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Reciprocal regulation of TEAD4 and CCN2 for the trophectoderm development of the bovine blastocyst

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Abstract

The first segregation at the blastocyst stage is the symmetry-breaking event to characterize two cell components; namely, inner cell mass (ICM) and trophectoderm (TE). TEA domain transcription factor 4 (TEAD4) is a well-known regulator to determine TE properties of blastomeres in rodent models. However, the roles of bovine TEAD4 in blastocyst development have been unclear. We here aimed to clarify the mechanisms underlining TE characterization by TEAD4 in bovine blastocysts. We first found that the *TEAD4* mRNA expression level was greater in TE than in ICM, which was further supported by TEAD4 immunofluorescent staining. Subsequently, we examined the expression patterns of TE-expressed genes; *CDX2*, *GATA2* and *CCN2*, in the *TEAD4*-knockdown (KD) blastocysts. These expression levels significantly decreased in the *TEAD4* KD blastocysts compared with controls. Of these downregulated genes, the *CCN2* expression level decreased the most. We further analyzed the expression levels of TE-expressed genes; *CDX2*, *GATA2* and *TEAD4* in the *CCN2* KD blastocysts. Strikingly, the *CCN2* KD blastocysts showed the downregulation of *CDX2*, *GATA2* and *TEAD4*. Furthermore, the ratio of TE-to-ICM cell numbers in the *CCN2* KD blastocysts significantly decreased compared to controls. To our knowledge, this is the first study showing the regulation of *CCN2* expression thorough *TEAD4* in mammalian embryos. Not only that, this study also provides evidence that reciprocal regulation of *TEAD4* and *CCN2* is required for TE development with appropriate gene expression in bovine blastocysts.

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Introduction

The blastocyst stage is a crucial period for mammalian preimplantation development because the first two lineages, namely the inner cell mass (ICM) and trophectoderm (TE), are distinctly established by this stage (Arnold & Robertson 2009). These cell lineages are fundamental for proper embryo and extraembryonic tissue formation (Arnold & Robertson 2009). As TE cells are derived from pluripotent undifferentiated blastomeres under the regulation of signal transduction pathways controlled strictly, the perturbation of these pathways could cause early embryonic lethal phenotype in mice (Chawiengsaksophak *et al.* 1997, Nichols *et al.* 1998, Mitsui *et al.* 2003).

TE is a monolayer of epithelial-like cell populations surrounding the outer part of the blastocyst, whose destination is determined by the expression level of a transcription factor, caudal type homeobox 2 (CDX2) (Strumpf *et al.* 2005). Hence, CDX2 is recognized as the most important factor in acquiring TE properties for the blastocyst development. As a potent evidence of this, ectopic expression of CDX2 in embryonic stem cells allows them to convert to trophoblast stem (TS) like cells (Niwa *et al.* 2005). One of the upstream regulators of CDX2 in mouse embryos is TEA domain transcription factor 4 (TEAD4) (Yagi *et al.* 2007). TEAD4 contributes to the establishment of a robust transcriptional network in TE by regulating not only *CDX2* but also many other genes critical for normal development, such as *Eomesodermin* (*Eomes*) (Yagi *et al.* 2007), *heart and neural crest derivatives expressed 1* (*Hand1*) (Nishioka *et al.* 2009) and *GATA-binding protein 3* (*Gata3*) (Ralston *et al.* 2010). Thus, TEAD4 is an essential mediator for the proper TE differentiation, whose loss leads to failure of TS cell line establishment in mice (Yagi *et al.* 2007). Although the important genes such as *CDX2* and *TEAD4* are commonly expressed at the blastocyst stage in most mammals (Berg *et al.* 2011, Home *et al.* 2012, Niakan & Eggan 2013, Bou *et al.* 2017), the regulatory mechanisms

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appear to vary among species (Berg *et al.* 2011, Kuijk *et al.* 2012). For example, bovine TEAD4 shows unique function to activate the ruminant-specific pregnancy recognition factor, interferon tau (Kusama *et al.* 2016). Therefore, to precisely evaluate the roles of TEAD4 in the first cell segregation at the blastocyst stage, the analyses dedicated to each species are required.

CCN family member 2 (CCN2) is well-known as one of potent downstream factors of TEAD4 in mammalian somatic cells, and this regulation of CCN2 expression by TEAD4 controls cell proliferation (Zhao *et al.* 2008). Further, we previously found *CCN2* mRNA was more predominantly expressed in TE cells than in ICM cells in the bovine blastocyst (Nagatomo *et al.* 2013). This site-predominant *CCN2* expression might be associated with the TE development through TEAD4 in cattle. However, this interaction between TEAD4 and CCN2 has not been proved in mammalian preimplantation embryos including cattle.

To elucidate the relationship between TEAD4 and CCN2 in the bovine blastocyst, we conducted the shRNA-mediated RNA interference for the suppression of *TEAD4* transcripts. Knockdown (KD) of *TEAD4* induced significant downregulation of TE-expressed genes, *CDX2*, *GATA2* and *CCN2*. Among these downregulated genes, the *CCN2* expression level was most altered in the *TEAD4* KD blastocysts. Next, we performed *CCN2* KD in bovine embryos, in which the expression levels of TE-expressed genes significantly decreased as well as in the *TEAD4* KD blastocysts. Notably, the *TEAD4* expression level in the *CCN2* KD blastocysts significantly decreased. The *CCN2* KD also decreased the ratio of TE to ICM cell numbers compared to the control blastocysts. Our results provide the first demonstration that the *CCN2* expression could be influenced by *TEAD4* in mammalian embryos. Not only that, reciprocal regulation of *TEAD4* and *CCN2* was required for stable expression of TE differentiation regulators, *CDX2* and *GATA2* in bovine blastocyst. Taken together, our findings offer the novel insights into the molecular circuitry governing the TE lineage differentiation through TEAD4 in bovine blastocyst development.

Materials and methods

All experimental protocols were approved by the Regulatory Committee for the Care and Use of Laboratory Animals, Hokkaido University.

Preparation of bovine embryos by in vitro *fertilization*

Bovine oocyte retrieval, *in vitro* oocyte maturation, fertilization and subsequent *in vitro* embryo culture were performed as described previously (Nagatomo *et al.* 2013). Briefly, cumulus-oocyte complexes (COCs) collected from slaughterhouse-derived ovaries were matured by culturing in TCM-199 medium (Thermo Fisher Scientific) at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 20–22 h. *In vitro*-matured oocytes were transferred to Brackett and Oliphant (BO) medium (Brackett & Oliphant 1975) containing 2.5 mM theophylline (Wako Pure Chemical Industries) and 7.5 µg/mL heparin sodium salt (Nacalai Tesque, Inc., Kyoto, Japan). Subsequently, frozen-thawed semen was centrifuged at 600 ***g*** for 7 min in BO medium, and the spermatozoa were added to the COCs at a final concentration of 5×10^6 cells/mL. After 12 h of incubation, the presumptive zygotes were denuded by pipetting and cultured in mSOFai medium (Aono *et al.* 2013) at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2 in air for 8 days.

Quantitative reverse-transcription PCR

Total RNA from five oocytes or embryos per biological replicate was isolated using the ReliaPrep RNA Cell Miniprep System (Promega), following the manufacturer's instructions. To obtain purely isolated ICM and TE for RNA preparation, blastocyst separation procedures were carried out according to our previous study (Nagatomo *et al.* 2013). Briefly, to evaluate the *TEAD4* mRNA expression level of only ICM, chemical isolation with detergent solution containing 0.2% (v/v) Triton X-100 in PBS was performed. This isolated ICM cell sample contained none of the TE cells as confirmed previously (Nagatomo *et al.* 2013). We separated TE from blastocysts using a micromanipulator equipped with a microsurgical blade (Feather, Osaka, Japan) under an inverted microscope (Olympus). The cDNA syntheses from ICM, TE, and whole embryo were conducted using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative PCR (qPCR) was performed after preparing the reaction mixtures in THUNDERBIRD SYBR qPCR Mix (Toyobo). The primer sets used for qPCR analysis were listed in Supplementary Table 1 (see section on supplementary data given at the end of this article). Thermal cycling conditions consisted of 1 cycle at 95°C for 30 s (denaturation), followed by 50 cycles at 95°C for 10 s (denaturation), annealing temperature corresponding to each primer set for 15 s (primer annealing) and 72°C for 30 s (extension). The TE-expressed genes examined in the present study were selected from the genes that played important roles for TE development in mouse blastocysts (Gulnaar *et al.* 1998, Niwa *et al.* 2005, Yagi *et al.* 2007, Ralston *et al.* 2010, Home *et al.* 2017) and were examined using TE of bovine blastocysts in our previous study (Nagatomo *et al.* 2013). Relative mRNA abundance was calculated by the ΔΔCt method, with *H2AFZ* (H2A histone family member Z) as the reference gene in each sample. The experiments were replicated three times.

Immunofluorescence and confocal microscopy

Primary antibodies included rabbit anti-CCN2 antibody (1:300, ab6992; Abcam), rabbit anti-CDX2 antibody (1:200, ab76541; Abcam) and mouse anti-TEAD4 antibody (1:1500, ab58310; Abcam). Secondary antibodies were Alexa Fluor 555 goat anti-rabbit IgG polyclonal (ab150082; Thermo Fisher Scientific, Inc.) and Alexa Fluor 488 goat anti-mouse IgG H&L (ab150113, Thermo Fisher Scientific, Inc.).

The zona pellucida of the oocytes and embryos were removed with 0.05% (w/v) Pronase (Sigma-Aldrich). Oocytes and embryos were fixed with 4% (w/v) paraformaldehyde (PFA; Wako Pure Chemical Industries) in PBS for 60 min, and then permeabilized for 60 min with 0.2% (v/v) Triton X-100 in PBS. Next, the oocytes and embryos were blocked for 45 min with Blocking One (1:5; Nacalai Tesque, Inc.) diluted in 0.05% (v/v) Tween 20 in PBS (blocking buffer). Temperature and incubation time during the primary antibody reaction varied depending on the target protein; namely, overnight at 4°C for CCN2, overnight at 37°C for CDX2 and 2 h at 20–22°C for TEAD4. After washes in 0.1% (v/v) Triton X-100 and 0.3% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS for 10 min five times, the oocytes and embryos were incubated for 30 min at room temperature with each secondary antibody diluted to 1:400 in blocking buffer. Nuclei were counterstained with 25 mg/mL Hoechst 33342 (Sigma-Aldrich) prepared in 0.2% (w/v) polyvinyl alcohol in PBS. Fluorescence signals were visualized using a TCS SP5II confocal laser-scanning microscope (Leica).

Whole-mount in situ *hybridization (WISH)*

WISH for *TEAD4* mRNA in bovine blastocysts was performed as described previously (Nagatomo *et al.* 2013). Briefly, bovine cDNA fragments encoding *TEAD4* transcripts were obtained by PCR amplifications and were transcribed *in vitro* with digoxigenin (DIG)-labeling using the T7 RNA polymerase for sense and antisense probes (DIG RNA Labeling Mix, Roche Diagnostics). The primer set to prepare sense and antisense probes was identical to that used for qPCR of *TEAD4*. The expanded blastocysts at day 8 were fixed and stored in 4% (w/v) PFA (Wako Pure Chemical Industries) in PBS with 0.1% (v/v) Tween 20 (PBT) at 4°C for 30 min to overnight. Fixed embryos were treated with proteinase K (Sigma-Aldrich) and placed in 4% (w/v) PFA, 0.2% (v/v) EM-grade glutaraldehyde (Sigma-Aldrich) in PBT at room temperature for 20 min. Subsequently, the blastocysts were placed in hybridization buffer (4× saline-sodium citrate (SSC) (pH 7.0), 50% (v/v) deionized formamide (Wako Pure Chemical Industries, Ltd.), 100 µg/mL heparin (Wako Pure Chemical Industries, Ltd.), 100 µg/mL salmon sperm DNA (Wako Pure Chemical Industries, Ltd.), 2× Denhardt's solution and 0.1% (v/v) Tween 20 (Wako Pure Chemical Industries, Ltd.)) at 60–70°C for 3–8 h. After these pretreatments, the blastocysts were subjected to WISH with DIG-labeled riboprobes in hybridization buffer overnight at the same temperature as prehybridization, followed by washing with buffer (50% (v/v) formamide, 2× SSC, 0.1% (v/v) Tween 20) 3 times at the hybridization temperature. According to the manufacturer's recommended procedure to detect DIG-labeled probes, samples were incubated in NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) solution (Roche) until coloration was obtained after incubation with anti-DIG antibodies (Roche). The hybridized embryos were observed in PBS containing 1 mM EDTA and 20% (v/v) glycerol for bright-field photographing under an inverted microscope (AMEX-1200, AMG, Bothell, WA, USA).

Microinjection of TEAD4 *and* CCN2 *shRNA expression vectors*

Construct preparation for RNA interference and subsequent microinjection into presumptive zygotes were performed as described previously (Akizawa *et al.* 2016). A shRNA containing antisense/sense regions, an 19-bp loop (5′-CTGTGAAGCCACAGATGGG) and a 6-bp terminator element (5′-TTTTTT) was designed to target nucleotides 913-932 of *TEAD4* mRNA (NCBI Reference Sequence XM_010827947) as well as target nucleotides 665–683 of *CCN2* mRNA (NCBI Reference Sequence NM_174030). The dsDNA was ligated downstream of the U6 promoter in the pBAsi/mU6 Neo vector (Stratagene, CA, USA). pBAsi/mU6 Neo plasmids targeting nucleotides 5957–5977 of enhanced green fluorescent protein (egfp) sequence (NCBI Reference Sequence NC_025025.1) (sh*egfp*) and plasmids lacking shRNA insert (empty vector) as used in the early studies from other laboratories (Orimo *et al.* 2005, Andey *et al.* 2014, Nelson *et al.* 2014, Li *et al.* 2015) and our previous reports (Nagatomo *et al.* 2015, 2016, Akizawa *et al.* 2016). Twelve hours after insemination, the synthesized shRNA expression constructs targeting *TEAD4*, *CCN2* and *egfp*, and empty vector (diluted to a final concentration of 10 ng/mL with mSOFai medium) were injected into the denuded presumptive zygotes using a FemtoJet injection device (Eppendorf, Hamburg, Germany). These presumptive zygotes were cultured to examine the effect of KD on embryonic development until the blastocyst stage.

Statistical analysis

All experimental data were presented as mean ± standard error of mean (S.E.M.). In the data on the *TEAD4* expression levels among whole embryos, ICM and TE, and the TE-expressed gene expression levels among uninjected, empty and sh*egfp* embryos, significant differences were compared using repeated-measures ANOVA followed by the Tukey's *post hoc* tests. Other than these experiments, data were statistically analyzed using unpaired Student's *t* test. Stat View (Abacus Concepts, Inc., Piscataway, NJ, USA) was used for analysis. $P<0.05$ was considered statistically significant.

Results

Site specificity of TEAD4 *expression in bovine blastocysts*

We first determined the site-dominant *TEAD4* expression by WISH and qPCR in bovine blastocysts. Although *TEAD4* mRNA was expressed both in TE and ICM (Fig. 1A), the expression level of *TEAD4* mRNA in TE was significantly higher than that in ICM (Fig. 1B). Moreover, whole-mount immunofluorescent staining revealed that TEAD4 proteins were predominantly expressed in nuclei of TE cells compared with nuclei of ICM cells (Fig. 1C). This expression pattern was consistent with previous report in cattle (Home *et al.* 2012), but was different from murine blastocysts (Nishioka *et al.* 2008).

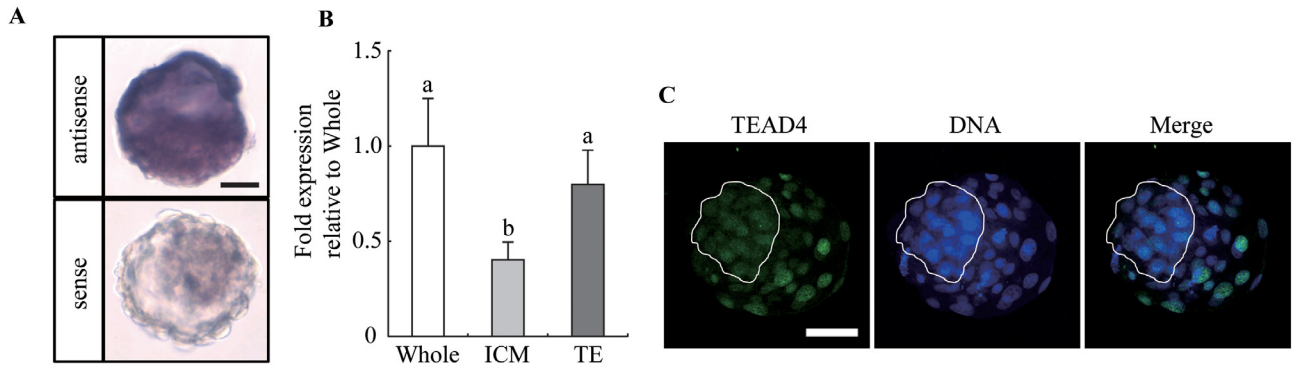

Figure 1 Site-dominant *TEAD4* expression status at the mRNA and protein levels in bovine blastocysts. (A) Whole-mount *in situ* hybridization for *TEAD4* mRNA. The results from DIG-labeled antisense and sense riboprobes were represented in the upper and lower panels, respectively. (B) The *TEAD4* mRNA expression level in whole embryo, chemically isolated ICM and mechanically isolated mural TE cell samples as described in the method section. The values represent relative expression levels to whole embryo. The results are shown as means ± S.E.M. (error bars) of three replicate experiments. (C) Immunofluorescent analysis of TEAD4 protein expression in the bovine blastocyst. The experiment was conducted three times, and at least five embryos were analyzed for each experiment. The white lines indicate ICM. (a and b) Different letters indicate significant differences between groups ($P<0.05$). Scale bar = 100 µm.

Effects of TEAD4 *KD on TE-expressed gene expression in bovine blastocysts*

To uncover the regulation of TE-expressed gene transcription by *TEAD4* in bovine embryos, we attempted to suppress *TEAD4* mRNA by shRNA-mediated interference. Before the *TEAD4* KD experiment, we confirmed that injection of empty or sh*egfp* vectors was non-effective for the expression levels of any genes examined in the subsequent experiments (Fig. 2). There were no significantly differences in expression levels of all the examined genes among uninjected, empty and sh*egfp* embryos, indicating that microinjection procedure and shRNA production have no impact on gene expression status in bovine embryos. Therefore, we regarded empty vector-injected embryos as controls of KD embryos in the subsequent experiments.

The efficiency of *TEAD4* KD was validated both at the mRNA and protein levels by qPCR and immunofluorescent staining respectively (Fig. 3A and B). The *TEAD4* expression level in the *TEAD4* KD blastocysts significantly decreased compared with empty vector-injected controls (Fig. 3A). Moreover, the immunofluorescent signals showing the TEAD4 proteins in the *TEAD4* KD embryos were obviously reduced (Fig. 3B). Moreover, to confirm the specificity of sh*TEAD4* vector, the expression level of *TEAD3* that is a member of TEAD family was investigated in the *TEAD4* KD embryos (Supplementary Fig. 1). As expected, the *TEAD3* in the *TEAD4* KD embryos was expressed at comparable level to that in controls (Supplementary Fig. 1). After these validations of KD efficiency, the *in vitro* development of *TEAD4* KD embryos was investigated until the blastocyst stage. There were no significant differences in the rates of cleaved embryos and blastocyst formation between *TEAD4* KD embryos and empty vector-injected controls (Table 1), which was consistent with previous report (Sakurai *et al.* 2017). The qPCR analyses revealed that the *TEAD4* KD blastocysts showed significantly suppressed expression levels of 3 TE-expressed genes; *CDX2*, *GATA2* and *CCN2* ($P<0.05$) (Fig. 3C). Among the downregulated genes in the *TEAD4* KD blastocysts, *CCN2* showed 11-fold decreased expression compared with that of empty vector-injected controls. In the

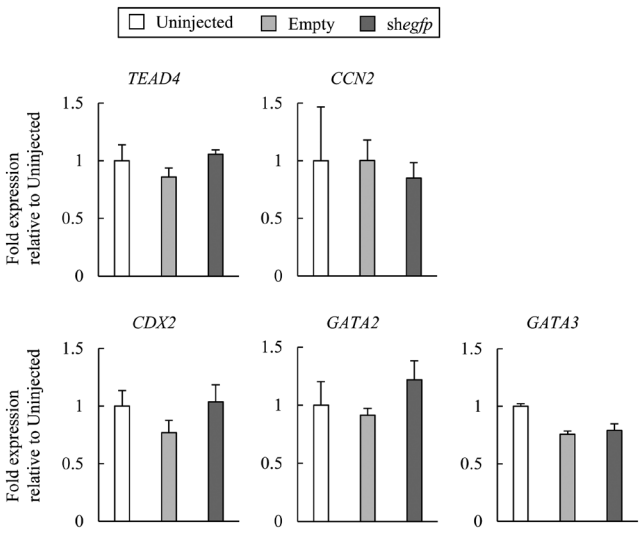

Figure 2 Validation of embryos injected with empty vectors as control in the shRNA-mediated RNA interference system. The mRNA expression levels of *TEAD4*, *CCN2*, *CDX2*, *GATA2* and *GATA3* were investigated among the following three types of embryos at the blastocyst stage: the uninjected embryos (Uninjected); the embryos that empty vectors were injected into (Empty) and the embryos that vectors targeting egfp sequence were injected into (sh*egfp*). The values represent relative expression levels to Uninjected. The results are shown as means ± S.E.M. (error bars) of four to five replicate experiments.

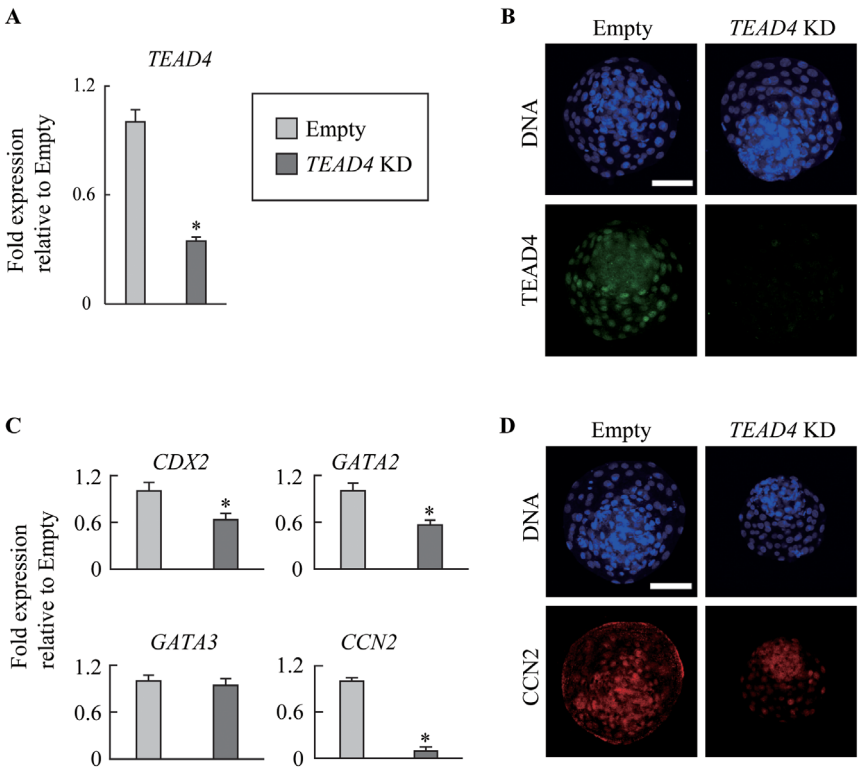

Figure 3 Effects of *TEAD4* knockdown (KD) on TE-expressed gene expression in bovine blastocysts. *TEAD4* KD efficiency was confirmed in blastocysts both at the mRNA (A) and protein (B) expression levels (34.5% compared to Empty). (C) The relative expression levels of four TE-expressed genes in empty vector-injected controls and *TEAD4* KD blastocysts (*CDX2*: 60.0%, *GATA2*: 56.2%, *GATA3*: 94.4%, and *CCN2*: 9.2% compared to Empty, respectively). (D) Immunofluorescent analysis of CCN2 protein expression in the *TEAD4* KD blastocyst. Ten embryos were analyzed for each experiment. The values represent relative expression levels to empty vector-injected controls. The results are shown as means ± S.E.M. (error bars) of three replicate experiments, $*P<0.05$. Scale bar = 100 µm.

protein level, CCN2 expression is suppressed in TE, but not in ICM (Fig. 3D).

Reciprocal regulation of TEAD4 *and* CCN2 *in bovine blastocysts*

Although there are several studies to demonstrate the essential roles of *CCN2* expression regulated by *TEAD4* in NIH-3T3, MCF10A (Zhao *et al.* 2008) and human mammary epithelial cells (Lai *et al.* 2011), the significance of *CCN2* expression during preimplantation development has remained unclear. Therefore, we next attempted to suppress *CCN2* mRNA by shRNA-mediated interference and validated the *CCN2* KD efficiency by assessing *CCN2* mRNA and protein expression. The *CCN2* expression level significantly decreased in the *CCN2* KD blastocysts (Fig. 4A), which was consistent with the reduction of fluorescent signals representing CCN2 proteins (Fig. 4B). Similar to *TEAD4* KD, *CCN2* KD had no effects on *in vitro* developmental rates of cleavage and the blastocyst formation (Table 2). Furthermore, we determined the expression levels of TE-expressed genes that were examined in the *TEAD4* KD blastocysts; *CDX2*, *GATA2* and *GATA3*, in the *CCN2* KD blastocysts. Besides, the *TEAD4* expression level was analyzed. Among these, the expression levels of *CDX2*, *GATA2* and *TEAD4*, in *CCN2* KD blastocysts significantly decreased compared to empty vector-injected controls, corresponding to *TEAD4* KD blastocysts ($P<0.05$) (Fig. 4C). Additionally, the fluorescent signals representing the TEAD4 proteins were reduced in the *CCN2* KD blastocysts (Fig. 4D).

Modulation of the ratio of TE to ICM cell numbers by CCN2 *in bovine blastocysts*

CCN2 could control cell and tissue growth in various circumstances (Nakanishi *et al.* 2000, Ivkovic *et al.* 2003, Dornhöfer *et al.* 2006). Further, the TEAD4-CCN2 regulation was involved in cell proliferation in human and mouse cell lines (Zhao *et al.* 2008). Considering these studies and our results, we performed immunofluorescent staining of CDX2 for the *CCN2* KD

Table 1 Effect of *TEAD4* KD on bovine preimplantation development *in vitro*.

Treatment	Number of putative zygotes tested	No of embryos developed to (% ± S.E.M.)	
		2-Cell	Blastocyst
Uninjected	105	75 (71.4 ± 9.79)	32 (30.5 ± 10.84)
Empty	111	66 (59.5 ± 4.48)	22 (19.8 ± 1.76)
sh*TEAD4*	172	102 (59.3 ± 2.33)	40 (23.3 ± 2.15)

Empty, embryos that vectors lacking shRNA sequence were injected into; S.E.M., standard error of the mean; sh*TEAD4*, embryos that vectors containing *TEAD4* shRNA sequence were injected into; uninjected, IVF embryos.

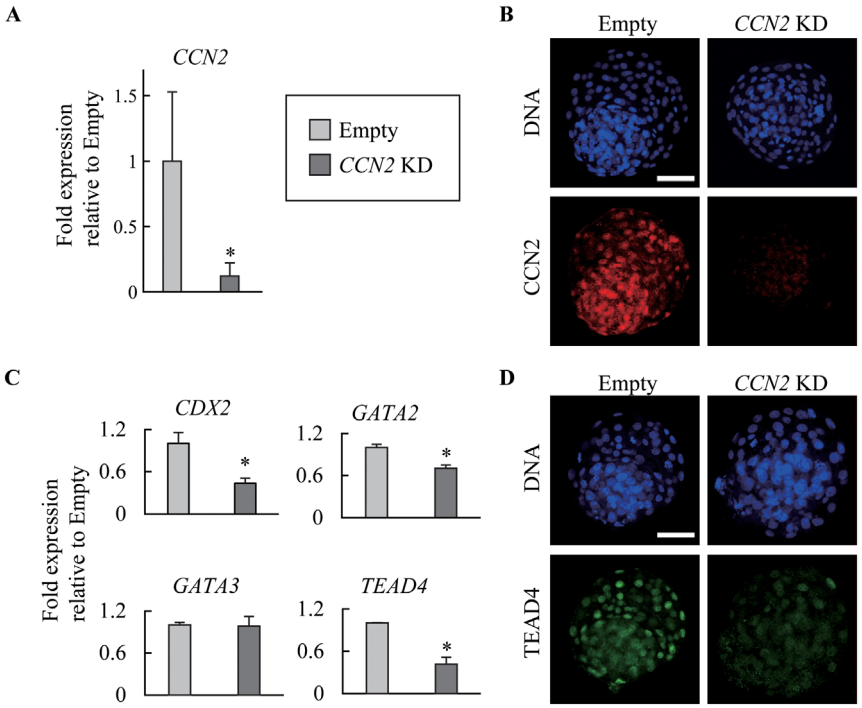

Figure 4 Effects of *CCN2* knockdown (KD) on TE-expressed gene expression in bovine blastocysts. *CCN2* KD efficiency was confirmed in blastocysts both at the mRNA (A) and protein (B) expression levels (12.2% compared to Empty). (C) The relative expression levels of four TE-expressed genes in empty vector-injected control and *CCN2* KD blastocysts (*CDX2*: 43.6%, *GATA2*: 70.2%, *GATA3*: 98.6%, and *TEAD4*: 41.7% compared to Empty, respectively). (D) Immunofluorescent analysis of TEAD4 protein expression in the *CCN2* KD blastocyst. Ten embryos were analyzed for each experiment. The values represent relative expression levels to empty vector-injected controls. The results are shown as means ± S.E.M. (error bars) of three replicate experiments, $*P<0.05$. Scale bar = 100 μm.

blastocysts to determine the effects of *CCN2* KD on the cell numbers of CDX2-positive TE and CDX2-negative ICM. Although there were no significant differences in the TE, ICM and total cell numbers between controls and the *CCN2* KD blastocysts (Fig. 5A), the ratio of TE to ICM cell numbers significantly decreased in the *CCN2* KD blastocysts ($P<0.05$) (Fig. 5B).

Discussion

It has been unclear whether the transcriptional modulation between *TEAD4* and *CCN2* is conserved in mammalian preimplantation embryos including cattle. We here attempted to dissect this by analyzing each *TEAD4* and *CCN2* KD bovine embryos. As expected, the *TEAD4* KD induced the *CCN2* suppression both at the mRNA and protein expression levels with decrease of the other genes required for TE cell characterization – *CDX2* and *GATA2*. The decrease of *CDX2* and *GATA2* expression was similarly observed in the *CCN2* KD blastocysts, indicating that the modulation of *CDX2* and *GATA2* expression by *TEAD4* was mediated through *CCN2*. Interestingly, the *TEAD4* expression was also suppressed in the *CCN2* KD blastocysts, suggesting that the *TEAD4* expression was sustained by *CCN2*. Our results demonstrate that *TEAD4* and *CCN2* modulate mutual expression during mammalian blastocyst development, which was essential for the stable expression of the TE-expressed genes including *CDX2*

Table 2 Effect of *CCN2* KD on bovine preimplantation development *in vitro*.

Treatment	Number of putative zygotes tested	No of embryos developed to (% ± S.E.M.)	
		2-cell	Blastocyst
Uninjected	171	129 (75.4 ± 9.10)	45 (26.3 ± 7.43)
Empty	182	143 (78.6 ± 5.79)	41 (22.5 ± 2.08)
sh*CCN2*	331	251 (75.8 ± 7.44)	83 (25.1 ± 5.11)

Empty, embryos that vectors lacking shRNA sequence were injected into; S.E.M., standard error of the mean; sh*CCN2*, embryos that vectors containing *CCN2* shRNA sequence were injected into; uninjected, IVF embryos.

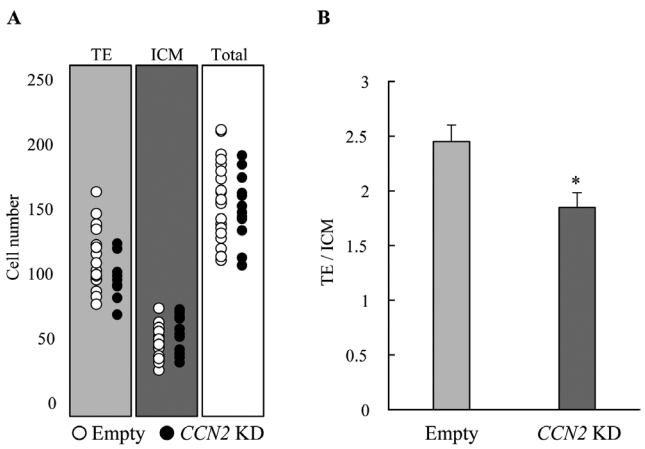

Figure 5 ICM/TE cell composition in the *CCN2* KD blastocyst. (A) The numbers of TE (left), ICM (middle) and total (right) cells in the *CCN2* KD blastocyst are shown. Each plot represents individual embryos; empty vector-injected controls (white) and *CCN2* KD blastocysts (black), respectively. (B) Ratio of TE cells to ICM cells in empty vector-injected controls and *CCN2* KD blastocysts. Error bar: S.E.M. $*P<0.05$.

and *GATA2*. For a better understanding of mammalian blastocyst development, it would be interesting to discuss comparing these findings in bovine embryos with advanced studies resulted from various genetic approaches in mouse embryos.

In the bovine blastocyst, the *TEAD4* mRNA expression was stronger in TE, and TEAD4 protein signals were also more distinct within nuclei in TE cells than in ICM. Characteristically, TEAD4 activates transcription of the target genes in cooperation with a transcriptional coactivator, Yes-associated protein (YAP) (Zhao *et al.* 2008). In mouse preimplantation embryos, YAP proteins localize into nuclei in outside blastomeres constructing TE, and thereby downstream factors of TEAD4 are specifically enhanced in TE (Nishioka *et al.* 2009). In bovine trophoblast CT-1 cells, YAP and TEAD4 coordinately regulate downstream factors (Kusama *et al.* 2016). As the *CDX2* expression was downregulated in *YAP* KD blastocysts (Negrón-Pérez & Hansen 2017), the regulatory mechanism for TE characterization through the YAP-TEAD4 axis may be conserved in bovine blastocyst as well as in mouse blastocysts. At least, the downregulation of *CDX2*, *GATA2* and *CCN2* in the *TEAD4* KD blastocyst support that *TEAD4* is associated with the expression of TE-expressed genes in the bovine blastocyst.

GATA3 is also one of the potent downstream targets of TEAD4 in mouse embryos (Ralston *et al.* 2010). However, in the present study, the *GATA3* expression level was unchanged both in the *TEAD4* and *CCN2* KD embryos. These observations suggest the presence of upstream regulator (s) of *GATA3* expression other than *TEAD4* in the bovine blastocyst. This *TEAD4*-independent regulation for *GATA3* expression has been adopted in mouse embryos. For example, transcription factor AP-2, gamma (TCFAP2C) and EOMES had been shown to directly bind to the *Gata3* promoter in mouse TS cells (Kidder & Palmer 2010). Another group also reported that *TEAD4* KD had no effects on *GATA3* expression in cattle (Sakurai *et al.* 2017). Overall, TEAD4 might not be essential for the *GATA3* mediation in the bovine blastocyst.

In several types of somatic cells, TEAD4 directly regulates the transcriptional activation of *CCN2*, resulting in cellular property alterations (Zhao *et al.* 2008, Lai *et al.* 2011). We also found that the *CCN2* downregulation was caused by the *TEAD4* suppression in bovine blastocysts. Furthermore, the TE-expressed genes downregulated and non-downregulated in the *TEAD4* KD and *CCN2* KD blastocysts were identical, suggesting that the sets of TE-expressed genes mediated by *CCN2* and *TEAD4* were overlapped. Nuclear localization of CCN2 could affect RNA transcription status in some cell types (Wahab *et al.* 2001). We also observed the CCN2 proteins within nuclei of bovine blastocysts as well as primary tissues in mice (Gray *et al.* 2007), and the protein expression was suppressed only in TE but not in ICM in the *TEAD4* KD blastocysts. This might suggest that the *CCN2* regulation through TEAD4 predominantly occurs in TE. Overall, this possible transcription regulation ability of CCN2 would play important roles for TE cell development in bovine blastocyst.

The significance of CCN2 has remained unclear in the blastocyst development. The analysis of TE, ICM and total cell numbers in the *CCN2* KD blastocysts revealed that the *CCN2* suppression significantly reduced the ratio of TE to ICM. We previously showed that the *CCN2* expression level was greater in TE than that in ICM of bovine blastocysts (Nagatomo *et al.* 2013) as well as *TEAD4* mRNA observed in the present study. This active *CCN2* expression in TE might affect the regulation of the cell composition in bovine blastocysts. Recently, another group reported that the supplementation of human recombinant *CCN2* into the culture medium for bovine embryos increased the ICM cell number (Kannampuzha-Francis *et al.* 2017). This apparent contradiction in effect on cell composition could be explained by the difference of CCN2 derivation. In the present study, we inhibited the endogenous *CCN2* transcripts to investigate the autocrine effects of embryonic *CCN2* expression on preimplantation development, which was inherently different from the addition of exogenous CCN2 proteins into the culture medium. As *CCN2* expression was confirmed in bovine endometrial epithelial cells (Munoz *et al.* 2017), the supplementation of CCN2 recombinants might reflect the effects of maternal CCN2 on preimplantation development. Further analyses would be required for a deeper understanding of bovine CCN2 from maternal and embryonic sources in a dual manner.

In conclusion, our results demonstrate that the stable expression patterns of TE-expressed genes are maintained through reciprocal regulation of both *TEAD4* and *CCN2* in cattle. To our knowledge, this is the first study showing *CCN2* expression mediated by *TEAD4* in mammalian embryos. Further analyses of interaction between *TEAD4* and *CCN2* in bovine blastocysts would expand the understanding of phenomena underling proper cell differentiation in preimplantation development.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-18-0043.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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