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—Original Article—

## The necessity of TEAD4 for early development and gene expression involved in differentiation in porcine embryos

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**Abstract.** TEA domain family transcription factor 4 (Tead4) is known to be important for the trophectoderm (TE) segregation in murine embryos. However, the role of TEAD4 in early development of porcine embryos is still unknown. We examined TEAD4 expression patterns and attempted to determine the functions of TEAD4 during porcine preimplantation development using RNA interference. *TEAD4* mRNA was upregulated from the 2–4-cell to 8–16-cell stages and then decreased to the blastocyst stage. Nuclear localization of TEAD4 protein was detected at the 16-cell stage, as well as at subsequent developmental stages. In porcine embryos injected with TEAD4 siRNA, transformation from morula to blastocyst was inhibited. Although *TEAD4* downregulation did not affect the expression levels of *POU class 5 homeobox 1 (OCT-4)*, transcription of *SRY-related HMG-box gene 2 (SOX2)* was detected at high level in *TEAD4*-downregulated embryos. It is possible that TEAD4 contributes to blastocyst formation in porcine embryos through downregulation of *SOX2* expression. Collectively, our results indicate that TEAD4 is an important factor for the preimplantation development of porcine embryos.

**Key words:** Early development, Gene expression, Porcine embryo, RNA interference, TEAD4

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In mammalian embryos, the first visible differentiation event is the segregation of the trophectoderm (TE) and inner cell mass (ICM) during the transition from the morula to the blastocyst stage. The TE, which is a single layer surrounding the fluid-filled cavity called the blastocoel, will provide the extraembryonic structures such as the placenta, whereas the ICM, which is attached to the inside of the TE, will develop into the fetus and extraembryonic tissues [1]. This ICM/TE differentiation is regulated by interaction between various transcriptional genes [2].

In mice, TEA domain family transcription factor 4 (Tead4) is detected in nuclei from the 4-cell to the blastocyst stage [3]. Murine embryos lacking *Tead4* expression fail to form a blastocoel and do not express *Caudal-related homeobox 2 (Cdx2)* which is required for TE development [3–5]. In addition, expression of ICM-specific genes such as *POU class 5 homeobox 1 (Oct-4)* and *SRY-related HMG-box gene 2 (Sox2)* are induced in these *Tead4*-deficient embryos [5, 6]. Therefore, *Tead4* is a key factor for TE segregation in murine embryos. In ICM progenitor cells, which are inside of embryos, the Hippo pathway is active, inducing cytoplasmic restriction of Yes-associated protein 1 (*Yap1*) via phosphorylation [7]. In contrast, the Hippo pathway is weakly activated in TE progenitor cells, which are outside of embryos [7]. In the outer cells, nuclear accumulation of *Yap1* leads to form *Tead4*–*Yap1* complex, and the complex induces

*Cdx2* expression [7]. Thus, *Tead4* regulates segregation of the TE lineage through the expression of *Cdx2* in murine embryos.

In murine embryos, *Cdx2* mutation leads to failure of TE maintenance [8–10], whereas *CDX2*-downregulated embryos of pigs and cows are able to develop normally to the blastocyst stage and form TE [11–13]. Furthermore, *CDX2*- and *TEAD4*-specific localization in the TE lineage starts from the ovoid stage, the next stage of the blastocyst [14]. On the other hand, *TEAD4* expression in porcine embryos has been observed from 4-cell stage, and *TEAD4* expression has been observed in both ICM and TE regions at the blastocyst stage [14, 15]. These results suggest that *TEAD4* controls the preimplantation development of porcine embryos through the expression of a specific factor other than *CDX2*. In porcine blastocysts, *OCT-4* is expressed in both ICM and TE regions [14, 16, 17], and we previously demonstrated that *OCT-4* is essential for porcine TE segregation [18, 19]. *Sox2* is a marker for pluripotency like embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [20–22]. In porcine blastocysts, *SOX2* expression is restricted to the ICM [23]. In murine ESCs, *Oct-4* interacts with *Sox2* by an *Oct*–*Sox* enhancer [24–26]. It is possible that *TEAD4* regulates segregation of the TE lineage through the expression of *OCT-4* and *SOX2* in TE precursor cells in porcine embryos.

In the present study, *TEAD4* expression was assessed at both mRNA and protein levels in porcine preimplantation embryos. In addition, we performed *TEAD4* knockdown using RNA interference targeted at *TEAD4* in order to investigate *TEAD4* function during early development of porcine embryos.

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**Table 1.** Primers and siRNA sequences

Name	Nucleotide sequences (5'–3')	Annealing temperature (°C)	Fragment size (bp)	GenBank accession no.
<i>TEAD-4</i>	F- TGGTGGAGAAAGTGGAGACC R- AAGTTCTCCAGCACGCTGTT	60	157	XM_605145
<i>OCT-4</i>	F- GTTCTCTTGGGAAGGTGTT R- ACACGCGGACCACATCCTTC	55.4	313	NM_001113060
<i>SOX2</i>	F- GCCCTGCAGTACAACCTCCAT R- GCTGATCATGTCCCCTAGGT	60	216	EU503117.1
<i>GAPDH</i>	F- TCGGAGTGAACGGATTG R- CCTGGAAGATGGTATGG	52	219	AF017079
TEAD4 siRNA-1	S- GCCAUUACUCCUACCGCAUTT AS- AUGCGGUAGGAGUAAUGGCTT	N/A	N/A	N/A
TEAD4 siRNA-2	S- CUGUGCGUCGCGUAUGUCUUC AS- AGACAUACGCGACGCACAGCA	N/A	N/A	N/A

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

## 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; Thermo Fisher Scientific, Kanagawa, Japan), 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) for 20 h in 500 µl of maturation medium, composed of a modified North Carolina State University (NCSU)-37 (mNCSU-37) [27] 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 (Serotropin, Aska Animal Health, Tokyo, Japan), and 10 IU/ml human chorionic gonadotropin (hCG; Gestron 1500, Kyoritsu seiyaku, Tokyo, Japan). The 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<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub>.

### *In vitro* fertilization and *in vitro* culture

After *in vitro* maturation, COCs were washed with modified Pig-FM (mPig-FM) [28], and 15–20 COCs were suspended in 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 [29]. 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  $5.0 \times 10^7$ /ml. COCs and sperm were incubated for 10 h at 39°C under a 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> atmosphere. Following microinjection of siRNA, the embryos were washed and cultured in PZM-5 [30] at 39°C under a 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> atmosphere until day 5 (IVF = day 0). Rates of embryo development were assessed on day 2 (2-cell ≤), day 3 (4-cell ≤), day 4 (16-cell and morula) and day 5 (blastocyst).

### Design of siRNA and microinjection into embryos

The target sights of the TEAD4 transcript were selected from porcine sequences (GenBank accession number: XM\_605145). Two types of specific siRNA (TEAD4 siRNA-1 and TEAD4 siRNA-2) were respectively designed using siRNA design software, BLOCK-iT RNAi Designer (<http://rnaidesigner.invitrogen.com/rnaiexpress/>) and Enhanced siDirect (<http://design.RNAi.jp/>). 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 [31], 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 FemtoJet (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

Oocytes and embryos at the appropriate developmental stage were treated with 0.1% protease in 1% PVP-PBS for 5 min and washed seven times in 1% PVP-PBS. Pools of ten (*in vitro* matured oocytes,

1-cell, 2–4-cell and 8–16-cell stage embryos) or five (morula and blastocyst stage embryos) oocytes or embryos were added to 5  $\mu$ l lysis buffer [0.8% Igepal (ICN Biomedicals, Aurora, OH, USA), 5 mM DTT (Thermo Fisher) and 1 U/ $\mu$ l of RNasin (Promega, Madison, WI, USA)], snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . RNA samples were heated to  $80^{\circ}\text{C}$  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  $\mu$ l. Real-time PCRs were performed using a StepOne<sup>TM</sup> system (Applied Biosystems, Tokyo, Japan), and products were detected with SYBR Green included in the QuantiTect SYBR Green PCR master mix (Qiagen). A 2  $\mu$ l aliquot of the RT product was used for each quantification. The amplification program was as follows: preincubation at  $95^{\circ}\text{C}$  for 15 min to activate HotStarTaq DNA Polymerase (Qiagen), followed by 45 cycles of denaturation at  $94^{\circ}\text{C}$  for 15 sec, annealing of primers at different temperatures (Table 1) for 30 sec, and elongation at  $72^{\circ}\text{C}$  for 30 sec. At the end of the last cycle, a melting curve was generated by starting fluorescence acquisition at  $60^{\circ}\text{C}$  and recording measurements at  $0.3^{\circ}\text{C}$  increments up to  $95^{\circ}\text{C}$ .

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 all real-time PCR runs. 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<sup>TM</sup> quantification software. Expression of the target gene in each run was normalized to the internal standard *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*.

#### Immunofluorescence staining

Oocytes or embryos at the appropriate developmental stage were fixed in 4% (w/v) paraformaldehyde in PBS (Wako Pure Chemical Industries, Osaka, Japan) for 20 min at room temperature, and then washed twice in PBS containing 0.1% (v/v) Triton X-100 (TXPBS) for 10 min each time. Samples were subsequently permeabilized in 0.2% (v/v) Triton X-100 in PBS for 30 min and then incubated in Image-iT FX Signal Enhancer (Thermo Fisher Scientific) for 30 min, followed by washing twice for 10 min in TXPBS. Blocking was performed by incubation for 1.5 h in 0.5% (w/v) BSA and 1% (w/v) skimmed milk in TXPBS for TEAD4 and SOX2 staining or in 7% (v/v) goat serum (Thermo Fisher Scientific) in TXPBS for OCT-4 staining, followed by washing in TXPBS for 5 min. The oocytes or embryos were incubated with an anti-TEAD4 primary antibody (1:1000; ab58310; Abcam, Cambridge, UK) in PBS supplemented with 0.5% (w/v) BSA and 0.05% (v/v) Triton X-100 at  $4^{\circ}\text{C}$  for 1 h, an anti-SOX2 primary antibody (1:50; sc-365823; Santa Cruz Biotechnology, Dallas, TX) in PBS supplemented with 0.5% (w/v) BSA and 0.05% (v/v) Triton X-100 at  $4^{\circ}\text{C}$  overnight or an anti-OCT-4 primary antibody (1:50; sc-9081; Santa Cruz Biotechnology) in PBS supplemented with 0.5% (w/v) BSA and 0.05% (v/v) Triton X-100

at  $4^{\circ}\text{C}$  overnight. The oocytes or embryos were washed four times in TXPBS for 15 min each time. The oocytes or embryos were then incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (A11029; Thermo Fisher Scientific) for TEAD4 and SOX2 staining or with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (A11034; Thermo Fisher Scientific) for OCT-4 staining at a dilution of 1:400 at room temperature for 1 h in PBS containing 0.5% (w/v) BSA and 0.05% (v/v) Triton X-100. Washing in TXPBS was done four times for 20 min each time and subsequently oocytes or embryos were mounted onto slides in a drop of VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindol (DAPI; Vector Laboratories, Burlingame, CA, USA). Fluorescent images were obtained using an inverted fluorescence microscope and digital camera systems (ECLIPSE Ti-U and DS-Fi2-L3; Nikon, Tokyo, Japan).

#### Statistical analysis

Percentage data for embryonic development and TEAD4, OCT-4 and SOX2 positive cell numbers was subjected to an arcsine transformation. The transformed values of embryonic development in experiment of TEAD4 siRNA-1 injection, OCT-4 and SOX2 positive cell numbers were analyzed using one-way analysis of variance (ANOVA), followed by multiple pairwise comparisons using the Tukey-Kramer method. The transformed developmental values for embryos that were injected with TEAD4 siRNA-2 were analyzed by the F-test, followed by the Student's *t*-test (day 2–4) or the Mann-Whitney's U test (day 5). Data for temporal *TEAD4* gene expression, the mRNA expression levels of *TEAD4* at the morula stage, the rate of TEAD4 positive cells and the total cell numbers were analyzed using the Kruskal-Wallis test, followed by multiple pairwise comparisons using the Scheffé method. The mRNA expression levels of *OCT-4* and *SOX2* at the morula stage were analyzed using one-way ANOVA followed by multiple pairwise comparisons using the Fisher's PLSD method. A difference with  $P < 0.05$  was regarded as statistically significant.

## Results

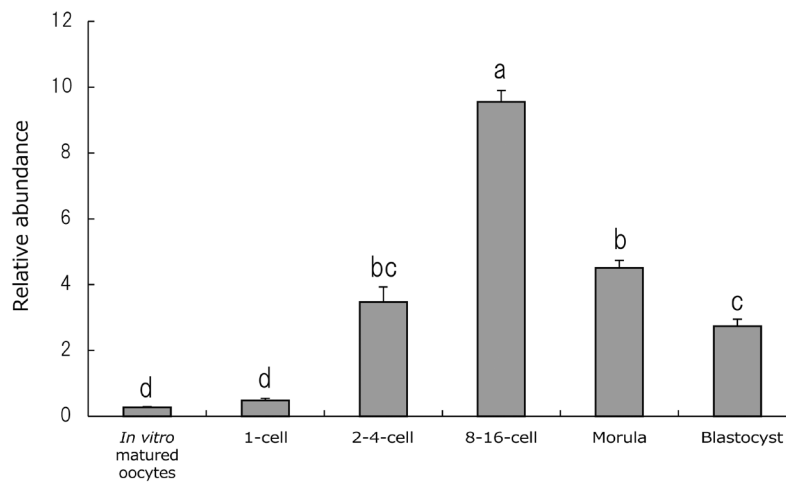
#### Temporal expression of *TEAD4* mRNA and protein in early embryos

Fig. 1 and Fig. 2 show the expression levels of *TEAD4* mRNA and expression patterns of TEAD4 protein in the *in vitro* matured oocytes and embryos at various developmental stages. *TEAD4* mRNA was significantly ( $P < 0.05$ ) upregulated from the 2–4-cell stage to the 8–16-cell stage, after which the expression level declined through the morula to the blastocyst stage.

Temporal expression status of TEAD4 protein was evaluated by immunofluorescence labeling (Fig. 2). A nuclear TEAD4 signal was detected from the 16-cell stage. At the blastocyst stage, nuclear TEAD4 signals were clearly observed in TE at least.

#### Effect of siRNA injection on *TEAD4* expression

Fig. 3 shows relative abundance of *TEAD4* in morulae that were uninjected, or that had been injected with control siRNA or injected with two specific TEAD4 siRNAs. The relative abundance of *TEAD4* in both TEAD4 siRNA-1- and TEAD4 siRNA-2-injected embryos was significantly ( $P < 0.05$ ) lower than that in uninjected and control



**Fig. 1.** Relative abundance (mean ± SEM) of *TEAD4* transcripts in porcine *in vitro* matured oocytes and 1-cell to blastocyst stages of embryos (n = 5). a, b, c, d Different superscripts indicate significant differences (P < 0.05).

siRNA-injected embryos. We selected *TEAD4* siRNA-1 for subsequent experiments.

The rate of *TEAD4* positive cells, which express *TEAD4* in nuclear, at the morula stage were calculated by immunofluorescence labeling (Fig. 4). It was difficult to detect nuclear *TEAD4* signals in the *TEAD4* siRNA-1-injected embryos compared with that in the uninjected and control siRNA-injected embryos. The rate of *TEAD4* positive cells in *TEAD4* siRNA-1-injected embryos (3.4%, n = 20) was significantly (P < 0.05) lower than that in the uninjected (19.8%, n = 20) and control siRNA-injected embryos (18.7%, n = 20). However, there were no differences in total cell numbers of these embryos among the experimental groups (uninjected embryos;  $19.2 \pm 1.6$ , control siRNA-injected embryos;  $19.8 \pm 1.6$ , *TEAD4* siRNA-1-injected embryos;  $14.6 \pm 1.2$ ).

#### Effect of *TEAD4* downregulation on the development of porcine embryos

We evaluated *in vitro* developmental competence in *TEAD4* siRNA-1-injected embryos (Table 2). No differences in development between experimental groups were observed until the morula stage on day 4. On day 5, the number of embryos developed to blastocyst stage in the *TEAD4* siRNA-1-injected embryos (0.4%) was significantly (P < 0.05) lower than that in the uninjected (24.5%)

and control siRNA-injected embryos (19.4%). Almost of all embryos obtained from the *TEAD4* siRNA injection could not developed to the blastocyst stage. In addition, we compared developmental rates between the *TEAD4* siRNA-2-injected and the control siRNA-injected group. The rate of development to blastocyst stage on day 5 in the *TEAD4* siRNA-2-injected embryos (2.7%) was significantly (P < 0.05) lower than that in the control siRNA-injected embryos (23.0%) although there were no differences in development until the morula stage on day 4.

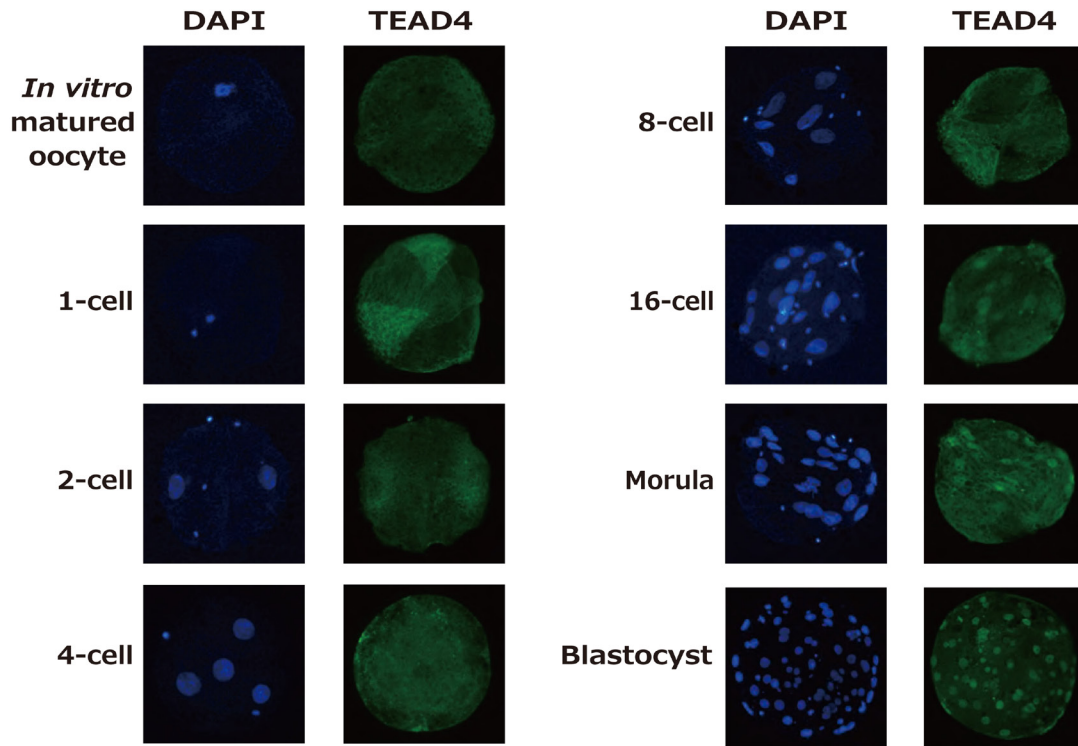
#### Relative expression levels of mRNA transcripts and protein expression in porcine embryos derived from *TEAD4* siRNA injection

To clarify the effects of *TEAD4* downregulation on gene expression involved in cell differentiation, *OCT-4* and *SOX2* transcript levels and protein expression in morulae were evaluated. There were no differences in *OCT-4* mRNA levels among the experimental groups (Fig. 5A). *TEAD4* downregulation also did not affect the rate of *OCT-4* positive cells (Fig. 5C, uninjected embryos; 90.1%, n = 10, control siRNA-injected embryos; 95.6%, n = 10, *TEAD4* siRNA-1-injected embryos; 88.7%, n = 10). The level of *SOX2* mRNA in *TEAD4* siRNA-1-injected embryos was significantly (P < 0.05) higher than that in the uninjected and control siRNA-injected embryos (Fig. 5B).

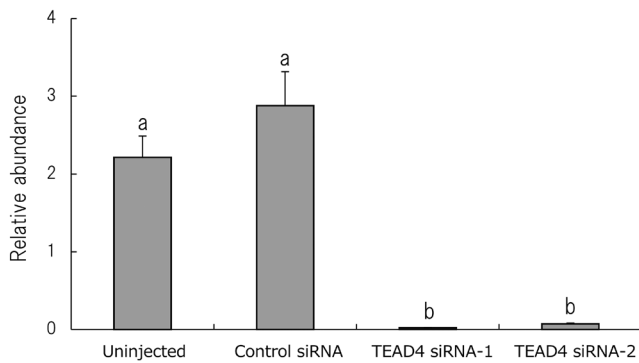
**Table 2.** Effect of *TEAD4* siRNA injection on *in vitro* development of porcine embryos \*

Treatment	Number of embryos cultured	No. (%) † of embryos developed to				
		Day 2	Day 3	Day 4		Day 5
		2-cell ≤	4-cell ≤	16-cell	Morula	Blastocyst
Uninjected	290	208 (71.7)	205 (70.7)	73 (25.2)	55 (19.0)	71 (24.5) <sup>a</sup>
Control siRNA	288	187 (64.9)	172 (59.7)	67 (23.3)	45 (15.6)	56 (19.4) <sup>a</sup>
<i>TEAD4</i> siRNA-1	282	175 (62.1)	161 (57.1)	68 (24.1)	46 (16.3)	1 (0.4) <sup>b</sup>

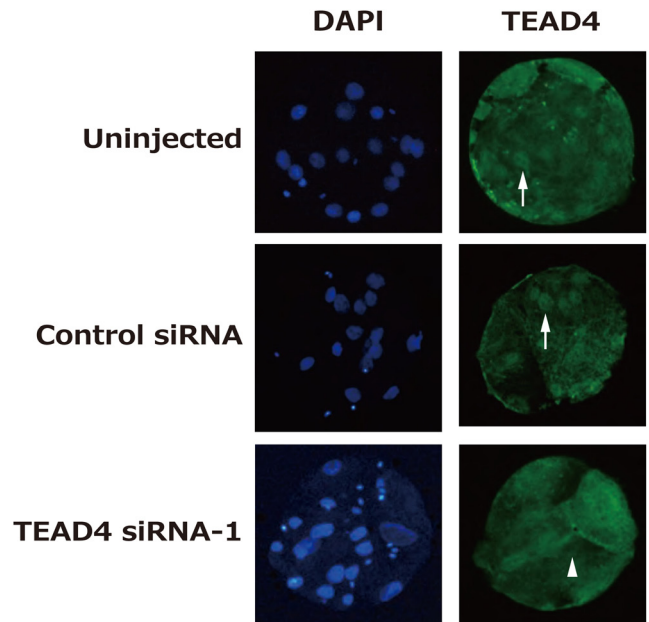
\* Experiments were replicated five times. † Percentages of the number of embryos cultured. <sup>a, b</sup> Values with different superscripts within each column differ significantly (P < 0.05).



**Fig. 2.** Representative photographs of TEAD4 protein expression in porcine *in vitro* matured oocytes and 1-cell to blastocyst stages of embryos. The oocyte and embryos are labeled for DAPI (blue) and TEAD4 (green).

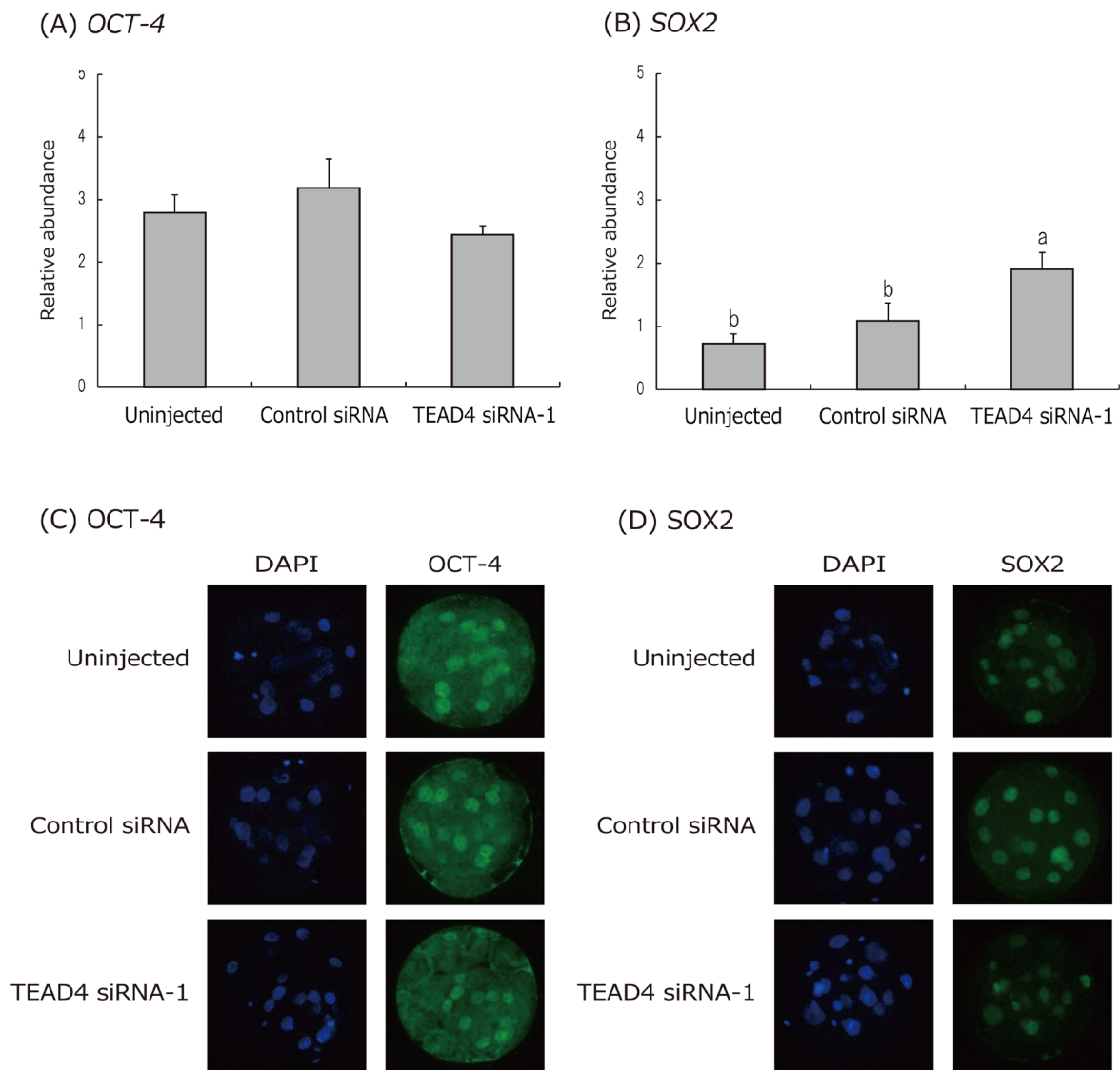


**Fig. 3.** Relative abundance (mean ± SEM) of *TEAD4* transcripts in porcine morula stage embryos obtained from Uninjected (n = 5), Control siRNA (n = 5) injection, TEAD4 siRNA-1 (n = 5) injection or TEAD4 siRNA-2 (n = 5) injection. <sup>a, b</sup> Different superscripts indicate a significant difference (P < 0.05).



**Fig. 4.** Representative photographs of TEAD4 protein expression in porcine morula stage embryos obtained from Uninjected, Control siRNA injection or TEAD4 siRNA-1 injection. Nuclear TEAD4 signals (indicated by arrows) were visible in Uninjected and Control siRNA-injected embryos. However, it was difficult to detect such signals in TEAD4 siRNA-1-injected embryos (shown by arrowhead). The embryos are labeled for DAPI (blue) and TEAD4 (green).

On the other hand, as shown in Fig. 5D, there were no differences in rates of SOX2 positive cells among the experimental groups (uninjected embryos; 95.8%, n = 10, control siRNA-injected embryos; 88.0%, n = 10, TEAD4 siRNA-1-injected embryos; 82.2%, n = 10).



**Fig. 5.** Relative abundance (mean ± SEM) of (A) *OCT-4* and (B) *SOX2* transcripts in porcine morula stage embryos obtained from Uninjected (n = 5), Control siRNA (n = 5) injection or TEAD4 siRNA-1 (n = 5) injection. <sup>a, b</sup> Different superscripts indicate a significant difference ( $P < 0.05$ ). Representative photographs of (C) *OCT-4* and (D) *SOX2* protein expressions in porcine morula stage embryos obtained from Uninjected, Control siRNA injection or TEAD4 siRNA-1 injection. The embryos are labeled for DAPI (blue) and *OCT-4* or *SOX2* (green).

## Discussion

*Tead4* controls TE segregation in murine embryos by promoting the expression of *Cdx2* [3–5]. In porcine embryos, *CDX2* is essential for development after blastocyst formation, such as hatching process, but *CDX2* downregulation has been shown not to affect blastocyst formation [11]. Thus, the role of TEAD4 in preimplantation development of porcine embryos is unclear. In the present study, we attempted to determine the necessity of TEAD4 for early development of porcine embryos by RNA interference techniques. In addition, expression of *OCT-4* and *SOX2* in *TEAD4*-downregulated embryos was evaluated. We recently showed that *OCT-4* is needed for blastocyst formation and

TE segregation in porcine embryos [18, 19, 32]. *Sox2* is involved in the maintenance of pluripotency and interacts with *Oct-4* in murine ESCs [21, 22, 25, 26]. These findings imply that TEAD4 plays a role in preimplantation development of porcine embryos through regulation of *OCT-4* and/or *SOX2* expression.

In murine embryos, *Tead4* transcription begins to be expressed at 2-cell stage, reaching a maximum between the 8-cell and the morula stages [4, 5]. In the present study, *TEAD4* mRNA expression in porcine oocytes and embryos was detected at all developmental stages. Furthermore, the level of *TEAD4* mRNA at the 8–16-cell stage was higher than at the other stages, as is the case with murine embryos. It has been reported that the level of *TEAD4* transcription in the ICM

region of porcine blastocyst is the same as in the TE region [14]. At the ovoid and filamentous stages, *TEAD4* expression levels in the TE region were higher than in the embryonic disc, which originates from the ICM [14]. On the other hand, nuclear signals of TEAD4 protein were detected beginning at the 16-cell stage, and TEAD4 signals were clearly observed in nuclei of TE at the blastocyst stage. TEAD4 is a transcriptional factor, and thus functions as a regulator of other genes in the cell nucleus. Therefore, TEAD4 may play a role in the development of porcine embryos from the 16-cell stage. Interestingly, in the present study, the downregulation of *TEAD4* expression in porcine embryos did not affect development up to the morula stage, but blastocyst formation was strongly inhibited. In experiments based on RNAi technology, it is well known that unexpected off-target effects can be induced. Thus, we designed another TEAD4 siRNA sequence and evaluated developmental competence of porcine embryos injected with the TEAD4 siRNA targeted another TEAD4 sequence. These embryos were also inhibited blastocyst development. These findings suggest that TEAD4 regulates the transition from the morula to the blastocyst through regulation of gene expression in porcine embryos.

In murine embryos, it is well known that *Tead4* regulates *Cdx2* expression, which is required for TE segregation [3–5]. However, *CDX2* mRNA expression in porcine morula stage embryos showed very late threshold cycles, and *TEAD4* downregulation had no influence on *CDX2* expression (data not shown). In porcine embryos, *CDX2* mRNA expression was increased at 16-cell stage embryos [11]. The cause of difference in expression levels of *CDX2* in porcine embryos is not clear. However, the expression level of *CDX2* in 16-cell stage embryos was significantly lower than that in the blastocyst embryos [11], and *CDX2* protein is also not detected in porcine 16-cell stage embryos [11]. Furthermore, *CDX2* is not necessary for blastocyst formation in porcine embryos [11]. Therefore, TEAD4 may control the blastocyst formation of porcine embryos by regulation of gene transcription other than *CDX2*. *Oct-4* is specifically expressed in ESCs and germ cells and is well known as a pluripotency factor [32–34]. Furthermore, *Oct-4* is important for the formation and maintenance of ICM, although it is dispensable for TE segregation in murine embryos [32]. On the other hand, our previous studies have demonstrated that direct *OCT-4* expression in the blastomere is essential for TE segregation in porcine embryos [18, 19]. Thus, we examined the expression of *OCT-4* to clarify the TEAD4 downstream factor. However, there was no change in *OCT-4* expression and localization when *TEAD4* was downregulated. This result suggests that TEAD4 and *OCT-4* may regulate TE segregation independently.

*Sox2* forms a complex with *Oct-4*, and this complex is required for the expression of pluripotency genes in ESCs [24–26]. *Sox2* is expressed in both ICM and TE in mice [35]. *Tead4*-deficient murine embryos express high levels of *Sox2* [6]. Home and coworkers [3] have also reported that the *Tead4* binding region is included in the binding motif of *Sox2*. Taken together, it is possible that *Tead4* directly suppresses *Sox2* expression in murine embryos. *SOX2* is located only in the ICM at the blastocyst stage in bovine and porcine embryos [23, 36]. Although downstream factors of TEAD4 is still unknown in porcine embryos, in the present study, *SOX2* expression was upregulated in TEAD4 siRNA-injected embryos, implying that there is a similar mechanism of *SOX2* repression by TEAD4 in

porcine embryos. However, in the present study, *SOX2* expression was detected in almost of all blastomeres in morula stage porcine embryos, thus the number of *SOX2* positive cells was not changed by *TEAD4* downregulation. It is possible that in porcine embryos, TEAD4 regulates expressions of other genes in addition to *SOX2*. In bovine embryos, blastocyst formation and TE expansion are not affected by *TEAD4* downregulation [37]. Furthermore, *TEAD4* downregulation in bovine embryos has no influence on the ICM-related genes, such as *OCT-4* and *NANOG* [37]. It is possible that necessity of TEAD4 for preimplantation development differs between animal species.

The Hippo pathway regulates nuclear localization of Yap, which is a transcriptional coactivator of Tead family genes [7, 38, 39]. When the Hippo pathway is active, Yap is phosphorylated by Lats and nuclear accumulation is prevented [38]. Thus, Yap does not play the role of transcriptional factor. In contrast, Yap localizes in the nucleus and forms a complex with Tead family transcriptional factors to regulate gene expression when the pathway is inactive [38]. In murine embryos, nuclear Yap1 expression is observed only in outer cells at the morula and the blastocyst stages [7, 40, 41], and it is known that the complex of Yap1 and Tead4 in the nucleus is important for the segregation of TE lineage. In porcine embryos, we have observed nuclear expression of YAP1 at the morula and the blastocyst stages (data not shown). In addition, our study indicated that TEAD4 regulates preimplantation development. Therefore, it is possible that the Hippo pathway controls TEAD4 function in porcine embryos. However, knowledge regarding the relationship between the Hippo pathway and TEAD4 in porcine embryos is limited, and further studies are needed to elucidate the molecular mechanism of TEAD4 function.

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