

OCT-4 expression is essential for the segregation of trophectoderm lineages in porcine preimplantation embryos

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Abstract. Oct-4, a member of the POU family of transcription factors, is a key factor that regulates the segregation of the inner cell mass (ICM) and the trophectoderm (TE) during the transition from morula to blastocyst in mice. However, little is known about its role in porcine early embryogenesis. To determine the function of OCT-4 in the ICM and TE segregation of porcine embryos, we studied the developmental morphology of porcine embryos using RNA interference technology. Our experiments demonstrated that when 1-cell stage embryos were co-injected with the small interfering RNA (siRNA) for targeted knockdown of *OCT-4* (OCT-4-siRNA) and tetramethylrhodamine isothiocyanate (TRITC)-dextran conjugate (Dx), they failed to form blastocysts. Therefore, in this study, we constructed chimeric embryos comprising blastomeres that either expressed *OCT-4* normally or showed downregulated *OCT-4* expression by co-injection of OCT-4-siRNA and Dx into one blastomere in 2- to 4-cell stage embryos. In control embryos, which were co-injected with control siRNA and Dx, Dx-positive cells contributed to the TE lineage in almost all the blastocysts examined. In contrast, Dx-positive cells derived from a blastomere co-injected with OCT-4-siRNA and Dx were degenerated in almost half the blastocysts. This was probably due to the inability of these cells to differentiate into the TE lineage. Real-time RT-PCR analysis revealed no difference in the levels of *SOX2*, *TEAD4*, *FGF4* and *FGFR1-IIIc*, all of which are known to be regulated by OCT-4, between the OCT-4-siRNA-injected morulae and the control ones. However, the level of *CDX2*, a molecule specifically expressed in the TE lineage, was significantly higher in the former than in the latter. Our results indicate that continuous expression of OCT-4 in blastomeres is essential for TE formation of porcine embryos.

Key words: Early development, OCT-4, Porcine embryo, RNA interference, Trophectoderm

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The first visible cell lineage segregation in early mammalian development occurs during the transition from the morula to the blastocyst stage. The blastocyst consists of two types of cells: trophectoderm (TE), an epithelial sheet surrounding the fluid filled cavity known as blastocoel, and inner cell mass (ICM), a group of cells attached to the inside of TE. TE cells are involved in implantation and form extraembryonic tissues, including the placenta. In contrast, the cells of the ICM are pluripotent and eventually give rise to the fetus and additional extraembryonic tissues. The segregation of ICM and TE is regulated by various genes. Oct-4 (encoded by *Pou5f1*), a member of the POU family of transcription factors, is specifically expressed in embryonic stem cells (ESCs) and germ cells [1, 2]. Furthermore, Oct-4 expression in mouse embryos is restricted to only the ICM [3]. Activation of Oct-4 expression is also used in the generation of induced pluripotent stem cells (iPSCs) [4]. Mouse embryos lacking *Oct-4* expression exhibited defective ICM formation, although they could differentiate into TE [2]. Therefore, Oct-4 is

thought to be important for the formation and maintenance of ICM [2]. On the other hand, the downregulation of *OCT-4* expression in 1-cell porcine embryos had no effect on development to the morula stage, but disrupted blastocyst formation, thus demonstrating the essential role of OCT-4 in the formation of ICM and TE in porcine embryos [5]. Since OCT-4 expression is detectable in both ICM and TE lineages of porcine embryos [3, 6, 7], it is likely that OCT-4 is expressed in TE precursors and directly regulates TE formation.

In mouse embryos, fibroblast growth factor 4 (*Fgf4*), a target of Oct-4 [8, 9], is required for TE cell proliferation [10–13]. *Fgf4* is highly expressed in ICM and activates the *Fgf* receptor expressed in the TE lineage [14, 15]. Thus, *Fgf4*–*Fgf* receptor signaling plays an important role in the proliferation and differentiation of TE cells [10, 16]. If there is a similar signaling pathway in porcine embryos, it is possible that OCT-4 is expressed in ICM precursors and plays a role in the segregation of ICM and TE, probably through downstream factors.

Recently, RNA interference (RNAi) has been shown to be functional in porcine preimplantation embryos [5]. Small interfering RNAs (siRNAs) silence specific genes via a targeted mRNA degradation and are widely used in molecular and cellular research [17]. Our previous study demonstrated that cytoplasmic injection of siRNA targeting *OCT-4* (hereafter referred to as OCT-4-siRNA) disrupted blastocyst formation [5], probably due to decreased *OCT-4* expression in all cells of the blastocyst. However, with this approach, it is impossible

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to trace the fate of blastomeres that exhibit downregulated *OCT-4* expression. Therefore, in the present study, we co-injected OCT-4-siRNA and tetramethylrhodamine isothiocyanate (TRITC)-dextran conjugate (hereafter referred to as Dx; used as a marker) into one blastomere of a 2- to 4-cell stage porcine embryo. Using this method, we generated embryos which have both *OCT-4*-downregulated and *OCT-4*-expressed cells, and observed whether the *OCT-4*-downregulated cells contribute to the TE lineage. We hypothesized that if the *OCT-4*-downregulated cells do not contribute to the TE lineage, then direct OCT-4 expression is essential for TE formation. In contrast, if the *OCT-4*-downregulated cells contribute to the TE lineage, it would show that some signaling from the cells normally expressing *OCT-4* likely drives TE formation, namely OCT-4 is essential for TE formation indirectly. In this study, to generate embryos that have both *OCT-4*-downregulated and *OCT-4*-expressed blastomeres, we injected the siRNA and Dx in one blastomere of the 2- to 4-cell stage embryos and traced that cell and its daughter cells. However, when using *in vitro* fertilization (IVF), we had several embryos at different developmental time points, including 1- to 8-cell stage embryos. To synchronize the number of 2- to 4-cell stage embryos at the time of injection, we opted to produce embryos by parthenogenesis.

In this study, we examined the mRNA expression patterns of *SRY-related HMG-box gene 2 (SOX2)*, *Caudal-related homeobox 2 (CDX2)* and *TEA domain family transcription factor 4 (TEAD4)* in porcine embryos. SOX2 expresses in the ICM of porcine blastocysts [18], and Fgf4 is activated by the Oct-4–Sox2 complex [8, 9]. On the other hand, in mouse embryos, mutual antagonism between Oct-4 and Cdx2 contributes to the segregation of ICM and TE, with Cdx2 restricted to the outer cells and Oct-4 restricted to the inner cells of the morula [19]. In addition, Tead4 is an important factor for TE development by acting upstream of *Cdx2* [20, 21].

As described above, we examined the role of OCT-4 in the segregation of TE lineage in porcine embryos using embryos constructed with *OCT-4*-downregulated and *OCT-4*-expressed cells.

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 separately cultured in four-well dishes (Thermo Fisher Scientific, Kanagawa, Japan) for 20 h in 500 μ l of maturation medium, which was composed of a modified North Carolina State University (NCSU)-37 (mNCSU-37) [22] solution containing 10% porcine follicular fluid, 0.6 mM cysteine, 0.05

mM β -mercaptoethanol, 1 mM dibutyl 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 27 h. The maturation culture was performed at 39°C in a humidified atmosphere comprising 5% CO₂, 5% O₂, and 90% N₂.

Parthenogenetic activation and *in vitro* culture

After *in vitro* maturation, the COCs were transferred to 0.1% hyaluronidase medium and freed from cumulus cells by pipetting. Denuded oocytes were washed with PZM-5 [23], and the oocytes were pulsed with single direct current electric pulse of 150 V/1 mm for 100 μ sec. Electro-stimulated oocytes were washed and cultured for 5 h at 39°C under a 5% CO₂, 5% O₂, and 90% N₂ atmosphere in modified PZM-5 (mPZM-5) containing 5 μ g/ml cytochalasin B and 10 μ g/ml cycloheximide. Following microinjection of siRNA, the embryos were washed and cultured in PZM-5 at 39°C under a 5% CO₂, 5% O₂, and 90% N₂ atmosphere until day 6 (PA = day 0). Rates of embryo development were assessed on day 2 (2-cell \leq), day 3 (8-cell \leq), day 4 (16-cell and morula), day 5 and day 6 (blastocyst).

siRNA microinjection into embryos

We used the same siRNA used for *OCT-4* knockdown, as described previously [5]. The target sites of the *OCT-4* transcript were selected from porcine sequences (GenBank accession number: NM_001113060). OCT-4-siRNA was synthesized by the outside company based on the sequences shown in Table 1. After parthenogenetic activation, embryos were subsequently transferred to a 20 μ l drop of modified TALP (mTALP) medium [24], containing 1 mg/ml BSA (fraction V) for microinjection. Approximately 10 μ l of 50 μ M specific siRNA duplexes with 4 mg/ml Dx (MW 70,000; Invitrogen) or specific siRNA without Dx was injected into the cytoplasm of each 1-cell stage embryo using a Transjector 5246 (Eppendorf, Hamburg, Germany). Approximately 10 μ l of 20 μ M nonsilencing siRNA (AllStars Negative Control siRNA, Qiagen, Tokyo, Japan) with Dx or nonsilencing siRNA without Dx was injected as control siRNA using the same method. To inject one blastomere of a 2- to 4-cell stage embryo, the embryos were cultured in PZM-5 for 20 h at 39°C under a 5% CO₂, 5% O₂, and 90% N₂ atmosphere after parthenogenetic activation. Either specific siRNA with Dx or nonsilencing siRNA with Dx was injected into the blastomere. The embryos were washed seven times immediately after microinjection, and cultured as described above.

Confocal microscopy and image analysis

On day 6, blastocysts that contained Dx-positive cells were incubated with 1 mg/ml calcein-AM (Invitrogen) in dimethyl sulfoxide at a concentration of 1:1000 at 39°C in a humidified atmosphere containing 5% CO₂ in air for 30 min in 0.5% BSA-PBS. Live cells were detected by calcein-AM staining. These embryos were fixed in 4% paraformaldehyde for 20 min at room temperature, and then washed in 0.5% BSA-PBS. Embryos were placed in PBS on a glass-bottom dish (Iwaki, Osaka, Japan) coated with Cell-Tak (Corning, Discovery Labware, Bedford, MA, USA). Fluorescent images were obtained using confocal laser scanning microscope systems (Nikon

Table 1. Primers and siRNA sequences

Name	Nucleotide sequences (5'–3')	Annealing temperature (C)	Fragment size (bp)	GenBank accession no.
<i>OCT-4</i>	F- GTTCTCTTTGGGAAGGTGTT R- ACACGCGGACCACATCCTTC	56	313	NM_001113060
<i>SOX2</i>	F- GCCCTGCAGTACAACCTCCAT R- GCTGATCATGTCCCGTAGGT	60	216	EU503117.1
<i>CDX2</i>	F- CAGGCCCTCTGAGAAGTGTC R- GGGGTCTTTCCTGAGGATTC	60	212	XM_003130908
<i>TEAD4</i>	F- TGGTGGAGAAAGTGGAGACC R- AAGTTCTCCAGCACGCTGTT	60	157	XM_605145
<i>FGF4</i>	F- GCGATGAGTGCAAGTTCAAA R- GAGGAAGTGGGTGACCTTCA	60	155	XM_003122418
<i>FGFR1-IIIc</i>	F- ACTGCTGGAGTTAATACCACCG R- GCAGAGTGATGGGAGAGTCC	60	125	AJ577088
<i>GAPDH</i>	F- TCGGAGTGAACGGATTG R- CCTGGAAGATGGTGATGG	52	219	AF017079
OCT-4-siRNA	S- GGGAAGGUGUUCAGCCAAATT AS- UUUGGUGAACACCUUCCCTT	N/A N/A	N/A N/A	N/A N/A

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

ECLIPSE Ti-E, C2si; Nikon, Tokyo, Japan) and were analyzed using the Nikon NIS Element AR 40006 64 bit software (Nikon).

Determination of the relative abundance of gene transcripts in porcine embryos

On day 4, the morulae, which were injected with or without siRNA or siRNA and Dx conjugate at the 1-cell stage, were treated with 0.1% protease in 1% PVP-PBS for 5 min and washed seven times in 1% PVP-PBS. Pools of five embryos were added to 5 µl of 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. Real-time PCRs were performed using a StepOne™ system (Applied Biosystems, Tokyo, Japan), and the 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, Kanagawa, Japan), 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 increased above the background for each sample. Final quantification was performed using a StepOne™ quantification software. Expression of the target gene in each run was normalized to that of the internal standard *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. Five samples were used for quantitative analysis, and each sample was run in duplicate for real-time PCR.

Immunofluorescence staining

On day 4, morulae 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. The embryos were then incubated with Alexa Fluor® 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. The embryos were then washed in TXPBS four times for 20 min each time. Samples were then mounted on slides in a drop of VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Fluorescent images

were obtained using inverted microscope systems (Nikon ECLIPSE Ti-U, Nikon DIGITAL SIGHT DS-L3, DS-Fi2; Nikon).

Statistical analysis

The percentage data for embryo development and contribution to TE were subjected to arcsine transformation. The transformed developmental values for embryos that were injected with siRNA at the 1-cell stage and the transcript levels of *SOX2*, *CDX2*, and *TEAD4* were analyzed by one-way analysis of variance (ANOVA), followed by multiple pairwise comparisons using the Tukey–Kramer method or the Fisher method. The transformed developmental values for embryos that were injected with siRNA at the 2- to 4-cell stage and values of contribution to TE were analyzed by the F-test, followed by the Student's *t*-test or the Mann–Whitney's U test. The transcript levels of *OCT-4*, *FGF4*, and *FGFR1-IIIc* were analyzed by the Kruskal–Wallis test, followed by multiple pairwise comparisons using the Scheffé method. 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 OCT-4-siRNA at the 1-cell stage were evaluated (Fig. 1A). The relative abundance of *OCT-4* in OCT-4-siRNA-injected embryos was significantly ($P < 0.001$) lower than that in uninjected and control siRNA-injected embryos.

Expression of the OCT-4 protein was evaluated in the morula stage embryos (Fig. 1B). OCT-4 signals were detected in cell nuclei in the uninjected and control siRNA-injected embryos, but were difficult to detect in the nuclei of OCT-4-siRNA-injected embryos.

Effect of *OCT-4* downregulation on the development of porcine embryos

In vitro developmental competence of embryos injected with OCT-4-siRNA at the 1-cell stage was evaluated (Table 2). On day 2, the rate of 2-cell \leq was significantly ($P < 0.05$) higher in uninjected embryos than in control siRNA-injected or OCT-4-siRNA-injected embryos. On day 3, the 8-cell \leq rate of OCT-4-siRNA-injected embryos was significantly ($P < 0.05$) lower than that of the uninjected embryos. On days 5 and 6, the blastocyst developmental rate of the OCT-4-siRNA-injected embryos was significantly ($P < 0.05$) lower than that of the uninjected and control siRNA-injected embryos.

On the other hand, no differences were observed in developmental rates between OCT-4-siRNA-injected and control siRNA-injected into one blastomere of 2- to 4-cell stage embryos (Table 3). On days 5 and 6, OCT-4-siRNA-injected embryos could develop to the blastocyst stage (22.4% and 24.4%, respectively).

Dx-positive blastomeres were detected in all developmental stages of embryos obtained from either OCT-4-siRNA or control siRNA injection at the 2- to 4-cell stage (Table 4). Detection rates of Dx-positive cells at the blastocyst stage (days 5 and 6) were 73.8% to 83.5%.

Fate of *OCT-4*-downregulated blastomeres after siRNA injection at the 2- to 4-cell stage

To investigate whether Dx-positive cells contribute to the TE lineage, blastocysts developing from 2- to 4-cell stage embryos that had received blastomere injection were inspected for fluorescence using confocal microscopy on day 6. Dx-positive cells were judged as live or dead cells based on calcein-AM staining: live cells show green fluorescence, as shown in Fig. 2B. According to the results of fluorescence imaging, we classified the stained blastocysts into three groups, namely TE, TE + Deg., and Deg. In the TE group, the number of blastocysts having all Dx-positive and calcein-AM-stained cells that are supposed to contribute to the TE lineage was significantly ($P < 0.05$; 57.9% vs. 20.0%) higher in blastocysts that received blastomere injection with the control siRNA than in those that received blastomere injection with the OCT-4-siRNA (Fig. 2A). In the Deg. group, the number of blastocysts having degenerated (as shown by the presence of non-calcein-AM-stained cells), but Dx-positive cells in almost a half of the embryos was significantly ($P < 0.01$; 46.7% vs. 5.3%) higher in the blastocysts that received blastomere injection with the OCT-4-siRNA than in those that received blastomere injection with the control siRNA (Fig. 2A). In the TE + Deg. group, there was no difference in the number of blastocysts having Dx-positive and calcein-AM-stained cells and Dx-positive but non-calcein-AM-stained cells between the OCT-4-siRNA- and the control siRNA-injected embryos (33.3% vs. 36.8%; Fig. 2A).

Relative expression levels of mRNA in porcine embryos derived from *OCT-4*-siRNA injection

To clarify the effects of *OCT-4* downregulation on the expression patterns of *SOX2*, *CDX2*, and *TEAD4*, all of which are regulated by OCT-4, real-time RT-PCR was performed using morulae that are derived from 1-cell stage embryos injected with the control siRNA or OCT-4-siRNA as well as using uninjected morulae. As shown in Fig. 3A and 3C, the relative abundances of *SOX2* and *TEAD4* mRNAs did not differ between the treatment groups. However, *CDX2* expression was significantly ($P < 0.05$) higher in OCT-4-siRNA-injected embryos than in control siRNA-injected embryos (Fig. 3B). Furthermore, we examined the mRNA level of *FGF4* and *FGFR1-IIIc* to investigate the effects of *OCT-4* downregulation on the FGF4 and FGF receptor. As shown in Fig. 4, the relative abundance of *FGF4* and *FGFR1-IIIc* transcripts did not differ between the treatment groups.

Discussion

OCT-4, the marker of pluripotent cells, is required *in vitro* for establishment and maintenance of ESCs and for reprogramming somatic cells to pluripotency [2, 4, 25]. In mouse embryos, Oct-4 is essential for the formation and maintenance of ICM [2]. In the present study, to clarify the necessity of OCT-4 for segregation of TE lineage in porcine embryos, we generated porcine embryos with blastomeres expressing *OCT-4* at reduced and normal levels and investigated the developmental morphology.

In murine embryos, although blastocyst formation was slightly reduced by Oct-4-siRNA injection [26], Oct-4-morpholino oligonucleotide-injected embryos failed to form blastocyst [27]. In porcine embryos derived from IVF, OCT-4 is required for embryonic

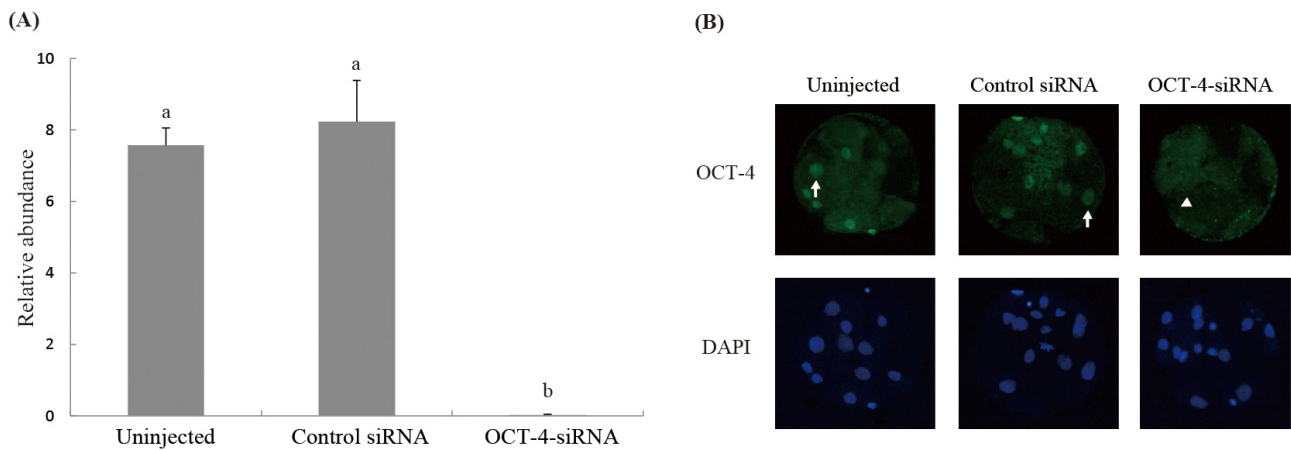


Fig. 1. *OCT-4* mRNA and protein expression in porcine embryos. (A) Relative abundance (mean \pm SEM) of *OCT-4* transcripts in uninjected porcine morula stage embryos ($n = 5$) or porcine morula stage embryos treated with control siRNA + Dx ($n = 5$), or with *OCT-4*-siRNA + Dx ($n = 5$). ^{a, b} Different superscripts indicate a significant difference ($P < 0.001$). (B) *OCT-4* signals (indicated by arrows) were visible in the uninjected morula as well as the morula derived from the control siRNA-injected 1-cell stage embryo. However, such signals were absent in the morula derived from the *OCT-4*-siRNA-injected 1-cell stage embryo (shown by arrowheads).

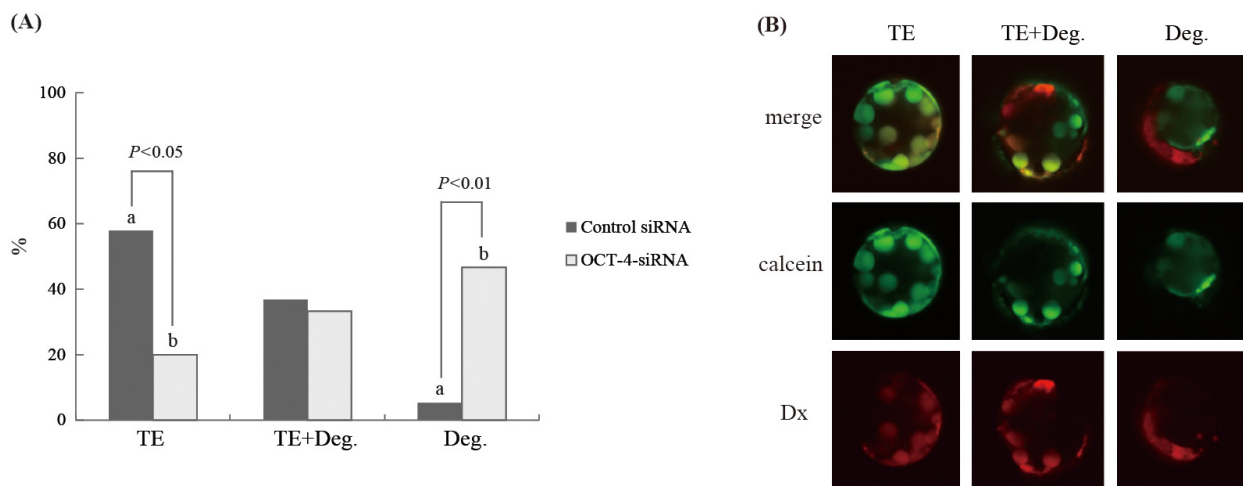


Fig. 2. Fate of *OCT-4*-downregulated blastomeres from embryos injected with siRNA and Dx at the 2- to 4-cell stage. (A) The percentage of TE formation in porcine blastocyst stage embryos following injection of one blastomere with control siRNA + Dx ($n = 38$) or with *OCT-4*-siRNA + Dx ($n = 30$) at the 2- to 4-cell stage. ^{a, b} Different superscripts indicate a significant difference. (B) Representative photographs of calcein-AM and Dx signals in porcine embryos. siRNA-injected blastomeres fluoresced red and live cells fluoresced green. All Dx-positive cells were stained by calcein-AM (TE), some Dx-positive cells were not stained by calcein-AM (TE+Deg.), and all Dx-positive cells were not stained by calcein-AM (Deg.).

development from the morula to the blastocyst stage [5]. In this study, *OCT-4* downregulation inhibited the transformation of morula to blastocyst in porcine embryos derived by parthenogenetic treatment. This result is similar to that of IVF embryos [5]. Thus, in porcine parthenogenetic embryos, *OCT-4* is essential for development from the morula to the blastocyst stage. On the other hand, in porcine embryos, *OCT-4*-downregulated embryos obtained using the CRISPR/CAS9 system developed to the blastocyst stage [28]. *OCT-4* downregulation by siRNA, both maternal and embryonic transcript of *OCT-4* may be downregulate. However, maternal *OCT-4* expression may remain using the CRISPR/CAS9 system. It is possible that maternal *OCT-4* expression is important for blastocyst formation of porcine embryos.

To clarify the role of maternal and embryonic *OCT-4* expression, combined RNA interference and CRISPR/CAS9 system should be necessary in future experiments. At the 2-cell and blastocyst stage, the developmental rates of *OCT-4*-siRNA-injected and control siRNA-injected embryos were significantly lower than those of uninjected embryos. The siRNAs were conjugated with Dx. Dextran-induced anaphylactic reactions have been observed in humans, although there are few reports of problems with experimental animals have appeared [29, 30]. Therefore, it is possible that Dx exerted some toxicity in the embryos. However, the blastocyst formation rate of embryos injected with *OCT-4*-siRNA and Dx was significantly lower than that in the embryos injected with control siRNA and Dx,

Table 2. Effect of OCT-4-siRNA injection at the 1-cell stage 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	201	160 (79.6) ^a	123 (61.2) ^a	47 (23.4)	49 (24.4)	91 (45.3) ^a	81 (40.3) ^a		
Control siRNA	197	135 (68.5) ^b	99 (50.3) ^{ab}	47 (23.9)	32 (16.2)	56 (28.4) ^b	50 (25.4) ^b		
OCT-4-siRNA	191	125 (65.4) ^b	80 (41.9) ^b	36 (18.8)	32 (16.8)	11 (5.8) ^c	9 (4.7) ^c		

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

Table 3. Effect of OCT-4-siRNA injection at the 2- to 4-cell stage 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		
Control siRNA	247	209 (84.6)	101 (40.9)	53 (21.5)	53 (21.5)	91 (36.8)	91 (36.8)		
OCT-4-siRNA	250	207 (82.8)	77 (30.8)	49 (19.6)	49 (19.6)	56 (22.4)	61 (24.4)		

* Experiments were replicated six times. [†] Percentages of the number of embryos cultured.

Table 4. Developmental competence of porcine embryos, which is constructed from Dx-positive and Dx-negative cells

Treatment	No. (%) [†] of embryos developed to							
	Day 2		Day 3		Day 4		Day 5	Day 6
	4-cell	8-cell	8-cell	16-cell	16-cell	Morula	Blastocyst	Blastocyst
Control siRNA	72/132 (54.5)	36/51 (70.6)	58/83 (69.9)	11/17 (64.7)	40/53 (75.5)	35/53 (66.0)	76/91 (83.5)	75/91 (82.4)
OCT-4-siRNA	89/147 (60.5)	36/41 (87.8)	52/62 (83.9)	11/15 (73.3)	38/49 (77.6)	38/49 (77.6)	42/56 (75.0)	45/61 (73.8)

[†] Percentage of the number of embryos cultured.

this developmental arrest was attributed to OCT-4-siRNA injection.

In the present study, although embryos injected with OCT-4-siRNA at the 1-cell stage failed to form blastocysts, embryos injected with OCT-4-siRNA into only one blastomere at the 2- to 4-cell stage developed into blastocysts. Furthermore, Dx-positive blastomeres were detected in embryos at all developmental stages. However, Dx-positive cells in almost all OCT-4-siRNA-injected embryos were degenerated. In most *OCT-4*-downregulated cells were difficult to contributing to the TE lineage and the blastocysts constructed from only *OCT-4*-expressed cells. Therefore, in porcine embryos, segregation of the TE lineage needs direct OCT-4 expression in the blastomeres. *OCT-4* transcripts were detected at high levels in porcine oocytes, followed by consistent degradation to the 8-cell stage and increased at the morula stage [31]. In porcine morulae, OCT-4 protein signal was observed in blastomeres derived from parthenogenetic treatment. In addition, almost all cells expressed OCT-4 at the blastocyst stage (data not shown). Thus, in porcine embryos, it is possible that OCT-4 expression in blastomeres at the morula stage is important for segregation of TE lineages. However, a few *OCT-4*-downregulated cells contributed to the TE lineage. These results indicate the possibility that OCT-4 downstream factors from the *OCT-4*-expressed cells exerted some effects on the *OCT-4*-downregulated cells, and this signaling pathway was involved in the differentiation of TE.

In this study, direct OCT-4 expression in the blastomere was

essential for segregation of the TE lineages. To identify the OCT-4 downstream factors, we examined the mRNA expression of candidate genes *SOX2*, *CDX2*, and *TEAD4*. Sox2 is required for the expression of pluripotency genes and for the repression of TE related genes in ESCs [32–35]. Sox2 acts cooperatively with Oct-4 and activates pluripotency factors such as Oct-4, Sox2, and Nanog [32, 35–39]. In porcine embryos, SOX2 specifically express in the ICM and epiblast [7, 18]. In mouse embryos, Sox2 is essential for the formation of both ICM and TE [40, 41]. In this study, the *SOX2* expression level in OCT-4-siRNA-injected embryos did not differ from that in control embryos. This result suggests that in porcine embryos, SOX2 expression is not under the influence of OCT-4.

In mouse embryos, Cdx2 is expressed by Tead4 and regulates TE segregation [20, 21, 42, 43]. In this study, the *CDX2* expression level in OCT-4-siRNA-injected embryos was higher than that in control siRNA-injected embryos. It is well known that Oct-4 and Cdx2 enhance their own expression, while mutually suppressing the expression of one another in ESCs [19]. Our result corresponded to the reported mutual antagonism between these two factors. Therefore, it is possible that OCT-4 regulates ICM/TE segregation through inhibition of CDX2 expression in porcine embryos. In contrast, *TEAD4* expression was unchanged in porcine morulae. In a previous study, *TEAD4* expression was observed in the 4-cell to blastocyst stages of porcine embryos, and differences in *TEAD4*

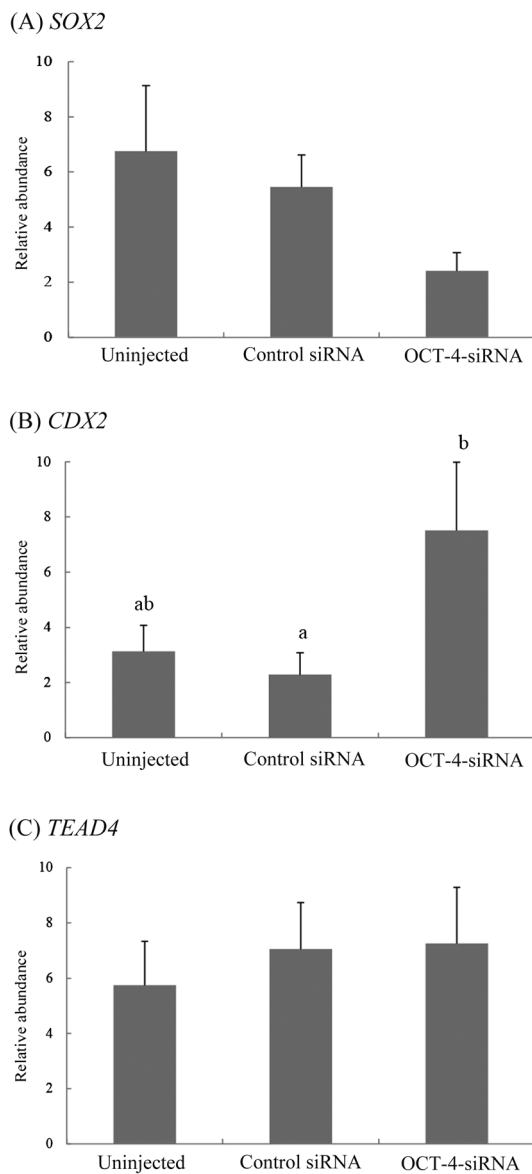


Fig. 3. Relative abundance (mean \pm SEM) of (A) *SOX2*, (B) *CDX2* and (C) *TEAD4* transcripts in uninjected porcine morula (n = 5) or porcine morula treated with control siRNA (n = 5) or with OCT-4-siRNA (n = 5). ^{a, b} Different superscripts indicate a significant difference ($P < 0.05$).

expression levels between ICM and TE lineages became appreciable in the elongation stages, such as the filamentous stage [6, 44]. These results suggested that *TEAD4* expression is not under the influence of OCT-4, but it is possible that *TEAD4* is a regulatory factor for the expression of TE related genes in porcine embryos.

Our findings are indicative of a signaling pathway involved in OCT-4 expression for TE formation. In mouse embryos, the *Fgf4* expressed in the ICM acts on TE cells through the *Fgf* receptor to maintain a proliferating population of TE cells [10, 13]. In addition, in *Oct-4* deficient mouse embryos, the *Fgf4* transcription level was

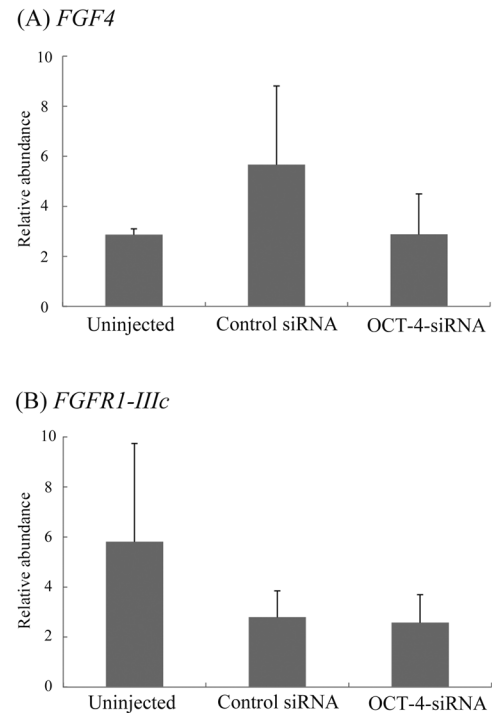


Fig. 4. Relative abundance (mean \pm SEM) of (A) *FGF4* and (B) *FGFR1-IIIc* transcripts in uninjected porcine morula (n = 5) or porcine morula treated with control siRNA + Dx (n = 5) or with OCT-4-siRNA + Dx (n = 5).

greatly reduced at the blastocyst stage [2]. *Fgf4* is activated by the Oct-4–Sox2 complex [8, 9]. These results suggest that *FGF4* expression is regulated by OCT-4 in ICM precursors and that *FGF4* is involved in the differentiation of the TE lineage in porcine embryos. Therefore, we examined the expression of *FGF4* and *FGFR1-IIIc* in porcine embryos injected with OCT-4-siRNA. However, the expression of both genes was unchanged in porcine embryos at the morula stage. In a previous study, *FGF4* expression was high in porcine ICM lineage in the blastocysts and the elongation stages [6]. Moreover, *FGF4* influenced the growth TE cells during the elongation of porcine embryos [45]. It is possible that *FGF4* expression is not under the influence of OCT-4 until completion of the ICM/TE segregation.

In conclusion, we showed that direct OCT-4 expression in the blastomere is essential for TE segregation in porcine embryos. This is the first study to demonstrate the critical importance of OCT-4 for TE formation by porcine embryos and these findings provide the basis for understanding the mechanism of early lineage segregation in porcine embryos. Further studies, such as ChIP analysis and knockdown of other target genes by siRNA, are needed to fully understand the molecular mechanism underlying TE lineage segregation in porcine embryos.

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References

- Yeom YI, Fuhrmann G, Ovitt CE, Brehm A, Ohbo K, Gross M, Hübner K, Schöler HR. Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development* 1996; **122**: 881–894. [Medline]
- Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 1998; **95**: 379–391. [Medline] [CrossRef]
- Kirchhof N, Carnwath JW, Lemme E, Anastassiadis K, Schöler H, Niemann H. Expression pattern of Oct-4 in preimplantation embryos of different species. *Biol Reprod* 2000; **63**: 1698–1705. [Medline] [CrossRef]
- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663–676. [Medline] [CrossRef]
- Sakurai N, Fujii T, Hashizume T, Sawai K. Effects of downregulating oct-4 transcript by RNA interference on early development of porcine embryos. *J Reprod Dev* 2013; **59**: 353–360. [Medline] [CrossRef]
- Fujii T, Sakurai N, Osaki T, Iwagami G, Hirayama H, Minamihashi A, Hashizume T, Sawai K. Changes in the expression patterns of the genes involved in the segregation and function of inner cell mass and trophectoderm lineages during porcine preimplantation development. *J Reprod Dev* 2013; **59**: 151–158. [Medline] [CrossRef]
- du Puy L, Lopes SM, Haagsman HP, Roelen BA. Analysis of co-expression of OCT4, NANOG and SOX2 in pluripotent cells of the porcine embryo, in vivo and in vitro. *Theoriology* 2011; **75**: 513–526. [Medline] [CrossRef]
- Ambrosetti DC, Basilico C, Dailey L. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol Cell Biol* 1997; **17**: 6321–6329. [Medline] [CrossRef]
- Yuan H, Corbi N, Basilico C, Dailey L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes Dev* 1995; **9**: 2635–2645. [Medline] [CrossRef]
- Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J. Promotion of trophoblast stem cell proliferation by FGF4. *Science* 1998; **282**: 2072–2075. [Medline] [CrossRef]
- Rappolee DA, Basilico C, Patel Y, Werb Z. Expression and function of FGF-4 in peri-implantation development in mouse embryos. *Development* 1994; **120**: 2259–2269. [Medline]
- Niswander L, Martin GR. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* 1992; **114**: 755–768. [Medline]
- Leunda-Casi A, de Hertogh R, Pampfer S. Control of trophectoderm differentiation by inner cell mass-derived fibroblast growth factor-4 in mouse blastocysts and corrective effect of FGF-4 on high glucose-induced trophoblast disruption. *Mol Reprod Dev* 2001; **60**: 38–46. [Medline] [CrossRef]
- Allen BL, Filla MS, Rapraeger AC. Role of heparan sulfate as a tissue-specific regulator of FGF-4 and FGF receptor recognition. *J Cell Biol* 2001; **155**: 845–858. [Medline] [CrossRef]
- Schlessinger J, Plotnikov AN, Ibrahim OA, Eliseenkova AV, Yeh BK, Yayon A, Linhardt RJ, Mohammadi M. Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol Cell* 2000; **6**: 743–750. [Medline] [CrossRef]
- Kunath T, Strumpf D, Rossant J. Early trophoblast determination and stem cell maintenance in the mouse—a review. *Placenta* 2004; **25**(Suppl A): S32–S38. [Medline] [CrossRef]
- Hannon GJ. RNA interference. *Nature* 2002; **418**: 244–251. [Medline] [CrossRef]
- Liu S, Bou G, Sun R, Guo S, Xue B, Wei R, Cooney AJ, Liu Z. Sox2 is the faithful marker for pluripotency in pig: evidence from embryonic studies. *Dev Dyn* 2015; **244**: 619–627. [Medline] [CrossRef]
- Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, Rossant J. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell* 2005; **123**: 917–929. [Medline] [CrossRef]
- Nishioka N, Yamamoto S, Kiyonari H, Sato H, Sawada A, Ota M, Nakao K, Sasaki H. Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mech Dev* 2008; **125**: 270–283. [Medline] [CrossRef]
- Yagi R, Kohn MJ, Karavanova I, Kaneko KJ, Vullhorst D, DePamphilis ML, Buonanno A. Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. *Development* 2007; **134**: 3827–3836. [Medline] [CrossRef]
- Petters RM, Wells KD. Culture of pig embryos. *J Reprod Fertil Suppl* 1993; **48**: 61–73. [Medline]
- Suzuki C, Yoshioka K. Effects of amino acid supplements and replacement of polyvinyl alcohol with bovine serum albumin in porcine zygote medium. *Reprod Fertil Dev* 2006; **18**: 789–795. [Medline] [CrossRef]
- Bavister BD, Leibfried ML, Lieberman G. Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biol Reprod* 1983; **28**: 235–247. [Medline] [CrossRef]
- Niwa H. How is pluripotency determined and maintained? *Development* 2007; **134**: 635–646. [Medline] [CrossRef]
- Shin MR, Cui XS, Jun JH, Jeong YJ, Kim NH. Identification of mouse blastocyst genes that are downregulated by double-stranded RNA-mediated knockdown of Oct-4 expression. *Mol Reprod Dev* 2005; **70**: 390–396. [Medline] [CrossRef]
- Foygel K, Choi B, Jun S, Leong DE, Lee A, Wong CC, Zuo E, Eckart M, Reijo Pera RA, Wong WH, Yao MW. A novel and critical role for Oct4 as a regulator of the maternal-embryonic transition. *PLoS ONE* 2008; **3**: e4109. [Medline] [CrossRef]
- Kwon J, Namgoong S, Kim NH. CRISPR/Cas9 as tool for functional study of genes involved in preimplantation embryo development. *PLoS ONE* 2015; **10**: e0120501. [Medline] [CrossRef]
- Ljungström KG, Renck H, Strandberg K, Hedin H, Richter W, Widerlöv E. Adverse reactions to dextran in Sweden 1970–1979. *Acta Chir Scand* 1983; **149**: 253–262. [Medline]
- Hedin H, Richter W, Ring J. Dextran-induced anaphylactoid reactions in man: role of dextran reactive antibodies. *Int Arch Allergy Appl Immunol* 1976; **52**: 145–159. [Medline] [CrossRef]
- Lee E, Lee SH, Kim S, Jeong YW, Kim JH, Koo OJ, Park SM, Hashem MA, Hossein MS, Son HY, Lee CK, Hwang WS, Kang SK, Lee BC. Analysis of nuclear reprogramming in cloned miniature pig embryos by expression of Oct-4 and Oct-4 related genes. *Biochem Biophys Res Commun* 2006; **348**: 1419–1428. [Medline] [CrossRef]
- Okumura-Nakanishi S, Saito M, Niwa H, Ishikawa F. Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. *J Biol Chem* 2005; **280**: 5307–5317. [Medline] [CrossRef]
- Kurosaka S, Eckardt S, McLaughlin KJ. Pluripotent lineage definition in bovine embryos by Oct4 transcript localization. *Biol Reprod* 2004; **71**: 1578–1582. [Medline] [CrossRef]
- Li J, Pan G, Cui K, Liu Y, Xu S, Pei D. A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. *J Biol Chem* 2007; **282**: 19481–19492. [Medline] [CrossRef]
- Chew JL, Loh YH, Zhang W, Chen X, Tam WL, Yeap LS, Li P, Ang YS, Lim B, Robson P, Ng HH. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol Cell Biol* 2005; **25**: 6031–6046. [Medline] [CrossRef]
- Wicklow E, Blij S, Frum T, Hirate Y, Lang RA, Sasaki H, Ralston A. HIPPO pathway members restrict SOX2 to the inner cell mass where it promotes ICM fates in the mouse blastocyst. *PLoS Genet* 2014; **10**: e1004618. [Medline] [CrossRef]
- Kuroda T, Tada M, Kubota H, Kimura H, Hatano SY, Suemori H, Nakatsuji N, Tada T. Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol Cell Biol* 2005; **25**: 2475–2485. [Medline] [CrossRef]
- Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH, Robson P. Transcriptional regulation of nanog by OCT4 and SOX2. *J Biol Chem* 2005; **280**: 24731–24737. [Medline] [CrossRef]
- Tomioka M, Nishimoto M, Miyagi S, Katayanagi T, Fukui N, Niwa H, Muramatsu M, Okuda A. Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. *Nucleic Acids Res* 2002; **30**: 3202–3213. [Medline] [CrossRef]
- Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev* 2003; **17**: 126–140. [Medline] [CrossRef]
- Keramari M, Razavi J, Ingman KA, Patsch C, Edenhofer F, Ward CM, Kimber SJ. Sox2 is essential for formation of trophectoderm in the preimplantation embryo. *PLoS ONE* 2010; **5**: e13952. [Medline] [CrossRef]
- Ralston A, Rossant J. Cdx2 acts downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryo. *Dev Biol* 2008; **313**: 614–629. [Medline] [CrossRef]
- Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* 2005; **132**: 2093–2102. [Medline] [CrossRef]
- Cao S, Han J, Wu J, Li Q, Liu S, Zhang W, Pei Y, Ruan X, Liu Z, Wang X, Lim B, Li N. Specific gene-regulation networks during the pre-implantation development of the pig embryo as revealed by deep sequencing. *BMC Genomics* 2014; **15**: 4. [Medline] [CrossRef]
- Valdez Magaña G, Rodríguez A, Zhang H, Webb R, Alberio R. Paracrine effects of embryo-derived FGF4 and BMP4 during pig trophoblast elongation. *Dev Biol* 2014; **387**: 15–27. [Medline] [CrossRef]