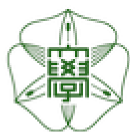
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Significance of *CCN2* expression in bovine preimplantation development

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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 Trophectoderm (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.

1 **Key words:** *CCN2*, pluripotency-related genes, bovine, blastocyst, cell-lineage specification.

1 **INTRODUCTION**

2 During early embryonic cleavage, blastomeres are morphologically indistinguishable, and
3 have the potential to differentiate into all cell lineages (Kelly 1977). As development progresses
4 however, the blastomeres gradually lose their developmental plasticity, and cell lineage segregation
5 begins, and is thereafter maintained via a fine-tuned transcriptional network (Arnold & Robertson
6 2009). By the blastocyst stage, embryos are comprised of two distinct cell lineages; namely, the
7 Inner Cell Mass (ICM), and the Trophectoderm (TE), which subsequently give rise to embryo-proper
8 and extraembryonic tissues, respectively. Hence, defective segregation of these two cell lineages
9 leads to diverse developmental defects.

10 The transcription factor *TEAD4* plays critical roles in TE differentiation by activating the
11 expression of core TE regulators, such as *Cdx2* in mice (Yagi *et al.* 2007; Nishioka *et al.* 2009).
12 Murine embryos lacking *TEAD4* expression exhibit failed blastocyst development, and resultantly, a
13 loss of trophoblast stem cells (Yagi *et al.* 2007). To evaluate whether *TEAD4* is similarly required for
14 bovine blastocyst formation, we previously knocked down *TEAD4* transcripts using short hairpin
15 RNA (shRNA)-mediated RNA interference in preimplantation embryos (Akizawa *et al.* 2018).
16 *TEAD4* knockdown (KD) significantly reduced the expression of important TE-expressed genes
17 such as *CDX2*, *GATA2*, and *CCN2*. Of these, *CCN2* was most markedly suppressed; however, while
18 it has been previously shown to be essential for fetal morphogenesis by the fact that *CCN2*-mutant

1 mice die soon after birth due to severe skeletal dysmorphisms (Ivkovic *et al.* 2003), its role in early
2 embryogenesis, including bovine preimplantation development, is not yet clear. Structurally, CCN2
3 is a signaling protein composed of four cysteine-rich domains, comprising an insulin-like growth
4 factor binding protein (IGFBP) domain, a von Willebrand type C (VWC) repeat, a thrombospondin
5 (TSP) repeat, and a C-terminal (CT) motif that contains a cysteine knot (Hall-Glenn & Lyons 2011).
6 In most mammals, CCN2 is uniformly expressed in the blastocyst ICM and TE (Gulnaar *et al.* 1998;
7 Moussad *et al.* 2002; Munoz *et al.* 2017), but its expression patterns vary between species after this
8 stage of development (Moussad *et al.* 2002; Oh *et al.* 2009). For example, CCN2 expression in pig
9 trophoblast cells diminishes soon after the blastocyst stage, while in humans, it is maintained until
10 implantation. CCN2 expression dynamics during bovine preimplantation development have not yet
11 been analyzed.

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

1 that CCN2 likely plays important roles not only in bovine TE formation, but also in the overall
2 development of the bovine blastocyst, including the ICM.

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

1 **MATERIALS and METHODS**

2 *Preparation of bovine embryos*

3 Bovine oocyte retrieval, and *in vitro* oocyte maturation, fertilization, and subsequent *in vitro*
4 embryo culture were performed as previously described (Nagatomo *et al.* 2013). Briefly,
5 cumulus-oocyte complexes (COCs) collected from slaughterhouse-derived ovaries were matured via
6 culture (20–22 h, at 38.5°C, in a humidified atmosphere with 5% CO_2 in air) in TCM-199 medium
7 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5% (v/v) FBS. *In vitro*-matured
8 oocytes were transferred to Brackett and Oliphant (BO) medium (Brackett & Oliphant 1975)
9 containing 2.5 mM theophylline (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 7.5
10 μg/mL Heparin Sodium Salt (Nacalai Tesque, Inc., Kyoto, Japan). Subsequently, frozen-thawed
11 semen was centrifuged at 600 × g for 7 min in BO medium, and the isolated spermatozoa were
12 added to the COCs at a final concentration of 5 × 106 cells/mL. After 12 h of incubation, the
13 presumptive zygotes were denuded by pipetting, and cultured (8 days, at 38.5°C, in a humidified
14 atmosphere with 5% CO_2 and 5% O_2 in air) in mSOFai medium (Aono *et al.* 2013). A Holstein cow
15 on campus of Hokkaido University was administered 25 mg of prostaglandin F2α (Pronalgon F;
16 Schering-Plough K.K., Tokyo, Japan) 20 days prior to artificial insemination (AI) followed by
17 intravaginal indwelling of the controlled intravaginal drug release (CIDR; 1.9 g of progesterone;
18 Eazi-Breed CIDR Cattle Insert; Pfizer Animal Health, New York, NY, USA) device from 9 to 3 days

1 prior to AI. Estradiol benzoate (2 mg; Ovahormon, Injection; Aska Animal Health, Tokyo) was
2 simultaneously injected with CIDR insertion. From 5 to 3 days prior to AI, follicle stimulating
3 hormone (Antrin R-10; Kyoritsu Seiyaku, Tokyo, Japan) consisted of twice-daily (morning and
4 afternoon) with a decreasing dose (6, 6, 5, 5, 4, and 4 AU per injection) were administered. At the
5 fifth and sixth FSH treatments, 30 and 20 mg of prostaglandin F2α were injected, respectively. On
6 the day of AI, 200 μg of gonadotropin releasing hormone (Conceral; ASKA Pharmaceutical Co.,Ltd.,
7 Tokyo, Japan) was administrated. The superstimulated cow was inseminated (day 0), and day-14
8 conceptuses were subsequently collected via non-surgical flushing in warm lactated Ringer's
9 solution (Nippon Zenyaku Kogyo Co.,Ltd, Fukushima, Japan).
10
11 *Quantitative Reverse-Transcription PCR*
12 The total RNA from five oocytes or embryos per biological replicate was isolated using the
13 ReliaPrep RNA Cell Miniprep System (Promega, Fitchburg, WI, USA), according to the
14 manufacturer's instructions. cDNA was then synthesized from the oocyte/embryo RNA using
15 ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative PCR (qPCR) was
16 performed using THUNDERBIRD SYBR qPCR Mix (Toyobo), and the specified primers
17 (Supplemental Table S1). The utilized thermal cycling conditions consisted of one cycle of 95°C for
18 30 s (denaturation), followed by 50 cycles of 95°C for 10 s (denaturation), the appropriate annealing

1 temperature for each primer set for 15 s (primer annealing), and 72°C for 30 s (extension). Relative
2 mRNA abundance was calculated using the ΔΔCt method, where *H2A histone family member Z*
3 *(H2AFZ)* was used as a reference gene for each sample. All experiments were performed in
4 triplicate.
5
6 *Immunofluorescence and Confocal Microscopy*
7 CCN2 immunofluorescence was analyzed in both bovine oocytes and embryos as
8 previously described (Akizawa *et al.* 2018). Briefly, the zona pellucida was removed from bovine
9 oocytes and embryos using 0.05% (w/v) Pronase (Sigma-Aldrich, St. Louis, MO, USA). Both
10 oocytes and embryos were fixed in 4% (w/v) paraformaldehyde (Wako Pure Chemical Industries,
11 Ltd.) in phosphate-buffered saline (PBS) for 60 min, and then permeabilized for 60 min using 0.2%
12 (v/v) Triton X-100 in PBS. They were then blocked for 45 min in Blocking One (1:5; Nacalai Tesque,
13 Inc.) solution diluted in 0.05% (v/v) Tween 20 in PBS (blocking buffer), and incubated in rabbit
14 anti-CCN2 antibody (1:300, ab6992; Abcam, Cambridge, UK) overnight at 4°C. After five 10-min
15 washes in 0.1% (v/v) Triton X-100 and 0.3% (w/v) bovine serum albumin (Sigma-Aldrich), the
16 oocytes and embryos were incubated with Alexa Fluor 555 goat anti-Rabbit IgG polyclonal
17 antibodies (diluted to 1:400 in blocking buffer; ab150082; Thermo Fisher Scientific, Inc.) for 30 min
18 at room temperature. Nuclei were counterstained with 25 mg/mL Hoechst 33342 stain

1 (Sigma-Aldrich) prepared in 0.2% (w/v) polyvinyl alcohol in PBS. Fluorescence signals were
2 observed using a TCS SP5II confocal laser-scanning microscope (Leica, Wetzlar, Germany). In each
3 experiment, at least five oocytes/ embryos were observed.
4
5 *Microinjection of CCN2-shRNA expression vectors into bovine embryos*
6 shRNA-mediated RNA interference (RNAi) for *CCN2* was induced as previously described
7 (Akizawa *et al.* 2018). Briefly, an shRNA containing antisense/sense regions, a 19-bp loop
8 (5′-CTGTGAAGCCACAGATGGG-3’), and a 6-bp terminator element (5′-TTTTTT-3’), was
9 designed to target nucleotides 665–683 of the *CCN2* mRNA sequence (NCBI Reference Sequence
10 NM_174030), and ligated downstream of the U6 promoter in the pBAsi/mU6 Neo vector (Stratagene,
11 CA, USA). Embryos injected with pBAsi/mU6 Neo plasmids lacking the shRNA insert (empty
12 vector) were used as controls, as previously described(Nagatomo *et al.* 2013; Akizawa *et al.* 2016).
13 Twelve hours after insemination, the synthesized shRNA-expression constructs (diluted to a final
14 concentration of 10 ng/mL with mSOFai medium) were injected into denuded presumptive zygotes
15 using a FemtoJet injection device (Eppendorf, Hamburg, Germany). These presumptive zygotes
16 were then cultured to the blastocyst stage to examine the effect of *CCN2* KD on the expression of
17 pluripotency-related genes.
18

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.

1 **RESULTS**

2 *CCN2 mRNA expression levels during preimplantation development*

3 The conducted qPCR analysis of *CCN2* mRNA expression dynamics in bovine oocytes and
4 embryos detected *CCN2* mRNA at all of the examined stages, (i.e. metaphase MII oocytes, and 2-, 4-,
5 8-, 16-, morula, and blastocyst-stage embryos) (Fig. 1A). Notably, *CCN2* expression significantly
6 decreased between the 16-cell and the blastocyst stages of development ($P < 0.05$) (Fig. 1A). Next,
7 elongating embryos were collected from cows at day 14 after artificial insemination via uterine
8 flushing, to investigate *CCN2* mRNA expression after the blastocyst stage. The results of this
9 analysis revealed that *CCN2* expression levels in the elongating conceptuses was significantly
10 increased compared to that observed in blastocyst-stage embryos ($P < 0.05$) (Fig. 1B).
11
12 *Subcellular CCN2 protein localization patterns during preimplantation development*
13 Next, immunostaining was performed to visualize and evaluate the subcellular localization
14 of CCN2 during early bovine embryogenesis. As for *CCN2* mRNA expression, CCN2 was detected
15 at all of the examined stages (Fig. 2), and shown to consistently localize to both the cytoplasm and
16 nucleus of each blastomere from the 2-cell to the blastocyst stage (Fig. 2). Notably, at the blastocyst
17 stage, the CCN2 fluorescent signals appeared strongest in the nucleus.
18

1 *Effects of CCN2 mRNA suppression on the expression of pluripotency-related genes in blastocysts*

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

1 **DISCUSSION**

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

10 Firstly, we revealed that *CCN2* mRNA is detectable throughout early bovine embryogenesis.
11 *CCN2* expression levels were higher in oocytes and embryos prior to major zygotic genome
12 activation (ZGA) (which occurs in cattle after the 8- to 16-cell stages) (Khan *et al.* 2012) than in the
13 subsequent-staged embryos. These results suggest that maternal *CCN2* mRNA may persist, and
14 direct bovine embryogenesis until ZGA, in a similar manner to maternal SMAD2/3 (which are
15 known to be upstream regulators of *CCN2* in bovine embryos) (Zhang *et al.* 2015). The fluorescent
16 signals for CCN2 proteins detected after the 16-cell stage in Fig. 2 might mainly be derived from
17 maternally expressed *CCN2* mRNA transcribed before the 8-cell stage. Conversely, we observed a
18 significant increase in *CCN2* expression in elongating conceptuses compared to blastocysts. The

1 elongation of bovine conceptus is accompanied by a remarkable proliferation of trophoblast cells
2 (Betteridge and Fléchon 1988). In fact, *CCN2* has been shown to be associated with cell proliferation
3 in both cattle and other mammals (Zhou *et al.* 2008); thus, it may mediate the elongation of bovine
4 embryos after the blastocyst stage.

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

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

1 essential for maintaining NANOG expression at the blastocyst stage (Simmet *et al.* 2018).
2 Consistent with this, the remarkable reduction of *NANOG* observed in the *CCN2* KD embryos in the
3 present study may be a result of downregulated *OCT4* expression. CCN2 has also been shown to
4 interact with various cell signaling pathways, including the FGFR and TGFB pathways, during
5 murine blastocyst-stage cell lineage segregation (Abreu *et al.* 2002; Crean *et al.* 2004). Further
6 studies are needed be elucidate the pathways by which CCN2 contributes to cell lineage segregation
7 in bovine embryos.

8 In conclusion, the present study demonstrates that *CCN2* is expressed throughout
9 development until implantation, and contributes to the transcriptional regulation of
10 pluripotency-related genes in both TE and ICM cell lineages in bovine blastocysts. In both cattle and
11 other mammals, the correct establishment and function of complex transcriptional networks enables
12 preimplantation embryos to develop to the blastocyst stage. Continued research is needed to decipher
13 the role(s) of *CCN2* in bovine-specific preimplantation after the blastocyst stage of development.
14

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7
8

1 **Figure legends**

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

10

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

16

17 **Figure 3. Effects of *CCN2* knockdown (KD) on the expression of pluripotency-related genes.**
18 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 ($P < 0.05$).

A

Fold expression relative to MII

1.4
1.2
1
0.8
0.6
0.4
0.2
0

ab abc a bc c c c

MII 2C 4C 8C 16C MO BL

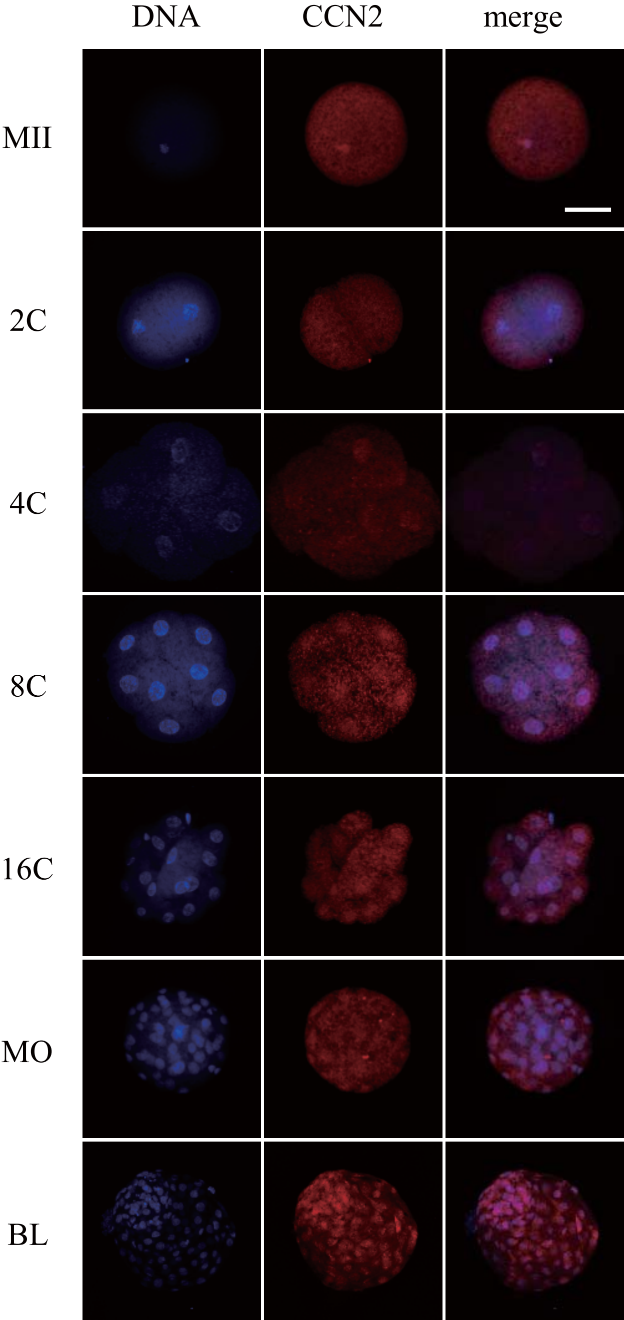
B

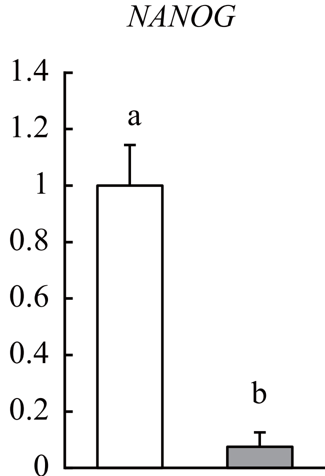
Fold expression relative to blastocyst

15
10
5
0

a b

Blastocyst D14


DNA
CCN2
merge
MII
2C
4C
8C
16C
MO
BL



OCT4
NANOG
Fold expression relative to Empty
1.2
1
0.8
0.6
0.4
0.2
0
1.4
a
b
Empty
CCN2KD

Supplemental Table S1 Primer sets for the quantitative RT-PCR analysis

Gene	Accession number	Primer sequence (5`-3`)	Annealing temperature	Product length (bp)
OCT4	NM_174580	F: ACATGTGTAAGCTGCGGCCC R: CTTTCGGGCCTGCACAAGGG	58	107
NANOG	NM_001025344	F: CTCGCAGACCCAGCTGTGTG R: CCCTGAGGCATGCCATTGCT	58	198
H2AFZ	NM_174809	F: AGAGCCGGTTTGCAGTTCCCG R: TACTCCAGGATGGCTGCGCTGT	58	116