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Journal

The Journal of Reproduction and Development, 63(2)

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Publication Date

2017-04-21

DOI

10.1262/jrd.2016-130

Peer reviewed

Effects of downregulating TEAD4 transcripts by RNA interference on early development of bovine embryos

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Abstract. Transcription factor TEA domain family transcription factor 4 (Tead4) is one of the key factors involved in the differentiation of the trophectoderm (TE) in murine embryos. However, knowledge on the roles of TEAD4 in preimplantation development during bovine embryos is currently limited. This study examined the transcript and protein expression patterns of TEAD4 and attempted to elucidate the functions of TEAD4 during bovine preimplantation development using RNA interference. *TEAD4* mRNA was found to be upregulated between the 16-cell and morula stages, and nuclear localization of the TEAD4 protein was detected at the morula stage, as well as in subsequent developmental stages. TEAD4 downregulation did not affect embryonic development until the blastocyst stage, and TEAD4-downregulated embryos were capable of forming the TE under both 5% and 21% O₂ conditions. Results of gene expression analysis showed that TEAD4 downregulation did not affect the expression levels of POU class 5 transcription factor 1 (*OCT-4*), *NANOG*, caudal-type homeobox 2 (*CDX2*), GATA binding protein 3 (*GATA3*), and interferon-tau (*IFNT*). In conclusion, TEAD4 might be dispensable for development until the blastocyst stage and TE differentiation in bovine embryos.

Key words: Bovine embryo, Preimplantation development, RNA interference, TEAD4

(J. Reprod. Dev. 63: 135–142, 2017)

The earliest differentiation event during mammalian embryonic development occurs between the morula and blastocyst stages. In this process, the outer cells of the morula differentiate into the trophectoderm (TE), a single layer of polarized epithelial cells surrounding the blastocoel that subsequently develops into the placenta. The inner cells of a morula differentiate into the inner cell mass (ICM), a population of pluripotent cells attached to the inside of the TE that give rise to the fetus and extra-embryonic tissues [1]. Successful segregation between ICM and TE and correct embryonic development depend on different transcriptional programs that comprise interaction among multiple genes. In mice, gene knockout studies showed that TEA domain family transcription factor 4 (Tead4) is essential for TE development and acts as a master regulator of the TE-specific transcriptional program [2, 3]. *Tead4*-deficient embryos fail to develop the TE and lack the expression of TE-specific factors such as caudal-type homeobox 2 (Cdx2) and GATA binding protein 3 (Gata3) [2–5]. Tead4 expression is detectable in both the ICM and TE lineages [2]. However, Tead4 upregulates TE-specific genes only in the outer cells [6]. In the inner cells, Hippo signaling modulates Tead4 function by preventing nuclear accumulation of a Tead4 coactivator, Yes-associated protein (Yap). In contrast, in the outer

cells, Yap can accumulate in the nucleus and form a complex with Tead4 to activate TE-specific genes [7]. A recent study showed that *Tead4*-null embryos can form a blastocoel and express Cdx2 and Gata3 under low oxidative stress conditions [8]. The authors concluded that Tead4 is involved in maintaining energy homeostasis, which dramatically changes between the morula and blastocyst stages in murine embryos [8].

We recently performed CDX2 downregulation in bovine embryos and reported that CDX2 functions in TE development and in regulating the molecular mechanism of TE [9]. In addition, CDX2 regulates the expression of interferon-tau (IFNT), which is secreted by TE lineage cells and acts as a pregnancy recognition factor [10]. TEAD4 is also expected to control TE differentiation in bovine embryos, but little is known about the roles of TEAD4 during embryonic development and the differentiation of ICM and TE. We previously performed mRNA expression analysis of *TEAD4* at the blastocyst and elongation stages, and results showed that *TEAD4* is expressed both in the ICM and TE [11]. Another study reported that nuclear localization of TEAD4 is observed only in the TE and that TEAD4 protein expression occurs only in the cytoplasm in ICM cells [12]. However, limited information is available on the temporal and spatial expression patterns of TEAD4 in bovine embryos, particularly at the developmental stages prior to blastocyst formation.

In the present study, we characterized the mRNA and protein expression patterns of *TEAD4* in bovine embryos until the hatched blastocyst stage. In addition, we performed TEAD4 knockdown and investigated its effects on the developmental competence and expression of genes speculated to be related in the differentiation of ICM and TE. The present study aims to investigate the roles of

Received: August 30, 2016

Accepted: November 25, 2016

Published online in J-STAGE: December 11, 2016

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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>TEAD4</i>	F- AAGTTCTGGGCAGACCTCAA R- GTGCTTCAGCTTGTGGATGA	60	249	XM_010827947.1
<i>CDX2</i>	F- GCCACCATGTACGTGAGCTACC R- ACATGGTATCCGCCGTAGTCCGG	55	140	NM_001206299.1
<i>GATA3</i>	F- ATGAAACCGAAACCCGATGG R- TTCACAGCACTAGAGAGACC	60	185	NM_001076804
<i>Oct-4</i>	F- GGTTCCTTTGGAAAGGTGTTC R- ACACTCGGACCACGTCTTTC	52	314	AF022987.1
<i>NANOG</i>	F- AATTCACAGCAGCAAATCAC R- CCCTTCCCTCAAATTGACAC	55	215	DQ069776
<i>IFNT</i>	F- GCAGTGCTTCAACCTCTTCC R- TCCTTCCCATGTCAGAGTCC	62	155	AF238611.1
<i>Histone H2A</i>	F- AGGACGACTAGCCATGGACGTGTG R- CCACCACCAGCAATTGTAGCCTTG	60	209	NM_174809
TEAD4 siRNA	S- GCUACGAGAAUGGCCACUATT AS- UAGUGGCCAUUCUCGUAGCTT	N/A N/A	N/A N/A	N/A N/A

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

TEAD4 during the early development of bovine embryos.

Materials and Methods

Chemicals

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

In vitro fertilization (IVF) and embryo culture

Bovine oocytes were obtained by aspirating ovaries collected at a local slaughterhouse, as previously described [11]. Cumulus–oocyte complexes (COCs) were matured in a drop of IVMD-101 medium (Research Institution for the Functional Peptides, Yamagata, Japan) [13] and covered with mineral oil at 39°C in a humidified atmosphere of 5% CO₂ in air for 20 h. After *in vitro* maturation, COCs were transferred to a drop of IVF-100 medium (Research Institution for the Functional Peptides). Subsequently, frozen-thawed semen was centrifuged twice at 600 × g for 5 min in IVF-100 medium, and the spermatozoa were added to the COCs at a final concentration of 5 × 10⁶ sperm per milliliter. COCs and spermatozoa were incubated at 39°C in a humidified atmosphere of 5% CO₂ in air for 6 h. After insemination, cumulus cells and excess spermatozoa were removed from presumptive zygotes by pipetting. Following microinjection of short interfering RNA (siRNA), the embryos were incubated in modified TALP (mTALP) medium [14] containing 1 mg/ml bovine serum albumin (BSA) at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂, or of 5% CO₂ in air. On Day 2 (IVF = Day 0), the embryos were transferred to mTALP medium supplemented with 3% (v/v) newborn calf serum (Thermo Fisher Scientific, Waltham, MA, USA) and cultured at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂, or of 5% CO₂ in air until Day 8. Embryonic developmental rates were assessed on Day 2 (2-cell ≤), Day 4 (16-cell ≤), Day 5 (32-cell ≤), Day 6 (morula ≤), Day 7

(blastocyst ≤), and Day 8 (blastocyst ≤ and hatching blastocyst ≤).

Design of siRNA and microinjection into embryos

The target site for *TEAD4* was selected from the bovine sequence (GenBank accession no. XM_010827947.1). Specific siRNA was designed using the online software BLOCK-iT™ RNAi Designer (Thermo Fisher Scientific; <http://rnaidesigner.thermofisher.com/rnaexpress/>). Both sense and antisense RNA sequences for siRNA were commercially synthesized (Table 1). After insemination, the denuded embryos were transferred to a drop of mTALP medium containing 1 mg/ml BSA for microinjection. Approximately 10 pl of 50 μM specific siRNA duplex was injected into the cytoplasm of each embryo using a FemtoJet (Eppendorf, Hamburg, Germany). Similarly, approximately 10 pl of 20 μM nonsilencing siRNA (AllStars Negative Control siRNA; Qiagen, Hilden, Germany) was injected as a control. The embryos were washed after microinjection and cultured as described above.

Determination of the relative abundance of gene transcripts

Total RNA was isolated from denuded metaphase II oocytes (confirmed based on the presence of one polar body), 1-cell, 2-cell, 4-cell, 8-cell, 16-cell, morula, blastocyst, expanded blastocyst, and hatched blastocyst-stage embryos. Oocytes and embryos at the appropriate developmental stage were treated with 0.1% (w/v) protease in phosphate-buffered saline (PBS) containing 1% (w/v) polyvinyl pyrrolidone for 5 min to remove the zona pellucida. Pools of 20 oocytes, 10 embryos from the 1-cell to the 8-cell stage, or 5 embryos from the other stages were added to 5 μl of lysis buffer [0.8% (v/v) Igepal (MP Biomedicals, LLC, Santa Ana, CA, USA), 5 mM dithiothreitol (Thermo Fisher Scientific) and 1 U/μl RNasin (Promega, Madison, WI, USA)], snap-frozen in liquid nitrogen, and subsequently stored at –80°C. For validation of siRNA, one expanded blastocyst per sample was collected on Day 7, and RNA

was extracted using the same method. RNA samples were heated at 80°C for 5 min and then subjected to reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The reaction mixture was then diluted with RNase free water to obtain a final volume of 31 μ l. Real-time quantitative PCR was performed using StepOne™ (Thermo Fisher Scientific), and products were detected using SYBR Green included in the QuantiTect SYBR Green PCR Master Mix Kit (Qiagen). The amplification program was as follows: preincubation at 95°C for 15 min to activate the HotStarTaq DNA Polymerase (Qiagen), followed by 45 cycles of denaturation at 94°C for 15 sec, primer-specific annealing (Table 1) for 30 sec, and elongation at 72°C for 30 sec. After completion of the last cycle, a melting curve was generated by starting fluorescence acquisition at 60°C and recording measurements in 0.3°C increments up to 95°C. A final quantification step was performed using StepOne™ quantification software (Thermo Fisher Scientific). Expression levels of the target gene in each run were normalized to the internal standard *Histone H2A*.

Immunofluorescent staining and assessment of cell numbers

Embryos at the appropriate developmental stages 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 for 10 min each in PBS containing 0.1% (v/v) Triton X-100 (TXPBS). Samples were subsequently permeabilized in 0.3% (v/v) Triton X-100 in PBS for 60 min, and washed twice in TXPBS for 10 min. Blocking was performed by incubation in 0.5% (w/v) BSA and 1% (w/v) skimmed milk in TXPBS for 90 min. Embryos were incubated with anti-TEAD4 primary antibody (1:1500; ab58310; Abcam, Cambridge, UK) in PBS supplemented with 0.5% (w/v) BSA and 0.05% (v/v) Triton X-100 at room temperature for 2 h, or with anti-CDX2 primary antibody (1:300; MU392A-UC; BioGenex, Fremont, CA, USA) in PBS supplemented with 0.5% (w/v) BSA and 0.05% (v/v) Triton X-100 at 37°C overnight. Next, the embryos were washed four times for 15 min each in TXPBS. The embryos were then incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (A-11029, Thermo Fisher Scientific) 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. The embryos were washed four times in TXPBS for 20 min and subsequently 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 a digital camera system (ECLIPSE Ti-U and DS-Fi2-L3; Nikon, Tokyo, Japan). Cell counting was performed using ImageJ (<https://imagej.nih.gov/ij/>).

Statistical analysis

Percentage data for embryonic development were subjected to an arcsine transformation. Transformed values were analyzed using one-way analysis of variance (ANOVA). Data for temporal *TEAD4* gene expression and the mRNA expression levels of *TEAD4* at the morula stage and *IFNT* were analyzed using the Kruskal-Wallis test, followed by multiple pairwise comparisons using Scheffé's method. The mRNA expression levels of *TEAD4* at the expanded blastocyst stage and of *CDX2*, *GATA3*, POU class 5 transcription

factor 1 (*OCT-4*), and *NANOG* were analyzed using one-way ANOVA followed by multiple pairwise comparisons using the Tukey-Kramer method. A P value < 0.05 was considered statistically significant.

Results

Temporal and spatial expression of TEAD4 mRNA and protein in early embryos

Metaphase II stage oocytes and *in vitro*-fertilized embryos were collected at different developmental stages from the 1-cell stage to hatched blastocyst, and analyzed expression levels of *TEAD4* mRNA and the expression pattern of TEAD4 protein using real-time quantitative PCR and immunofluorescent staining, respectively (Fig. 1). *TEAD4* mRNA showed very late threshold cycles until the 8-cell stage, indicating low starting quantities (Fig. 1A, data from the metaphase II stage to the 4-cell stage not shown). *TEAD4* expression slightly increased ($P = 0.09$) from the 8-cell to the 16-cell stage and exhibited approximately fourfold upregulation from the 16-cell to the morula stage. After the morula stage, *TEAD4* was consistently and strongly expressed until the hatched blastocyst stage.

To characterize TEAD4 protein expression dynamics, we stained bovine embryos from the 8-cell stage to the hatched blastocyst stage, using TEAD4-specific antibody (Fig. 1B). A diffuse TEAD4 signal was consistently observed in the cytoplasm until the 16-cell stage. A nuclear TEAD4 signal was detected in the morula stage. After the blastocyst stage, strong nuclear TEAD4 signals were observed in almost all blastomeres.

Effect of siRNA injection on TEAD4 expression

To determine the efficacy of TEAD4 downregulation using RNA interference, we examined the mRNA and protein expression levels of *TEAD4* in uninjected, control siRNA-injected, and *TEAD4*-specific siRNA (*TEAD4* siRNA)-injected morula-stage embryos on Day 5 (Fig. 2). The relative abundance of *TEAD4* mRNA in the embryos injected with *TEAD4* siRNA was significantly lower ($P < 0.01$) than that in uninjected and control siRNA-injected embryos (Fig. 2A). Protein expression of TEAD4 was evaluated via immunofluorescent staining. Nuclear signals from *TEAD4* siRNA-injected embryos were difficult to detect at the morula stage (Fig. 2B).

Effect of TEAD4 downregulation on the development of bovine embryos

We evaluated the *in vitro* developmental competence of *TEAD4* siRNA-injected embryos (Table 2). There was no significant difference in the rate of development during all the developmental stages until Day 8 among the experimental groups. The embryos with *TEAD4* downregulation could form blastocoels, expand themselves, and hatch from the zona pellucida. To determine whether siRNA injection can maintain TEAD4 downregulation beyond the morula stage on Day 5, we evaluated *TEAD4* expression at the expanded blastocyst stage on Day 7 (Fig. 3). The relative abundance of *TEAD4* mRNA in the embryos injected with *TEAD4* siRNA was significantly lower ($P < 0.01$) than that in the uninjected and control siRNA-injected embryos (Fig. 3A). Although TEAD4 signals were observed in some nuclei of *TEAD4* siRNA-injected embryos, the signals were weaker compared to those in uninjected and control siRNA-injected embryos

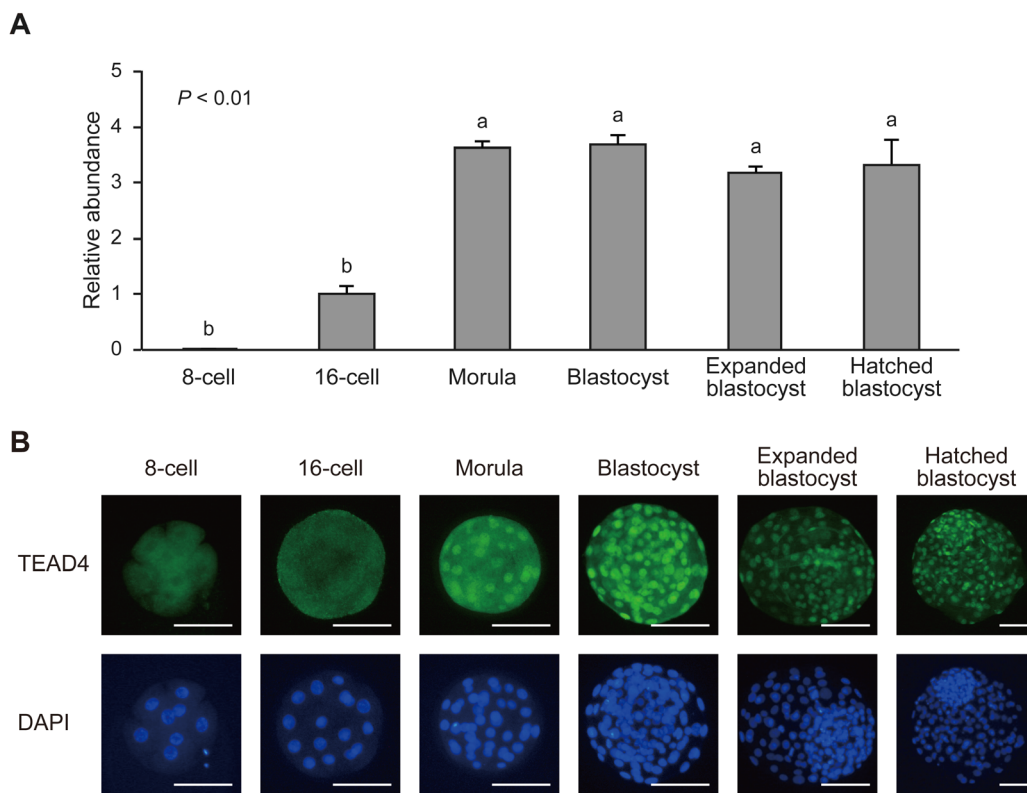


Fig. 1. *TEAD4* mRNA and protein profiles during the bovine preimplantation development. (A) Relative abundance (mean ± SEM; n = 6) of *TEAD4* transcripts in 8-cell, 16-cell, morula, blastocyst, expanded blastocyst, and hatched blastocyst. ^{a, b} Different characters indicate a significant difference ($P < 0.01$). (B) Representative images of *TEAD4* protein expression. Embryos were labeled with *TEAD4* antibody (green) and DAPI for DNA (blue). Scale bars, 100 μm .

(Fig. 3B). Therefore, we concluded that the blastocyst formation could have given rise under the *TEAD4*-downregulated condition in bovine embryos.

Next, to evaluate the effect of *TEAD4* downregulation on cell composition of ICM and TE cell numbers, we performed immunostaining against *CDX2*, which is exclusively expressed in TE cells, and subsequently counted total cell number and *CDX2*-positive cell number. ICM cell number was calculated by subtracting the counts of *CDX2*-positive cells from the total cell count. However, there was no significant difference in ICM/TE ratios among uninjected (0.43, n = 24), control siRNA-injected (0.37, n = 20), and *TEAD4* siRNA-injected embryos (0.40, n = 21).

Quantitative PCR gene expression analysis

To clarify the effect of *TEAD4* downregulation on gene expression, we evaluated the mRNA levels of *CDX2*, *GATA3*, *OCT-4*, and *NANOG* at the morula stage (Fig. 4) and of *CDX2*, *GATA3*, *OCT-4*, and *IFNT* at the expanded blastocyst stage (Fig. 5). There was no significant difference in all transcript levels of the genes among the experimental groups at the morula stage on Day 5. At the expanded blastocyst stage on Day 7, the relative abundances of *CDX2*, *GATA3*, *OCT-4*, and *IFNT* in *TEAD4*-downregulated embryos remained unchanged compared to the uninjected and control siRNA-injected

embryos. Although *CDX2* mRNA level in the control siRNA-injected embryos was significantly lower ($P < 0.05$) than that in the uninjected embryos, no significant difference was observed between control siRNA-injected embryos and *TEAD4* siRNA-injected embryos.

Developmental rates under the high O_2 condition

Tead4-deficient murine embryos successfully formed the TE, that is, form the blastocyst cavity under the low O_2 (5%) cultural condition. However, the embryos failed to develop to blastocyst in atmospheric O_2 (21%) [8]. For the cultural condition in cows, embryos are generally cultured at 5% O_2 . In the present study, *TEAD4*-downregulated embryos that were able to form the blastocyst cavity were cultured at 5% O_2 . Therefore, we evaluated the developmental competence of *TEAD4*-downregulated embryos cultured in 21% O_2 (Table 3). However, no significant difference in the rate of development during all the developmental stages was observed among the experimental groups.

Discussion

In the present study, we analyzed *TEAD4* expression patterns during early embryonic development. To elucidate the mechanisms underlying ICM/TE differentiation, we downregulated *TEAD4* expression in

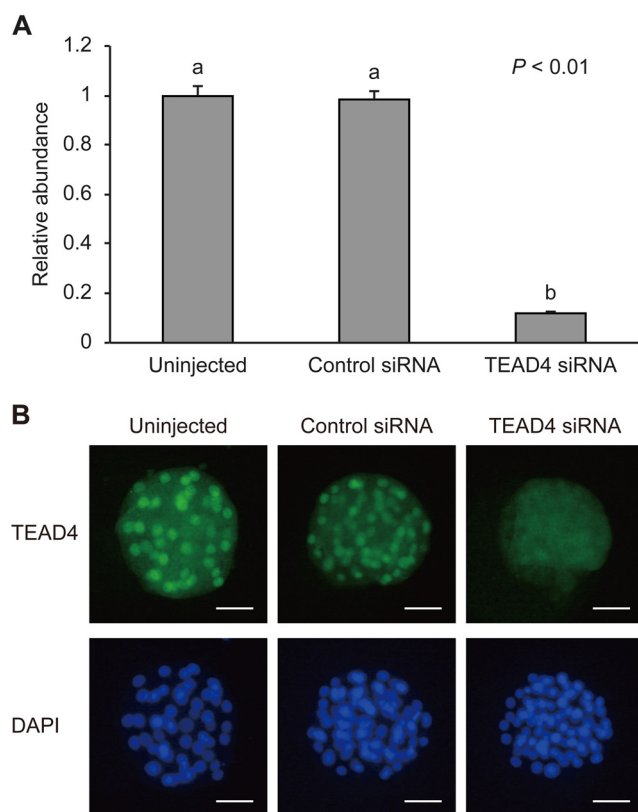


Fig. 2. Validation of TEAD4 siRNA at the morula stage on Day 5. (A) Relative abundance (mean ± SEM) of *TEAD4* transcripts in uninjected ($n = 6$), control siRNA-injected ($n = 5$), or TEAD4 siRNA-injected embryos ($n = 6$). ^{a, b} Different characters indicate a significant difference ($P < 0.01$). (B) Representative images of TEAD4 immunofluorescent staining of morula-stage uninjected, control siRNA-injected, or TEAD4 siRNA-injected embryos. Embryos were labeled with TEAD4 antibody (green) and DAPI for DNA (blue). Scale bars, 50 μm .

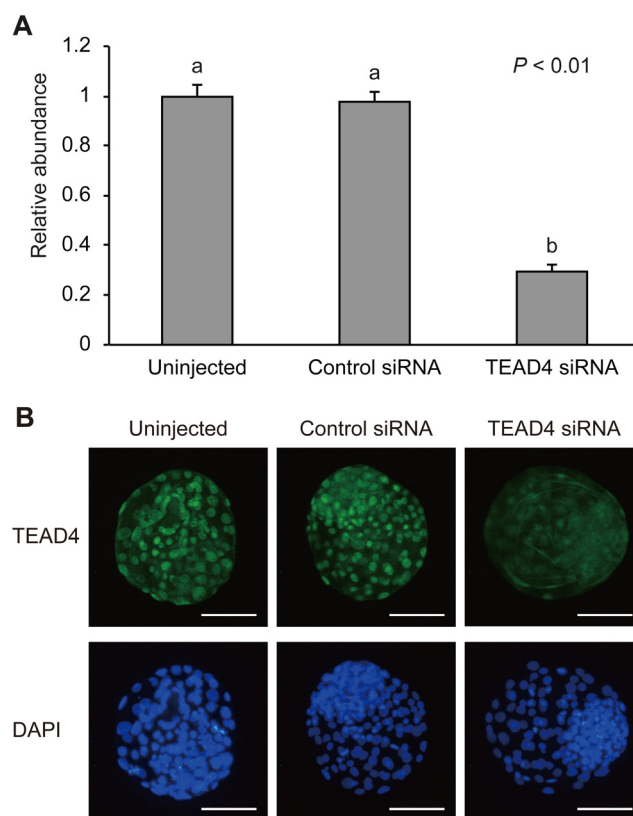


Fig. 3. Validation of TEAD4 siRNA at the expanded blastocyst stage on Day 7. (A) Relative abundance (mean ± SEM; $n = 12$) of *TEAD4* transcripts in uninjected, control siRNA-injected, or TEAD4 siRNA-injected embryos. ^{a, b} Different characters indicate a significant difference ($P < 0.01$). (B) Representative images of TEAD4 immunofluorescent staining of expanded blastocyst-stage uninjected, control siRNA-injected, or TEAD4 siRNA-injected embryos. Embryos were labeled with TEAD4 antibody (green) and DAPI for DNA (blue). Scale bars, 100 μm .

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

Treatment	Number of embryos cultured	No. (%) [†] of embryos developed to						
		Day 2	Day 4	Day 5	Day 6	Day 7	Day 8	
		2-cell ≤	16-cell ≤	32-cell ≤	Morula ≤	Blastocyst ≤	Blastocyst ≤	Hatching blastocyst ≤
Uninjected	230	196 (85.2)	153 (66.5)	138 (60.0)	135 (58.7)	137 (59.6)	134 (58.3)	63 (27.4)
Control siRNA	248	213 (85.9)	159 (64.1)	140 (56.5)	138 (55.6)	137 (55.2)	135 (54.4)	59 (23.8)
TEAD4 siRNA	241	192 (79.7)	143 (59.3)	122 (50.6)	115 (47.7)	114 (47.3)	124 (51.5)	50 (20.7)

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

bovine embryos using RNA interference. We attempted to analyze *TEAD4* mRNA expression dynamics using real-time PCR and observed very late threshold cycles until the 8-cell stage. However, *Histone H2A* expression, which was used as the internal standard, also showed very late threshold cycles until the 4-cell stage. Although the results obtained until the 4-cell stage were insufficient to evaluate *TEAD4* mRNA dynamics during the earlier developmental stages, results of

this study showed that *TEAD4* transcripts are weakly expressed at the 8-cell and 16-cell stages but become upregulated starting from the morula stage. In the present study, nuclear TEAD4 expression was detected beginning at the morula stage and was observed in almost all cells, including the ICM, in blastocoel-formed embryos. In murine embryos, *Tead4* mRNA was not detected in oocytes and 1-cell-stage embryos [3]. *Tead4* is expressed prior to *Cdx2*, which

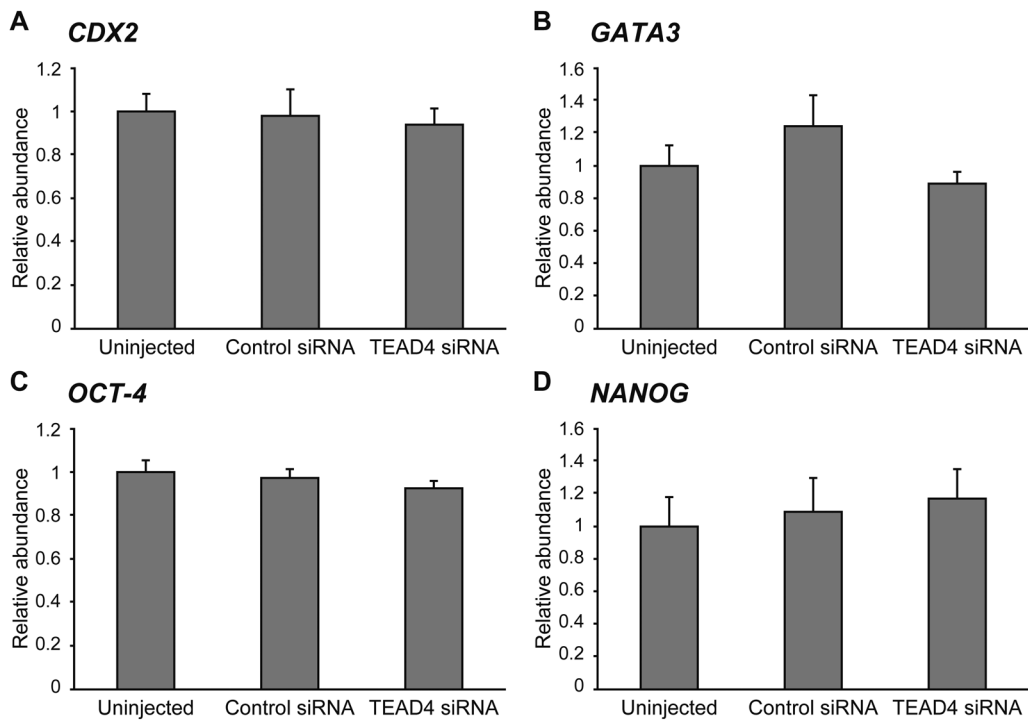


Fig. 4. Relative abundance (mean ± SEM) of (A) *CDX2*, (B) *GATA3*, (C) *OCT-4*, and (D) *NANOG* transcripts at the morula stage in uninjected (n = 6), control siRNA-injected (n = 5), and TEAD4 siRNA-injected embryos (n = 6).

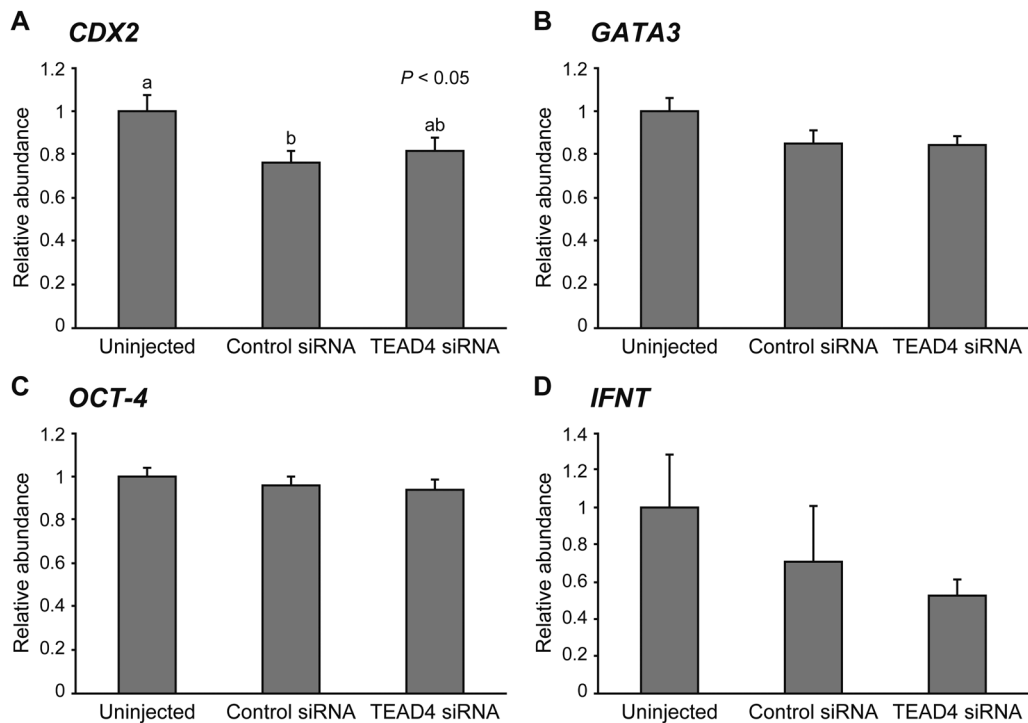


Fig. 5. Relative abundance (mean ± SEM) of (A) *CDX2*, (B) *GATA3*, (C) *OCT-4*, and (D) *IFNT* transcripts at the expanded blastocyst stage in uninjected (n = 12), control siRNA-injected (n = 12), and TEAD4 siRNA-injected embryos (n = 12). ^{a, b} Different characters indicate a significant difference ($P < 0.05$).

Table 3. Effect of TEAD4 siRNA injection on *in vitro* development under 21% O₂ conditions*

Treatment	Number of embryos cultured	No. (%) [†] of embryos developed to						
		Day 2	Day 4	Day 5	Day 6	Day 7	Day 8	
		2-cell ≤	16-cell ≤	32-cell ≤	Morula ≤	Blastocyst ≤	Blastocyst ≤	Hatching blastocyst ≤
Uninjected	228	198 (86.8)	149 (65.4)	106 (46.5)	99 (43.4)	93 (40.8)	84 (36.8)	12 (5.3)
Control siRNA	212	179 (84.4)	131 (61.8)	106 (50.0)	99 (46.7)	102 (48.1)	94 (44.3)	5 (2.4)
TEAD4 siRNA	217	196 (90.3)	143 (65.9)	117 (53.9)	104 (47.9)	88 (40.6)	93 (42.9)	9 (4.1)

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

is expressed from the 8-cell stage and is regulated by Tead4. *Tead4* expression begins at the 2-cell stage and continues until the 4-cell stage, after which expression levels reach a peak between the 8-cell and morula stages [2, 3]. In bovine embryos, *TEAD4* mRNA and protein expression drastically changed before and after the morula stage and resembled the corresponding expression dynamics observed in murine embryos.

There are two conflicting reports regarding the distribution of Tead4 protein at the morula and blastocyst stages in mice. One report demonstrated that although Tead4 is expressed in all cells, Tead4 cannot induce *Cdx2* expression in the inner cells. This is because the role of Tead4 as a transcription factor depends on the existence of its coactivator, Yap, in the nucleus [6]. In the inner cells of a morula and the ICM, the transition of Yap into the nucleus is blocked by position-dependent Hippo/Lats signaling, and Tead4 does not regulate the expression of TE-related genes at this location. The other report proposed that Tead4 is not localized in a nucleus of the inner cells of a morula and the ICM and that the TE-specific transcriptional program is not activated in the inner cells [12]. Similarly, Home and coworkers [12] performed immunofluorescent staining for TEAD4 in bovine blastocysts and showed that nuclei of ICM lacked the protein expression of TEAD4. However, in the present study, we observed a TEAD4 nuclear signal in the ICM and thus do not agree with the results of the previous study. Although the exact reasons for the contrasting results are unclear, one plausible reason might be due to the process during immunofluorescent staining. In fact, other groups have demonstrated that nuclear localization of Tead4 in the murine ICM could be detected using the anti-Tead4 antibody that was used in the study by Home and coworkers [12] and in the present study [15, 16].

Tead4 is known as one of the most crucial factors for TE differentiation. Besides Tead4, genetic evidence suggests that *Cdx2* and *Gata3* also have regulatory roles in TE differentiation. However, loss of Tead4 leads to more severe phenotypes than loss of *Cdx2* and *Gata3* [17, 18] and results in arrested embryonic development at the morula stage [2, 3]. Therefore, Tead4 is thought to be an upstream regulator of TE differentiation. In bovine embryos, *CDX2* downregulation does not prevent blastocyst formation but causes aberrations in expansion, proliferation, and elongation of the TE lineage [9, 19, 20]. If TEAD4 plays a role in regulating the expression of TE-specific genes in bovine embryos, TEAD4 downregulation is expected to result in more serious defects compared to *CDX2* downregulation. However, in the present study, TEAD4-downregulated embryos correctly formed blastocoels and showed normal TE expansion. Furthermore, TEAD4

downregulation had no influence not only on the ICM-related genes, *OCT-4* and *NANOG*, but also on the TE-related genes, *CDX2*, *GATA3*, and *IFNT*. *CDX2* expression level in control siRNA-injected embryos was significantly lower than that in uninjected embryos. However, in our previous study [9], injection of control siRNA did not result in *CDX2* downregulation. Although the reason for the above result remains unclear, we suspect that injection of control siRNA will not downregulate *CDX2* expression. These results suggest that in contrast to murine embryos, TEAD4 might be not an indispensable factor for the formation and differentiation of TE in bovine embryos.

Kaneko and DePamphilis [8] reported that *Tead4* deficient murine embryos are capable of forming blastocyst when cultured under low oxidative stress conditions. They also proposed that Tead4 is required to prevent oxidative stress during early embryonic development. Glucose and O₂ consumption are increased in murine embryos between the morula and blastocyst stages [21–23], implying that generation of mitochondrial reactive oxygen species (ROS) is also sharply increased [24]. Tead4 is involved in preventing excess accumulation of ROS in murine embryos [8]. In bovine embryos, the similar patterns of glucose and O₂ consumption have been reported [25–27]. In general, 5% O₂ is adopted as a standard culture condition for bovine embryos to protect them from oxidative stress [28]. Although TEAD4-downregulated embryos were able to develop to the blastocyst stage under a low O₂ condition, it is possible that problems that occur in TEAD4-downregulated embryos were rescued by low O₂ tension. We therefore observed the developmental capacity of TEAD4-downregulated embryos under the atmospheric oxygen condition. However, there was no significant effect of TEAD4 downregulation on embryonic development, which implies that TEAD4 might not exert protective effect from oxidative stress in bovine embryos. However, knowledge regarding the relationship between TEAD4 and energy homeostasis in mammalian embryos is limited, and further studies are required to investigate this relationship.

In the present study, we demonstrated that TEAD4 might be dispensable for embryonic development and TE differentiation in bovine embryos. Results suggest that mechanisms regulating embryonic development until the blastocyst stage and differentiation mechanisms of ICM and TE vary among species. TE differentiation in bovine embryos remains incomplete at the blastocyst stage, but is completed at the elongation stage [19]. Although we did not determine the exact function of TEAD4 until the blastocyst stage, investigating the role of TEAD4 from the blastocyst to elongation stage is necessary. In the present study, we downregulated TEAD4 expression using RNA interference. However, this method does not

completely eliminate the expression of the target gene. Therefore, the possibility that the remaining TEAD4 expression was sufficient to maintain the normal development and differentiation cannot be ruled out. Further studies are required to elucidate functions of TEAD4 in bovine embryos.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 26292162.

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