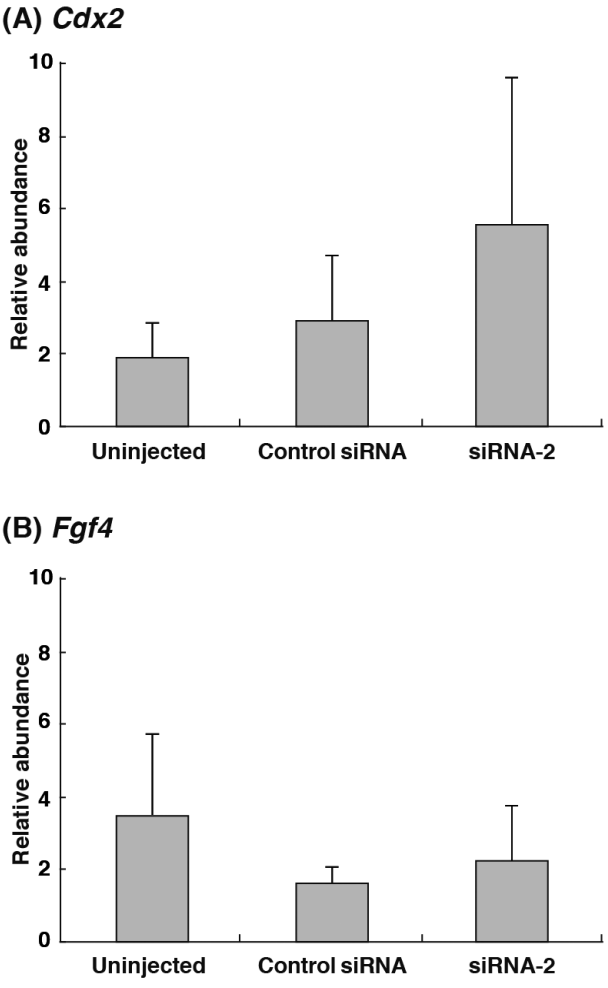


Fig. 4. Relative abundance (mean ± SEM) of (A) *Cdx2* and (B) *Fgf4* transcripts in uninjected porcine morula stage embryos (n=6), or porcine morula stage embryos treated with the control siRNA (n=6) or with siRNA-2 (n=6).

Namely, porcine embryos with downregulated Oct-4 expression failed to advance to blastocyst formation from the morula stage.

Cdx2 and Fgf4 transcripts in porcine embryos derived from Oct-4 siRNA injection

To clarify the effect of Oct-4 downregulation on transcripts of genes, which correlated with Oct-4, mRNA expressions of *Cdx2* and *Fgf4* at the morula stage were examined. As shown in Fig. 4A, the relative abundances of *Cdx2* did not differ between treatment groups. Similarly, there was no difference in *Fgf4* transcript levels among the three experimental groups (Fig. 4B).

Discussion

Oct-4 was first identified in the mouse as an embryonic stem cell and is the earliest expressed transcription factor that is known to be crucial for murine preimplantation development [4–6]. Furthermore, Oct-4 is a central regulator of pluripotency [11] and is also included in the essential factors needed to induce pluripotency for differentiated cells [29]. In the present study, we evaluated the role of Oct-4 in porcine preimplantation embryos using RNAi technology and suggested that Oct-4 is of critical importance for blastocyst formation in porcine embryos.

In the mouse, Oct-4 protein accumulation in oocytes is present until

the 2-cell stage, and zygotic *Oct-4* expression begins at the 4-cell to 8-cell stages [5, 11, 30]. Strong nuclear localization of Oct-4 is detected from the 8-cell stage, and the level of Oct-4 increases suddenly at the morula stage [5, 11, 30]. On the other hand, high levels of Oct-4 transcripts are detectable in the porcine oocyte before fertilization and in 2-cell stage embryos followed by consistent degradation of maternally inherited mRNAs until the 4- to 8-cell stages [31, 32]. The levels of *Oct-4* transcripts increase in both morula and blastocyst stage embryos [31]. In the present study, cytoplasmic localization of the Oct-4 protein in porcine embryos was detected until the 8-cell stage. Nuclear localization of the Oct-4 protein began at the 16-cell stage, and was also clearly detected in morula stage embryos. Our observations, taken together with leading studies, indicate that the Oct-4 protein detected until the 8-cell stage is derived from the oocyte and/or transcription using maternal mRNA, and that zygotic *Oct-4* expression in porcine embryos begins from the 16-cell stage. Moreover, Oct-4 is expressed at higher levels in porcine embryos at the morula stage. The morula stage is the starting point of ICM/TE segregation for blastocyst formation in mammalian embryos: downregulation of Oct-4 expression at the morula stage is indispensable for evaluating the role of Oct-4 in the early development of porcine embryos. In the present study, injection of siRNA-2 downregulated Oct-4 expression in porcine embryos during the 8-cell to morula stage. Therefore, siRNA-2 was adequate to clarify for the role of Oct-4 in porcine embryos. A difference in *Oct-4* gene silencing efficiency was observed between the two target siRNA sequences in this study. It is well known that siRNA-based RNAi in mammalian cells varies considerably depending on the target sequences selected [33, 34]. Thus, the difference in knockdown competency may have resulted from the siRNA sequences selected for siRNA-1 and siRNA-2.

In our study, the downregulation of Oct-4 expression in porcine embryos had no effect on development to the morula stage, but blastocyst formation was inhibited. Some embryos derived from Oct-4 siRNA injection developed to blastocyst stage, and Oct-4 signals were detected in these embryos. These results indicated that Oct-4 is essential for blastocyst formation of porcine embryos. On the other hand, the *Oct-4* transcript level in siRNA-1-injected embryos was in between those of siRNA-2-injected and control embryos. The blastocyst formation rate of siRNA-1-injected embryos was also in between those of siRNA-2-injected and control embryos (data not shown). Therefore, it is possible that inhibitory effect on blastocyst formation is proportional to the *Oct-4* expression levels and that the Oct-4 transcript level in the siRNA-1-injected embryos represents the lower limit of the inhibitory effect. Nichols *et al*. [11] reported that although partial cavitation occurred in murine embryos defective for Oct-4 expression, these embryos were rarely fully expanded. Similarly, murine embryos injected with siRNA targeting Oct-4 appeared condensed at the morula stage [35]. Furthermore, both Oct-4 knockout and knockdown murine embryos did not contain a recognizable ICM [11, 35]. These results suggest that Oct-4 is essential for segregation of the ICM in murine embryos. Therefore, it is possible that the failure of blastocyst formation in porcine embryos injected with siRNA-2 resulted from inhibition of ICM segregation in embryos with downregulated Oct-4 expression. In porcine embryos, *Oct-4* expression is detected in both ICM and TE lineages [17, 19]. In the present study, total cell numbers of siRNA-2-injected embryos did not increase after morula stage. This observation indicated that cellular proliferation of porcine embryos is inhibited by Oct-4 downregulation. If Oct-4 expression is also involved in segregation and/or proliferation of the TE lineage in porcine embryos, even partial cavitation as observed in mouse embryos may be difficult in porcine embryos downregulated Oct-4 expression. On the other hand, in murine embryos, arrest of blastocyst formation or cavitation is also induced by downregulation of *Sox2* gene [36]. Sox2 acts cooperatively with Oct-4 at promoters activating transcription of several genes, which play important roles for embryo development [13, 37, 38]. Although the reason for blastocyst formation arrest by Oct-4 downregulation is not clear, it is possible that decreasing Oct-4 expression levels influence several transcription factors and induce developmental arrest.

Nganvongpanit *et al*. [39] reported that there was no significant difference in the bovine blastocyst rate between Oct-4 dsRNA injected and uninjected embryos, but a longer culture period was needed for their blastocyst formation. In the present study, we evaluated blastocyst formation at day 5 and day 6, and the blastocyst rates in control groups peaked at day 5. A lower rate of blastocyst formation in siRNA-2-injected embryos was found at both day 5 and day 6, almost all embryos had collapsed structures at day 6. These findings indicated that the phenotype of Oct-4 downregulation in porcine embryos is robust inhibition of blastocyst formation, not retardation of blastocyst formation. The reason for the difference in response to Oct-4 downregulation between bovine and porcine embryos is unclear. However, it was reported that the number of ICM cells in blastocysts of Oct-4 dsRNA injected bovine embryos was reduced compared with control embryos [39]. Thus, Oct-4 may be also involved in bovine embryonic development during the morula to blastocyst stage.

Oct-4 and Cdx2 are initially coexpressed throughout the murine embryo prior to blastocyst formation [10, 30, 40]. Mutual antagonism between these two factors may contribute to the eventual segregation of their expression domains [9], with Cdx2 restricted to the outer cells and Oct-4 restricted to the inner cells of the morula embryos. Therefore, we hypothesized that if there is a reciprocal inhibition between Oct-4 and Cdx2 in porcine embryos, *Cdx2* expression may be increased by Oct-4 downregulation. However, the *Cdx2* expression level in siRNA-2-injected embryos did not differ from that in control embryos. Nishioka *et al*. [41] reported that TEA domain transcription factor 4 (Tead4) induces Cdx2 expression in murine embryos. We have previously observed *Tead4* expression in porcine blastocyst embryos, and differences in *Cdx2* and *Tead4* expression levels between ICM and TE lineages became appreciable at elongation stages such as the ovoid or filamentous stage [17]. Therefore, it is possible that Tead4 is a regulatory factor for Cdx2 expression in porcine embryos.

In murine embryos, it is established that the Fgf4 signaling pathway is required for maintaining the proliferation of TE cells [15, 42, 43]. *Fgf4* is highly expressed in the ICM and epiblast, and activates *membrane-associated Fgf receptor 2* (*Fgfr2*) expressed by the TE lineage [44, 45]. Embryos with an *Fgf4* or *Fgfr2* mutation show peri-implantation lethality resulting from defects in functional ICM formation as well as a placental defect [46, 47], which together indicate the importance of the Fgf4 signaling pathway for proliferation and

differentiation of both ICM and TE cells. In *Oct-4* deficient murine embryos, the *Fgf4* mRNA transcript level was greatly reduced at the blastocyst stage [11]. Similarly, bovine blastocysts obtained from Oct-4 dsRNA injection showed lower *Fgf4* expression levels [39]. These results suggest that Fgf4 expression is regulated by Oct-4 during early embryo development. Therefore, we examined *Fgf4* expressions in porcine embryos injected with Oct-4 siRNA. However, *Fgf4* expression was unchanged in porcine embryos at the morula stage. In a previous study, we detected higher *Fgf4* expression in bovine and porcine ICM lineages at the blastocyst and elongated stages [16, 17]. Moreover, in murine embryos, Fgf4 expression was strongly detected in the ICM at the blastocyst stage [15]. It is possible that Fgf4 expression is not under the influence of Oct-4 until completion of ICM/TE segregation at the blastocyst stage.

In conclusion, we found that Oct-4 is essential for blastocyst formation of porcine embryos. The present study is the first to demonstrate the critical importance of Oct-4 for early development of porcine embryos and may also provide the basis to understand the mechanism of early lineage segregation in porcine embryos.

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