1 Significance of CCN2 expression in bovine preimplantation development

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17 Running title: CCN2 expression dynamics in bovine blastocysts
ABSTRACT

In mammalian preimplantation development, the first cell lineage segregation occurs during the blastocyst stage, when the Inner Cell Mass and Trophoderm (TE) differentiate. Species-specific analyses are essential to elucidate the molecular mechanisms that underlie this process, since they differ between various species. We previously showed that the reciprocal regulation of CCN2 and TEAD4 is required for proper TE differentiation in bovine blastocysts; however, the function of CCN2 during early embryogenesis has remained otherwise elusive. The present study assessed the spatiotemporal expression dynamics of CCN2 in bovine embryos, and evaluated how changes to CCN2 expression (using a CCN2 knockdown (KD) blastocyst model) regulate the expression of pluripotency-related genes such as OCT4 and NANOG. The conducted quantitative PCR analysis revealed that CCN2 mRNA was expressed in bovine oocytes (at the metaphase stage of their second meiosis), and embryos. Similarly, immunostaining detected both cytoplasmic and nuclear CCN2 at all analyzed oocyte and embryonic stages. Finally, both OCT4 and NANOG expression levels were shown to be significantly reduced in CCN2 KD blastocysts. Together, these results demonstrate that bovine CCN2 exhibits unique expression patterns during preimplantation development, and is required for the proper expression of key regulatory genes in bovine blastocysts.
**Key words:** CCN2, pluripotency-related genes, bovine, blastocyst, cell-lineage specification.
 INTRODUCTION

During early embryonic cleavage, blastomeres are morphologically indistinguishable, and have the potential to differentiate into all cell lineages (Kelly 1977). As development progresses however, the blastomeres gradually lose their developmental plasticity, and cell lineage segregation begins, and is thereafter maintained via a fine-tuned transcriptional network (Arnold & Robertson 2009). By the blastocyst stage, embryos are comprised of two distinct cell lineages; namely, the Inner Cell Mass (ICM), and the Trophoderm (TE), which subsequently give rise to embryo-proper and extraembryonic tissues, respectively. Hence, defective segregation of these two cell lineages leads to diverse developmental defects.

The transcription factor TEAD4 plays critical roles in TE differentiation by activating the expression of core TE regulators, such as Cdx2 in mice (Yagi et al. 2007; Nishioka et al. 2009).

Murine embryos lacking TEAD4 expression exhibit failed blastocyst development, and resultantly, a loss of trophoblast stem cells (Yagi et al. 2007). To evaluate whether TEAD4 is similarly required for bovine blastocyst formation, we previously knocked down TEAD4 transcripts using short hairpin RNA (shRNA)-mediated RNA interference in preimplantation embryos (Akizawa et al. 2018). TEAD4 knockdown (KD) significantly reduced the expression of important TE-expressed genes such as CDX2, GATA2, and CCN2. Of these, CCN2 was most markedly suppressed; however, while it has been previously shown to be essential for fetal morphogenesis by the fact that CCN2-mutant
mice die soon after birth due to severe skeletal dysmorphisms (Ivkovic et al. 2003), its role in early embryogenesis, including bovine preimplantation development, is not yet clear. Structurally, CCN2 is a signaling protein composed of four cysteine-rich domains, comprising an insulin-like growth factor binding protein (IGFBP) domain, a von Willebrand type C (VWC) repeat, a thrombospondin (TSP) repeat, and a C-terminal (CT) motif that contains a cysteine knot (Hall-Glenn & Lyons 2011).

In most mammals, CCN2 is uniformly expressed in the blastocyst ICM and TE (Gulnaar et al. 1998; Moussad et al. 2002; Munoz et al. 2017), but its expression patterns vary between species after this stage of development (Moussad et al. 2002; Oh et al. 2009). For example, CCN2 expression in pig trophoblast cells diminishes soon after the blastocyst stage, while in humans, it is maintained until implantation. CCN2 expression dynamics during bovine preimplantation development have not yet been analyzed.

Similarly, little is known about the function of CCN2 in bovine preimplantation development. Previously, we demonstrated that CCN2 KD blastocysts exhibit significantly reduced TEAD4 mRNA expression, suggesting that TEAD4 and CCN2 may function together (i.e. non-redundantly) to direct correct TE differentiation in bovine embryos. Interestingly, immunofluorescent staining of CCN2 production at the blastocyst stage showed that CCN2 localizes to both TE and ICM cells; however, TEAD4 KD-mediated CCN2 suppression occurs in the TE, but not the ICM. Thus, while the underlying molecular mechanisms are not yet clear, these data suggest
that CCN2 likely plays important roles not only in bovine TE formation, but also in the overall
development of the bovine blastocyst, including the ICM.

In the present study, we first investigated CCN2 expression dynamics during bovine
preimplantation development via both a quantitative PCR analysis, and an immunostaining assay for
CCN2. We then examined the effects of CCN2 KD on the expression of pluripotency-related genes,
and showed that in CCN2 KD blastocysts, the expression levels of the core pluripotency related
transcription factors OCT4 and NANOG are significantly reduced. Taken together, these findings
support that CCN2 is required for proper lineage segregation in bovine blastocysts, to regulate the
expression of both TE-expressed, and core pluripotency-related genes.
MATERIALS and METHODS

Preparation of bovine embryos

Bovine oocyte retrieval, and in vitro oocyte maturation, fertilization, and subsequent in vitro embryo culture were performed as previously described (Nagatomo et al. 2013). Briefly, cumulus-oocyte complexes (COCs) collected from slaughterhouse-derived ovaries were matured via culture (20–22 h, at 38.5°C, in a humidified atmosphere with 5% CO2 in air) in TCM-199 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5% (v/v) FBS. In vitro-matured oocytes were transferred to Brackett and Oliphant (BO) medium (Brackett & Oliphant 1975) containing 2.5 mM theophylline (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 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 isolated spermatozoa were added to the COCs at a final concentration of 5 × 106 cells/mL. After 12 h of incubation, the presumptive zygotes were denuded by pipetting, and cultured (8 days, at 38.5°C, in a humidified atmosphere with 5% CO2 and 5% O2 in air) in mSOFai medium (Aono et al. 2013). A Holstein cow on campus of Hokkaido University was administered 25 mg of prostaglandin F2α (Pronalgon F; Schering-Plough K.K., Tokyo, Japan) 20 days prior to artificial insemination (AI) followed by intravaginal indwelling of the controlled intravaginal drug release (CIDR; 1.9 g of progesterone; Eazi-Breed CIDR Cattle Insert; Pfizer Animal Health, New York, NY, USA) device from 9 to 3 days
prior to AI. Estradiol benzoate (2 mg; Ovahormon, Injection; Aska Animal Health, Tokyo) was
simultaneously injected with CIDR insertion. From 5 to 3 days prior to AI, follicle stimulating
hormone (Antrin R-10; Kyoritsu Seiyaku, Tokyo, Japan) consisted of twice-daily (morning and
afternoon) with a decreasing dose (6, 6, 5, 5, 4, and 4 AU per injection) were administered. At the
fifth and sixth FSH treatments, 30 and 20 mg of prostaglandin F2α were injected, respectively. On
the day of AI, 200 μg of gonadotropin releasing hormone (Conceral; ASKA Pharmaceutical Co., Ltd.,
Tokyo, Japan) was administrated. The superstimulated cow was inseminated (day 0), and day-14
conceptuses were subsequently collected via non-surgical flushing in warm lactated Ringer's
solution (Nippon Zenyaku Kogyo Co., Ltd, Fukushima, Japan).

Quantitative Reverse-Transcription PCR

The total RNA from five oocytes or embryos per biological replicate was isolated using the
ReliaPrep RNA Cell Miniprep System (Promega, Fitchburg, WI, USA), according to the
manufacturer’s instructions. cDNA was then synthesized from the oocyte/embryo RNA using
ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative PCR (qPCR) was
performed using THUNDERBIRD SYBR qPCR Mix (Toyobo), and the specified primers
(Supplemental Table S1). The utilized thermal cycling conditions consisted of one cycle of 95°C for
30 s (denaturation), followed by 50 cycles of 95°C for 10 s (denaturation), the appropriate annealing
temperature for each primer set for 15 s (primer annealing), and 72°C for 30 s (extension). Relative
mRNA abundance was calculated using the ΔΔCt method, where \( H2A \) histone family member \( Z \)
(\( H2AFZ \)) was used as a reference gene for each sample. All experiments were performed in
triplicate.

**Immunofluorescence and Confocal Microscopy**

CCN2 immunofluorescence was analyzed in both bovine oocytes and embryos as
previously described (Akizawa et al. 2018). Briefly, the zona pellucida was removed from bovine
oocytes and embryos using 0.05% (w/v) Pronase (Sigma-Aldrich, St. Louis, MO, USA). Both
oocytes and embryos were fixed in 4% (w/v) paraformaldehyde (Wako Pure Chemical Industries,
Ltd.) in phosphate-buffered saline (PBS) for 60 min, and then permeabilized for 60 min using 0.2%
(v/v) Triton X-100 in PBS. They were then blocked for 45 min in Blocking One (1:5; Nacalai Tesque,
Inc.) solution diluted in 0.05% (v/v) Tween 20 in PBS (blocking buffer), and incubated in rabbit
anti-CCN2 antibody (1:300, ab6992; Abcam, Cambridge, UK) overnight at 4°C. After five 10-min
washes in 0.1% (v/v) Triton X-100 and 0.3% (w/v) bovine serum albumin (Sigma-Aldrich), the
oocytes and embryos were incubated with Alexa Fluor 555 goat anti-Rabbit IgG polyclonal
antibodies (diluted to 1:400 in blocking buffer; ab150082; Thermo Fisher Scientific, Inc.) for 30 min
at room temperature. Nuclei were counterstained with 25 mg/mL Hoechst 33342 stain
(Sigma-Aldrich) prepared in 0.2% (w/v) polyvinyl alcohol in PBS. Fluorescence signals were observed using a TCS SP5II confocal laser-scanning microscope (Leica, Wetzlar, Germany). In each experiment, at least five oocytes/embryos were observed.

Microinjection of CCN2-shRNA expression vectors into bovine embryos

shRNA-mediated RNA interference (RNAi) for CCN2 was induced as previously described (Akizawa et al. 2018). Briefly, an shRNA containing antisense/sense regions, a 19-bp loop (5′-CTGTGAAGCCACAGATGGG-3′), and a 6-bp terminator element (5′-TTTTTT-3′), was designed to target nucleotides 665–683 of the CCN2 mRNA sequence (NCBI Reference Sequence NM_174030), and ligated downstream of the U6 promoter in the pBAsi/mU6 Neo vector (Stratagene, CA, USA). Embryos injected with pBAsi/mU6 Neo plasmids lacking the shRNA insert (empty vector) were used as controls, as previously described (Nagatomo et al. 2013; Akizawa et al. 2016). Twelve hours after insemination, the synthesized shRNA-expression constructs (diluted to a final concentration of 10 ng/mL with mSOFai medium) were injected into denuded presumptive zygotes using a FemtoJet injection device (Eppendorf, Hamburg, Germany). These presumptive zygotes were then cultured to the blastocyst stage to examine the effect of CCN2 KD on the expression of pluripotency-related genes.
Statistical analysis

In the result of CCN2 mRNA expression dynamics (Fig. 1A), data were statistically analyzed using a one-way analysis of variance followed by Tukey’s multiple comparison test. Excluding Fig. 1A, significant differences were compared using Student’s t test. Statistical analyses were conducted using StatView statistical-analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). A P value < 0.05 was considered to indicate statistical significance.
RESULTS

CCN2 mRNA expression levels during preimplantation development

The conducted qPCR analysis of CCN2 mRNA expression dynamics in bovine oocytes and embryos detected CCN2 mRNA at all of the examined stages, (i.e. metaphase MII oocytes, and 2-, 4-, 8-, 16-, morula, and blastocyst-stage embryos) (Fig. 1A). Notably, CCN2 expression significantly decreased between the 16-cell and the blastocyst stages of development (P < 0.05) (Fig. 1A). Next, elongating embryos were collected from cows at day 14 after artificial insemination via uterine flushing, to investigate CCN2 mRNA expression after the blastocyst stage. The results of this analysis revealed that CCN2 expression levels in the elongating conceptuses was significantly increased compared to that observed in blastocyst-stage embryos (P < 0.05) (Fig. 1B).

Subcellular CCN2 protein localization patterns during preimplantation development

Next, immunostaining was performed to visualize and evaluate the subcellular localization of CCN2 during early bovine embryogenesis. As for CCN2 mRNA expression, CCN2 was detected at all of the examined stages (Fig. 2), and shown to consistently localize to both the cytoplasm and nucleus of each blastomere from the 2-cell to the blastocyst stage (Fig. 2). Notably, at the blastocyst stage, the CCN2 fluorescent signals appeared strongest in the nucleus.
Effects of CCN2 mRNA suppression on the expression of pluripotency-related genes in blastocysts

We previously constructed a CCN2 KD system in bovine embryos (Akizawa et al. 2018), which demonstrated that CCN2 KD is sufficient to disrupt the expression patterns of primary TE-expressed genes, such as CDX2, GATA2, and TEAD4. Since CCN2 was found in the present study to be ubiquitously (i.e. in both the ICM and TE) expressed throughout the analyzed embryos (Fig. 2), we evaluated the effects of CCN2 KD on the blastocyst expression of pluripotency-related genes. The results of this analysis showed that the expression of both OCT4, (which is normally detected uniformly in both ICM and TE blastomeres), and NANOG, (which is predominantly expressed in ICM blastomeres), were significantly decreased in CCN2 KD blastocysts (P < 0.05) (Fig. 3). These factors are known to be required for ICM-cell self-renewal and pluripotency in both mice and cattle (Wu et al. 2016; Bogliotti et al. 2018); thus, our results indicate that CCN2 expression mediates OCT4 and NANOG expression in bovine blastocysts.
DISCUSSION

CCN2 has been shown to be strongly involved in several major biological disorders, such as fibrosis, and various malignancies; thus, it has been extensively studied as a possible therapeutic target for the last few decades (Kubota & Takigawa 2015). However, because homozygous mutant-CCN2 neonates are recovered at the expected Mendelian ratio (Ivkovic et al. 2003), the function(s) of CCN2 in preimplantation development have thus far been overlooked. Thus, the present study is the first to evaluate both CCN2 mRNA and protein expression dynamics, and the contribution of CCN2 to the proper expression of pluripotency-related genes in bovine preimplantation embryos.

Firstly, we revealed that CCN2 mRNA is detectable throughout early bovine embryogenesis. CCN2 expression levels were higher in oocytes and embryos prior to major zygotic genome activation (ZGA) (which occurs in cattle after the 8- to 16-cell stages) (Khan et al. 2012) than in the subsequent-staged embryos. These results suggest that maternal CCN2 mRNA may persist, and direct bovine embryogenesis until ZGA, in a similar manner to maternal SMAD2/3 (which are known to be upstream regulators of CCN2 in bovine embryos) (Zhang et al. 2015). The fluorescent signals for CCN2 proteins detected after the 16-cell stage in Fig. 2 might mainly be derived from maternally expressed CCN2 mRNA transcribed before the 8-cell stage. Conversely, we observed a significant increase in CCN2 expression in elongating conceptuses compared to blastocysts. The
elongation of bovine conceptus is accompanied by a remarkable proliferation of trophoblast cells (Betteridge and Fléchon 1988). In fact, CCN2 has been shown to be associated with cell proliferation in both cattle and other mammals (Zhou et al. 2008); thus, it may mediate the elongation of bovine embryos after the blastocyst stage.

Immunostaining revealed that CCN2 localized within both the cytoplasm and nucleus in bovine embryos. CCN2 is secreted, and generally detected in the Golgi apparatus (Zhou et al. 2008), supporting that it performs important roles in the cytoplasm. However, CCN2 is detected in both cytoplasmic and nuclear fractions in some somatic cells and nuclear-localized CCN2 has been shown to modulate RNA transcription (Wahab et al. 2001). Although the mechanisms by which nuclear translocation of CCN2 is enhanced in bovine embryos are not yet clear, the results of the conducted knockdown experiments in the present study strongly suggest that nuclear CCN2 regulates transcriptional activity in blastomeres during bovine preimplantation development.

We previously demonstrated that CCN2 KD affects the expression of TE-expressed genes, and decreases the ratio of TE to ICM cells in bovine embryos, indicating that CCN2 mediates TE development (Akizawa et al. 2018). However, to date, the role(s) played by CCN2 in the establishment of the complex transcriptional networks required for blastocyst formation have not yet been elucidated. In the present study, CCN2 KD suppressed both OCT4 and NANOG expression in bovine blastocysts. A recent OCT4 knockout study in bovine embryos revealed that OCT4 is
essential for maintaining NANOG expression at the blastocyst stage (Simmet et al. 2018).

Consistent with this, the remarkable reduction of *NANOG* observed in the CCN2 KD embryos in the present study may be a result of downregulated *OCT4* expression. CCN2 has also been shown to interact with various cell signaling pathways, including the FGFR and TGFB pathways, during murine blastocyst-stage cell lineage segregation (Abreu et al. 2002; Crean et al. 2004). Further studies are needed to elucidate the pathways by which CCN2 contributes to cell lineage segregation in bovine embryos.

In conclusion, the present study demonstrates that *CCN2* is expressed throughout development until implantation, and contributes to the transcriptional regulation of pluripotency-related genes in both TE and ICM cell lineages in bovine blastocysts. In both cattle and other mammals, the correct establishment and function of complex transcriptional networks enables preimplantation embryos to develop to the blastocyst stage. Continued research is needed to decipher the role(s) of *CCN2* in bovine-specific preimplantation after the blastocyst stage of development.

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for the Northern Biosphere, Hokkaido University, Sapporo, Japan, for their support during the collection of bovine embryos.
References


Figure 1. CCN2 mRNA expression dynamics in bovine oocytes and embryos. (A) CCN2 expression levels were assessed in oocytes undergoing metaphase during their second meiosis (MII), and in embryos at the 2- (2C), 4- (4C), 8- (8C), 16-cell (16C), morula (MO), and blastocyst (BL) stages, via quantitative RT-PCR (qPCR). Three independent experiments were performed using five oocyte/embryo samples per assay. (B) CCN2 expression levels were compared between blastocysts and day-14 conceptuses (D14), (where day 0 = artificial insemination). Data are presented as the mean ± SEM (error bars) of three replicate experiments. Superscript letters indicate statistically significant differences between groups (P < 0.05).

Figure 2. CCN2 protein localization in bovine oocytes and preimplantation embryos.

Representative confocal images showing bovine oocytes (in metaphase of their second meiosis (MII)), and embryos (at the 2- (2C), 4- (4C), 8- (8C), 16-cell (16C), morula (MO), and blastocyst (BL) stages) in which CCN2 was visualized via immunofluorescent staining (middle column). Nuclei (left column) were visualized via staining with Hoechst 33342. Bar = 75 μm.

Figure 3. Effects of CCN2 knockdown (KD) on the expression of pluripotency-related genes.

The relative expression levels of the pluripotency-related genes NANOG and OCT4 in empty
vector-injected control, and CCN2 KD blastocysts (NANOG, 7.5%; and OCT4, 39.1% compared to the empty control vector, respectively) are shown. Three independent experiments were performed using five embryo samples per assay. Data are presented as the mean ± SEM (error bars) of three replicate experiments. Superscript letters indicate statistically significant differences between groups (P < 0.05).
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