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Comparing spatial expression dynamics of bovine blastocyst under three different procedures: in-vivo, in-vitro derived, and somatic cell nuclear transfer embryos

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Abstract

There has been no work on spatiotemporal transcriptomic differences of blastocysts using in vivo- and in vitro-derived, and somatic cell nuclear transfer (SCNT) embryos. Here, we first compared the lineage-differentially transcriptomic profiles of in vivo- and in vitro-derived embryos by microarray analysis using divided into inner cell mass (ICM)-and trophectoderm (TE)-side samples, as well as those derived from SCNT in order to explore lineage-differentially expressed genes that are associated with preimplantation development in cattle. The transcriptomic profiles of the ICM-specific and TE-specific genes were similar between in vitro-derived embryos and in vivo-derived embryos, whereas SCNT embryos exhibited unusual lineage-differentially gene expression regulation at the blastocyst stage. The genes expressed in a spatiotemporal manner between developmentally normal in-vivo derived blastocysts and developmentally abnormal SCNT blastocysts might play critical roles for preimplantation development. Comparing spatial expression dynamics of bovine blastocyst under three different procedures revealed that *CIITA* was expressed in ICM-side

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samples of all the embryo types. *CIITA* is known as the master regulator of major histocompatibility complexes (MHC) class II genes that express in antigen-presenting cells but its biological function in preimplantation embryo is still unknown in mammals. Knock-down of *CIITA* expression in in vitro-derived embryos did not affect cleavage, but disrupted development of embryos into the blastocyst stage. These findings provide the novel transcriptomic information on blastocyst formation, raising the possibility that immune function-related gene directly plays important roles in bovine preimplantation development.

Key Words: cattle, blastocyst, cell lineage specification, gene expression

Introduction

Blastocyst formation is undoubtedly the first developmental milestone in mammalian ontogeny. The formation of the blastocyst by cavitation of an originally solid cell mass or morula is a crucial stage of normal development because it marks the primary differentiated state of dimorphic cells—i.e., the inner cell mass (ICM) and the trophectoderm (TE). The ICM generates the embryo proper and a portion of extra-embryonic tissues, while the TE gives rise to the embryonic part of the placenta. Differential transcriptional regulation in blastomeres toward either the ICM or TE lineage occurs until the blastocyst stage¹⁹. Impairment of transcriptional regulations underlying binary cell specification events leads to well-documented defects in embryonic viability, such as those observed in *Oct3/4* and *Cdx2* mutant mice^{15,16}.

With the development of transcriptome analyses such as microarrays, the number of global gene expression studies of bovine blastocysts have been increasing gradually. Comparison between in vivo- and in vitro-derived blastocysts revealed that the expression profile of in vitro-derived blastocysts was distinct from that of in vivo-derived blastocysts^{4,7,11}. To assess reprogramming of somatic nuclei, global gene expression analyses have also been performed on somatic-cell nuclear transfer (SCNT) embryos^{2,18,20,21}. However, these analyses were evenly performed by using whole blastocysts; therefore, our knowledge regarding the manner in which transcripts contribute to

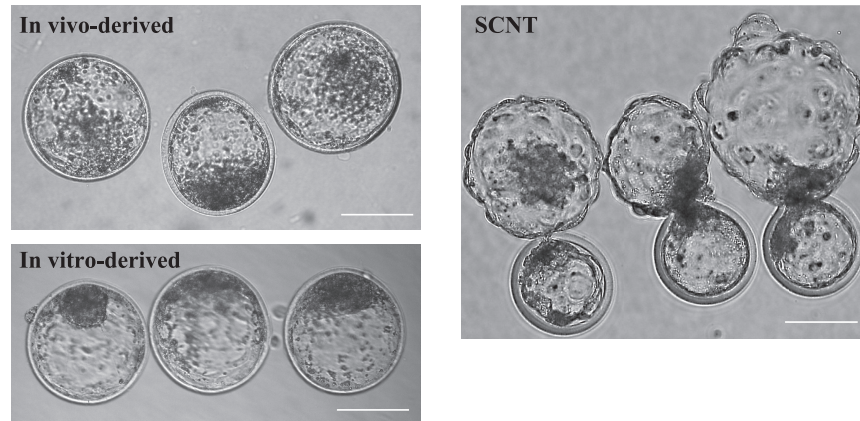
the first cell lineage decision under various conditions—i.e., in vivo-derived, in vitro-derived, and SCNT embryos—is limited.

Recently, we reported an assessment technique to determine which genes are expressed in a lineage-specific manner in the ICM and TE by using in vitro-derived blastocysts dissected using a micromanipulator equipped with a microsurgical blade¹⁴. Through the use of our experimental procedure, we here performed more precise transcriptional profiling of ICM- and TE-side samples under three different embryo types; in vivo-derived, in vitro-derived, and SCNT embryos to explore the gene critical for blastocyst formation in cattle.

Materials and Methods

Preparation of in vivo-derived embryos: Japanese black cows were synchronized using the controlled intravaginal drug release (CIDR; 1.38 g of progesterone; Eazi-Breed CIDR Cattle Insert; Pfizer Animal Health, New York, NY, USA) device for 13 d, and 500 µg of prostaglandin F_{2α} (Schering-Plough K.K., Tokyo, Japan) was administered 2 d prior to device removal. Superovulation was induced by injecting 18 AU of follicle-stimulating hormone (Antrin-R10; Kawasaki Mitaka Pharmaceutical Co., Tokyo, Japan) prior to artificial insemination. On day 7, fertilized embryos at the blastocyst stage were recovered by uterine flushing. To collect the expanded blastocysts (Fig. 1A), the recovered

A



B

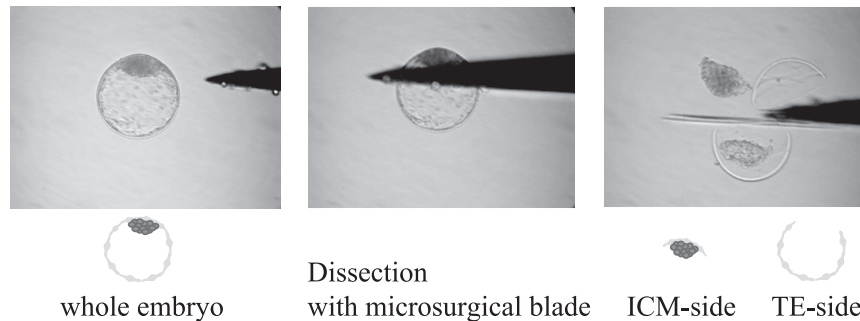


Fig. 1. Preparation of ICM- and TE-side samples by dissecting bovine blastocysts. (A) Mechanical dissection of blastocysts into ICM- and TE-side samples from in vivo-derived, in vitro-derived, and SCNT embryos at the expanded blastocyst stage. Only blastocysts possessing clear ICMs were dissected. Since zona pellucida of SCNT blastocyst had a slit made during micromanipulation, SCNT blastocysts were not hatching from zona pellucida by themselves. Bar = 150 μ m. (B) Each blastocyst was dissected into the ICM and TE sides by using a micromanipulator equipped with a microscurgical blade under an inverted microscope.

embryos were subjected to in vitro culture for 24 h by using the same medium as IVF embryos in our previous report¹⁴, modified synthetic oviduct fluid medium (mSOFaa) supplemented with 10 μ g/mL insulin (Sigma Aldrich, St. Louis, MO, USA), 1 mg/mL polyvinyl alcohol (Sigma Aldrich), and 10 μ M cysteamine (Sigma Aldrich) at 38.5°C in a humidified atmosphere of 5% CO₂. All the animal experiments in this study were approved by the Institutional Animal Care and Use Committee, Hokkaido University, and were performed in accordance with National University Corporation Hokkaido University Regulations on

Animal Experimentation.

Preparation of in vitro-derived embryos: Bovine oocyte retrieval, in vitro oocyte maturation, fertilization, and subsequent in vitro bovine embryo culture were performed according to the methods described in a previous study¹⁴. Briefly, cumulus-oocyte complexes (COCs) collected from slaughterhouse-derived ovaries were matured in TCM-199 (Gibco, Grand Island, NY, USA) and were cultured at 38.5°C in a humidified atmosphere of 5% CO₂ and air for 20–22 hours. In vitro matured oocytes were transferred to Brackett

and Oliphant (BO) medium³⁾ containing 2.5 mM theophylline (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Subsequently, frozen-thawed semen was centrifuged at $600 \times g$ for 8 minutes in BO medium, and the spermatozoa were added to the COCs at a final concentration of 5×10^6 cells/mL. After 18 hours of incubation, presumptive zygotes were denuded and cultured in mSOFaa described above at 38.5°C in a humidified atmosphere of 5% CO₂. The embryos were cultured until expanded blastocysts at day 8 were harvested and used for subsequent experiments.

Preparation of SCNT embryos: The SCNT procedure was performed as described previously²³⁾. Bovine fibroblast cells were obtained from the ear of a 20-d-old Japanese black male calf. The biopsied tissue was minced into small pieces (2 mm²) that were washed 3 times in Dulbecco Phosphate buffered Saline (Gibco, Grand Island, NY, USA) plus 100 U/mL of penicillin (Nacalai Tesque, Inc, Kyoto, Japan) containing 100 µg/mL of streptomycin (Nacalai Tesque, Inc). Tissue explants were then cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco) plus 5% (v/v) FCS at 37.5°C in a humidified atmosphere of 5% CO₂. Culture medium was changed every 2 d, and after 1 week, primary fibroblast cells had assembled around the tissue explants. Explants were then removed and the fibroblast cells were cultured to confluence (passage 0). To passage the fibroblasts, cells were disaggregated by incubating them in PBS plus 0.05% (w/v) trypsin and 0.02% (w/v) ethylenediaminetetraacetic acid (Nacalai Tesque) for 5 min at 38.5°C. The cells were then split 1:3, and confluence was achieved after 6 d. For long-term storage, cells were collected after trypsin treatment, frozen in DMEM supplemented with 20% (v/v) FCS and 10% (v/v) dimethyl sulfoxide, and stored in liquid nitrogen. When used as donor cells for SCNT, they were thawed and cultured in DMEM containing 5% (v/v) FCS and passaged 5–10 times (based on an estimate of two cell doublings per passage). For use as

donor nuclei, cells were cultured for 4–5 d past confluence.

Donor cells were trypsinized, washed by centrifugation, and resuspended in TCM199 supplemented with 0.5% (w/v) bovine serum albumin (BSA) (Sigma Aldrich). Enucleated oocytes at metaphase II of the second meiosis were combined with donor cells in 30 µL of TCM199 supplemented with 0.5% (w/v) BSA, and donor cells were introduced into the perivitelline space of the recipient oocytes through the opening made during enucleation. Doublets were transferred into Zimmerman cell fusion medium²⁴⁾ and induced with two direct current 20-V/mm electrical pulses for 10 µs, with a 0.1 sec interval, using an LF101 cell fusion unit (TR Tech, Tokyo, Japan) equipped with a needle electrode. The electrical pulses simultaneously induced initial oocyte activation. Fused oocytes were further incubated for activation in mSOFaa supplemented with 5% (v/v) FCS, 10 µg/mL cycloheximide (Sigma Aldrich), and 5 µg/mL cytochalasin D (Sigma Aldrich) for 1 h, followed by incubation in 10 µg/mL cycloheximide for 4 h. After washing with mSOFaa, the SCNT embryos were subjected to in vitro culture for 8 d under the same condition as the in vivo- and in vitro-derived embryos for 8 days (Fig. 1A).

Preparation of bovine blastocyst ICM- and TE-side samples: Mechanical dissection of blastocysts into ICM- and TE-side samples was performed for all the embryo types, in vivo-derived, in vitro-derived, and SCNT as described previously¹⁴⁾. Blastocysts were placed in 10 µL of PBS and dissected using a micromanipulator equipped with a microsurgical blade (Feather, Osaka, Japan) under an inverted microscope (Olympus, Tokyo, Japan) as shown in Fig. 1B. After dissection, PBS supplemented with 5% FBS was gently added via a glass capillary, and the dissected cell samples were collected, washed with PBS, and stored in –80°C until use.

Global gene expression analysis: To compare the

gene expression profiles of in vitro-derived blastocysts with those of the other cell samples, we used our previous data that were collected from divided cell samples prepared under the same culture and animal-strain conditions, and using the same bull sperm that was used for the production of in vivo-derived embryos¹⁴). For each analysis, 3 sample types were obtained from 15 different blastocysts per replicate: 5 whole, 5 ICM-side samples, and 5 TE-side samples. We performed microarray analysis ($n = 3$) by using small quantities of total RNA extracted from each sample as described previously¹⁴). Total RNA was extracted in 11 μ L RNase-free water using an RNeasy Micro Kit (Qiagen, Valencia, CA, USA). The Two-Cycle Eukaryotic Target Labeling Kit (Affymetrix, Santa Clara, CA, USA) was used to synthesize cRNA from 9 μ L of total RNA solution. The first cycle of amplification was performed in a 65- μ L reaction volume. After the quality of the amplified product was verified using Experion capillary electrophoresis (Bio-Rad, Hercules, CA, USA), 10 μ g of fragmented cRNA was hybridized to a GeneChip Bovine Genome 430 2.0 Array (Affymetrix) containing 24,027 probe sets. GeneChip Operating Software version 1.3 (Affymetrix) output files were then loaded into GeneSpring version 7.3 (Agilent Technologies, Santa Clara, CA) with per-chip normalization to the 50th percentile and per-gene normalization to the median expression level of the control blastocysts. The filtered genes were analyzed using one-way analysis of variance (ANOVA) and Tukey post-hoc test for significant differences. The cutoff value used to identify differentially expressed genes in our study was a false discovery rate of 5%. Principal component analysis (PCA) was used to analyze the gene expression patterns of the samples. Hierarchical clustering was performed using the Pearson correlation for measurement of the similarity between genes and a clustering algorithm with average linkage. Gene ontology analysis was performed using FatiGO at Babelomics (www.fatigo.org). Three independent experiments were replicated for each sample type

(whole embryos, ICM-side samples, and TE-side samples).

Ingenuity Pathway Analysis: Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc., Red Wood, CA, USA) version 3.0 was used to identify the possible biological pathways and the inter-relationship network between subsets of candidate genes that had interesting patterns of expression as reported previously¹⁰). A detailed description of IPA can be found at www.ingenuity.com. Data sets containing the Affymetrix gene identifiers and their corresponding expression fold change values were uploaded as Excel files. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base.

Network building was performed using the program for querying the Ingenuity Pathways Knowledge Base for interactions between the genes of interest and all the other gene objects stored in the database. A set of networks was then generated, and IPA software was used to compute a score for each network according to the fit of the network to the set of focus genes.

Microinjection of CIITA shRNA expression vectors: An RNA interference hairpin with antisense/sense regions, 12-bp loop (GCTTCCTGTCAC), and a 6-bp terminator element (TTTTTT) was designed to target nucleotides 3222–3242 of *CIITA* mRNA (GenBank XM_585540.6). The top and bottom strand oligonucleotides (Sigma-Aldrich Japan, Tokyo, Japan) were denatured at 95°C for 5 min, and were gradually cooled to 25°C for annealing. The double-strand DNA was ligated downstream of the U6 promoter in the pBasi/mU6 Neo vector (Stratagene, La Jolla, CA). The following sequence was used: 5'-GAT CCGCTTGTACAATAACTGTATCTGCTTCCT GTCACAGATACAGTTATTGTACAAGCTTTT TTA-3' (underlined and double-underlined portions indicate stem loop region and terminator element, respectively). The short hairpin RNA expression vector (pBasi/mU6/CIITA-I) targeting *CIITA*

mRNA (*CIITA* shRNA) was prepared using the EZgene EndoFree Plasmid Isolation Mini Kit (Biomiga, INC., San Diego, CA). Twelve hours after the completion of insemination, zygotes were centrifuged at 15000 rpm for 15 min to visualize their pronuclei. The synthesized *CIITA* shRNA were then injected into cytoplasm of each bovine zygote by using a FemtoJet injection device (Eppendorf, Hamburg, Germany). After *CIITA* knockdown, the *CIITA* mRNA expression at the 2- to 4-cell stage was confirmed by quantitative RT-PCR (qPCR) under the same procedure as reported previously¹⁴. The zygotes injected with *CIITA* shRNA were cultured to examine the effect of *CIITA* knockdown on subsequent embryonic development into the blastocyst stage. In this experiment, zygotes were injected with the pBasi/mU6 Neo without *CIITA* shRNA insert as a control.

Statistical analysis: Statistical analyses of all data for comparison were carried out using analysis of one-way analysis of variance (ANOVA) Fisher PLSD test by using the statistical analysis software Statview (Abacus Concepts, Inc., Berkeley, CA). A *P* value of < 0.05 was considered significant.

Results

Transcriptomic comparison of in vivo-derived, in vitro-derived, and SCNT blastocysts

According to the methods used for in vitro-derived embryos in our previous study¹⁴, we prepared both the ICM- and TE-side samples from in vivo-derived and SCNT blastocysts, and subjected them to global gene expression analysis. Based on the obtained expression data, we performed hierarchical clustering by using GeneSpring GX7.3 software and constructed a dendrogram for each sample (Fig. 2A). In terms of cell lineage, the expression patterns of the TE-side samples were markedly different from those of the whole embryo and ICM-side samples.

With respect to the procedure used to generate embryos, the expression patterns of both ICM- and TE-side samples derived from SCNT embryos differed from those derived from in vivo-derived embryos. Therefore, the cell samples of SCNT embryos exhibited gene expression patterns that substantially differed from those of embryos generated using the other two procedures. Next, the gene expression pattern data obtained for the three different embryo types were compared according to the three cell types (whole embryos, ICM-side samples, and TE-side samples) by performing PCA (Fig. 2B). When the expression patterns of whole embryo samples were compared among the three embryo types, each of them was different from the other two. The expression patterns of in vivo- and in vitro-derived embryos clustered in a similar manner for the ICM- and TE-side samples (Fig. 2B). However, the expression profiles of both ICM- and TE-side samples from SCNT embryos were definitely distant from those of in vivo- and in vitro-derived embryos (Fig. 2B). Thus, our dataset for each separated cell sample clearly showed that the transcriptional profiles of in vivo- and in vitro-derived embryos were similar, whereas SCNT embryos exhibited unique patterns of gene transcription, which were related to the specification of cells into ICM and TE lineages at the blastocyst stage. After GeneSpring normalization, we performed an ANOVA and post-hoc test at a false discovery rate of 5%. The results revealed that 984, 2279, and 2599 genes showed significantly differential expression between the ICM- and TE-side samples from in vivo-derived, in vitro-derived, and SCNT embryos, respectively (Fig. 2C). These genes are listed in Supplemental tables 1-3 (VIVO, VITRO, and SCNT lists).

Gene ontology classification of transcripts differentially expressed in ICM- and TE-sides of blastocysts

To determine the biological roles of the differentially expressed genes, we used FatiGO

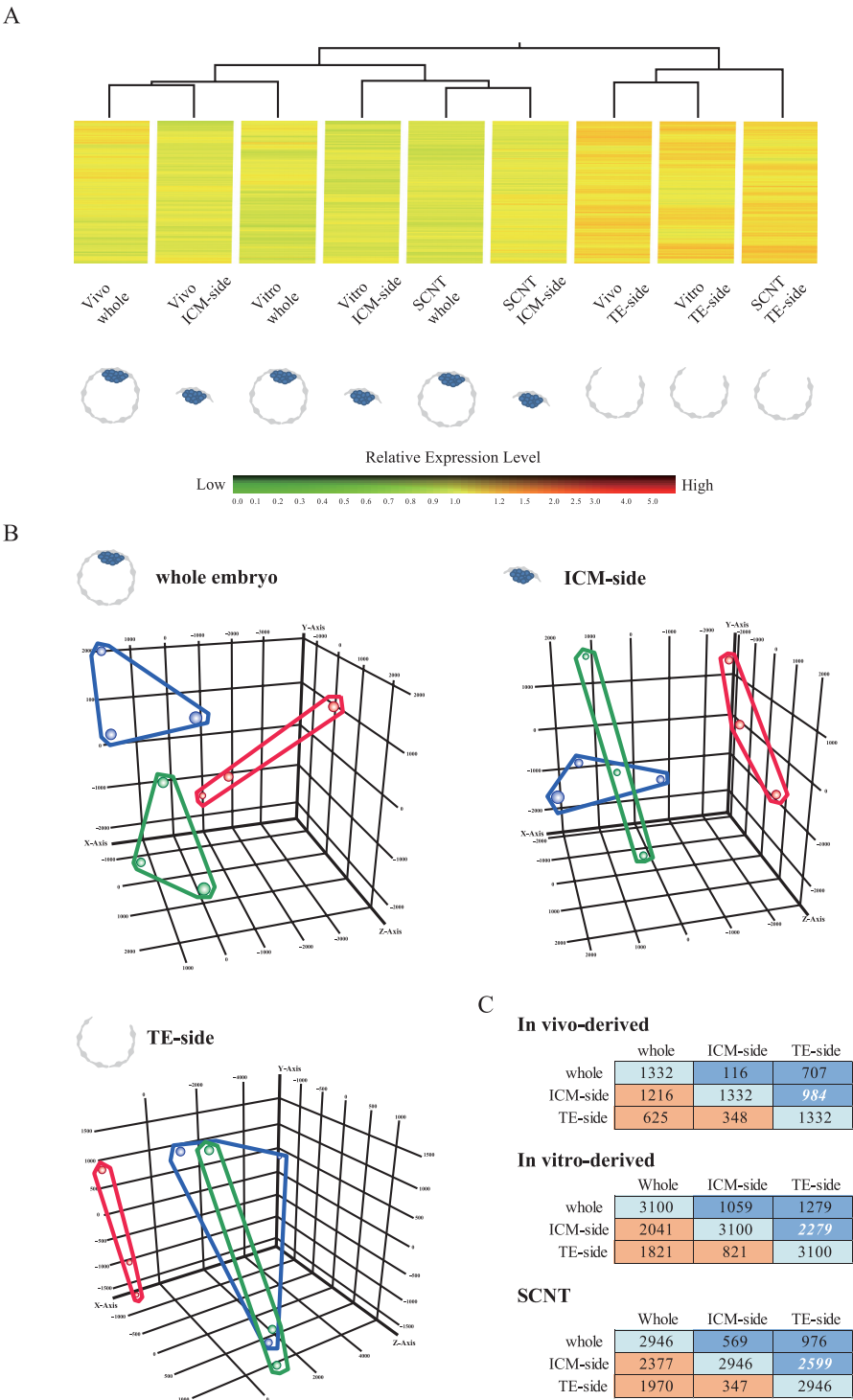


Fig. 2. Transcriptomic analysis for whole embryos, as well as ICM and TE-side samples from in vivo-derived, in vitro-derived, and SCNT blastocysts. (A) Hierarchical clustering of whole embryos, ICM-side, and TE-side samples from in vivo-derived, in vitro-derived, and SCNT blastocysts. Colors correspond to the relative RNA abundance of more than 23,000 transcripts. (B) PCA of gene expression in all the samples subjected to hierarchical clustering analysis (in vivo-derived, blue; in vitro-derived, green; SCNT, red). (C) One-way ANOVA and post-hoc testing for the three types of samples from in vivo-, in vitro-derived, and SCNT blastocysts. Boxes show the number of genes that are statistically different (blue) or similar (orange) based on a group-to-group comparisons.

at Babelomics (www.fatigo.org) to perform an ontological comparison of the probe sets segregated using the post-hoc test (Supplemental figures 1–3). Gene ontology (GO) classification provides controlled vocabularies for the descriptions of the molecular functions, cellular components, and biological processes of analyzed genes. In the molecular function category, 1, 9, and 4 terms were significantly different between the ICM- and TE-side samples from in vivo-derived, in vitro-derived, and SCNT embryos, respectively (Supplemental figure 1), whereas 13, 26, and 21 terms were significantly different in the cellular component category, respectively (Supplemental figure 2). In the biological process category, 9, 16, 18 terms were significantly different between the ICM- and TE-side samples from in vivo-derived, in vitro-derived, and SCNT embryos, respectively (Supplemental figure 3).

We examined the molecular function terms associated with ICM-enriched genes from embryos generated using the three procedures and found a number of terms related to transcriptional modulation (Supplemental figure 1), such as nucleic acid binding (GO: 0003676), in embryos generated using all the three procedures; as well as structural constituent of ribosome (GO: 0003735) and RNA binding (GO: 0003723) in both in vitro-derived and SCNT embryos. Meanwhile, in TE-enriched genes were associated with a number of terms related to carriers and transporters (Supplemental figure 1), such as carrier activity (GO: 0005386), in both in vitro-derived and SCNT embryos, as well as ion transporter activity (GO: 0015075) in the in vitro-derived embryos. Therefore, the results of this analysis did not appear to reflect the functional and transcriptional differences between ICM- and TE-side samples among three procedure backgrounds. Interestingly, in both the cellular component and biological process categories, every GO term for in vivo-derived embryos was included in the list of GO terms for in vitro-derived embryos, suggesting that the transcriptional dynamics resulting from ICM and TE lineage

specification were homologous between in vivo- and in vitro-derived embryos (Supplemental figure 2A and B, Supplemental figure 3A and B). However, a number of GO terms for SCNT embryos were entirely distinct from other fertilized embryos, including non-membrane-bound organelle (GO: 0043228), plasma membrane (GO: 0005886), intracellular organelle (GO: 0043229), plasma membrane part (GO: 0044459), intracellular organelle part (GO: 0044446), intracellular non-membrane-bound organelle (GO: 0043232), and proton-transporting two-sector ATPase complex (GO: 0016469) in the cellular component category (Supplemental figure 2C). Furthermore, in the biological process category (Supplemental figure 3C), many GO terms were specific in SCNT embryos as follows, catabolic process (GO: 0009056), cellular catabolic process (GO: 0044248), group transfer coenzyme metabolic process (GO: 0006752), cofactor biosynthetic process (GO: 0051188), DNA metabolic process (GO: 0006259), cellular macromolecule catabolic process (GO: 0044265), RNA processing (GO: 0006396), coenzyme biosynthetic process (GO: 0009108), mRNA processing (GO: 0006397), RNA splicing (GO: 0008380), and regulation of small GTPase mediated signal transduction (GO: 0051056). In general, GO terms associated with catabolism were characteristically expressed in ICM cells in SCNT embryos (Supplemental figure 3C).

Expression of lineage-differential genes in embryos generated using various procedures and their role in preimplantation bovine development

To gain a deeper understanding of the significance of lineage-differential transcription for cell specification at the blastocyst stage, we performed IPA for all the genes that showed significant differential expression in the ICM- and TE-side samples from in vivo-derived, in vitro-derived, and SCNT embryos (Supplemental tables 1–3). Based on their known biological relationships, which had been described previously¹⁰⁾, the genes in these three lists were

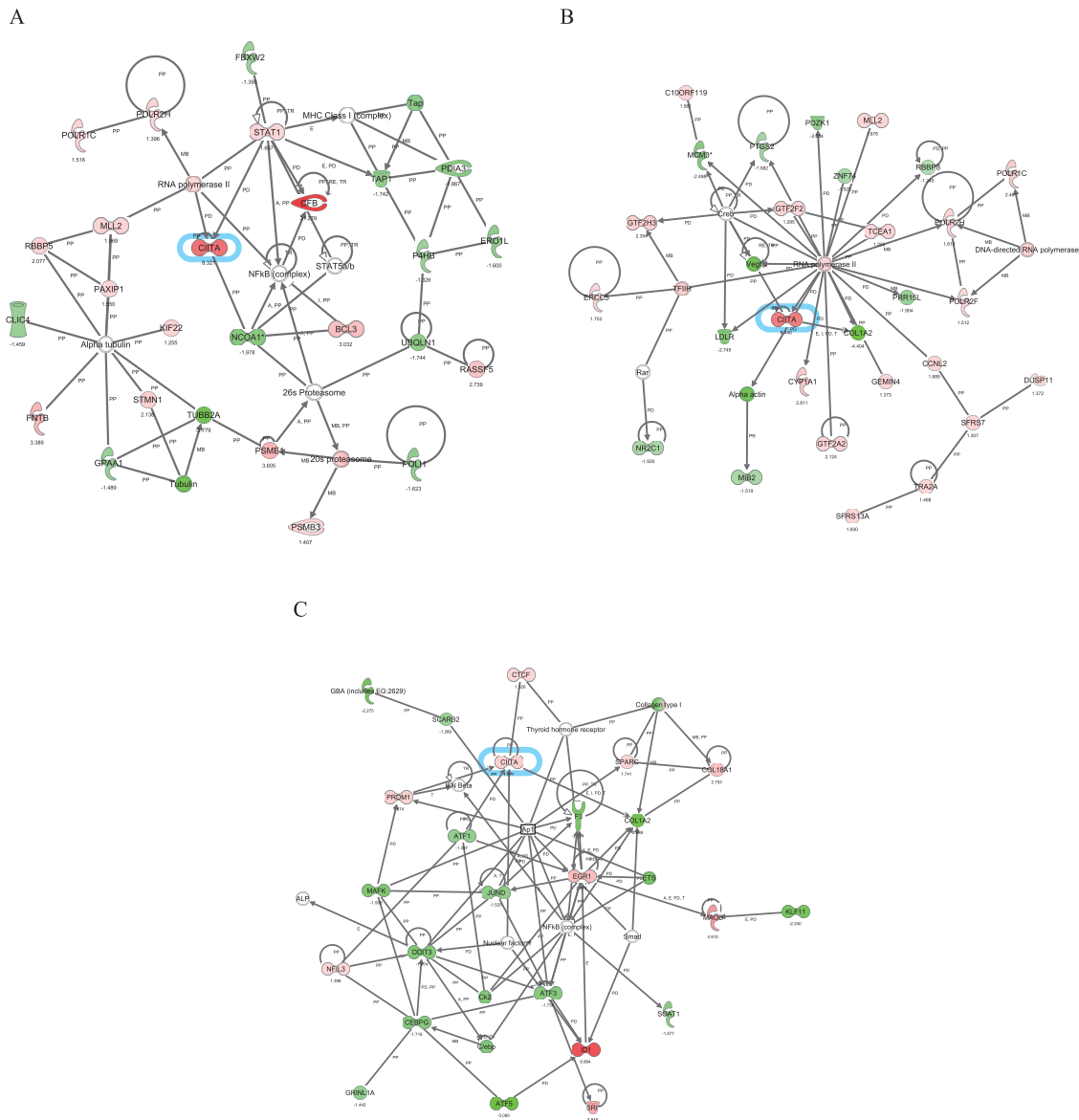


Fig. 3. ICM-exclusively expressed gene in all embryo types evaluated using IPA and its roles during preimplantation development. We constructed functional networks by integrating the genes identified in Fig. 2C (Supplemental figures 4–6). We focused on three networks containing *CIITA*, which was consistently expressed in the three embryo types; in vivo-derived (A), in vitro-derived (B), and SCNT (C) (corresponding to Supplemental figures 4_3P, 5_1C, and 6_3N). Genes marked in red and green are upregulated and downregulated in the ICM, respectively. The network is displayed graphically as nodes (gene or gene product) and edges (the biological relationships between nodes, including the functional or physical interactions). The shape of the nodes (e.g., circle and diamond) indicates whether the type of protein (structural protein, transcription factor, etc.) (refer to Supplemental figure 7).

integrated into IPA pathways. The results of the integration showed that 16, 21, and 14 networks were produced from the VIVO, VITRO, and SCNT gene lists, respectively (Supplemental figures 4–7).

Of these genes, we focused on *CIITA*, which

was only the transcription regulator and consistently expressed in ICM-side samples from all three types (Fig. 3A–C). The genes detected in all the embryo types may play important roles in bovine blastocyst formation, because it is highly probable that genes expressed in a spatiotemporal

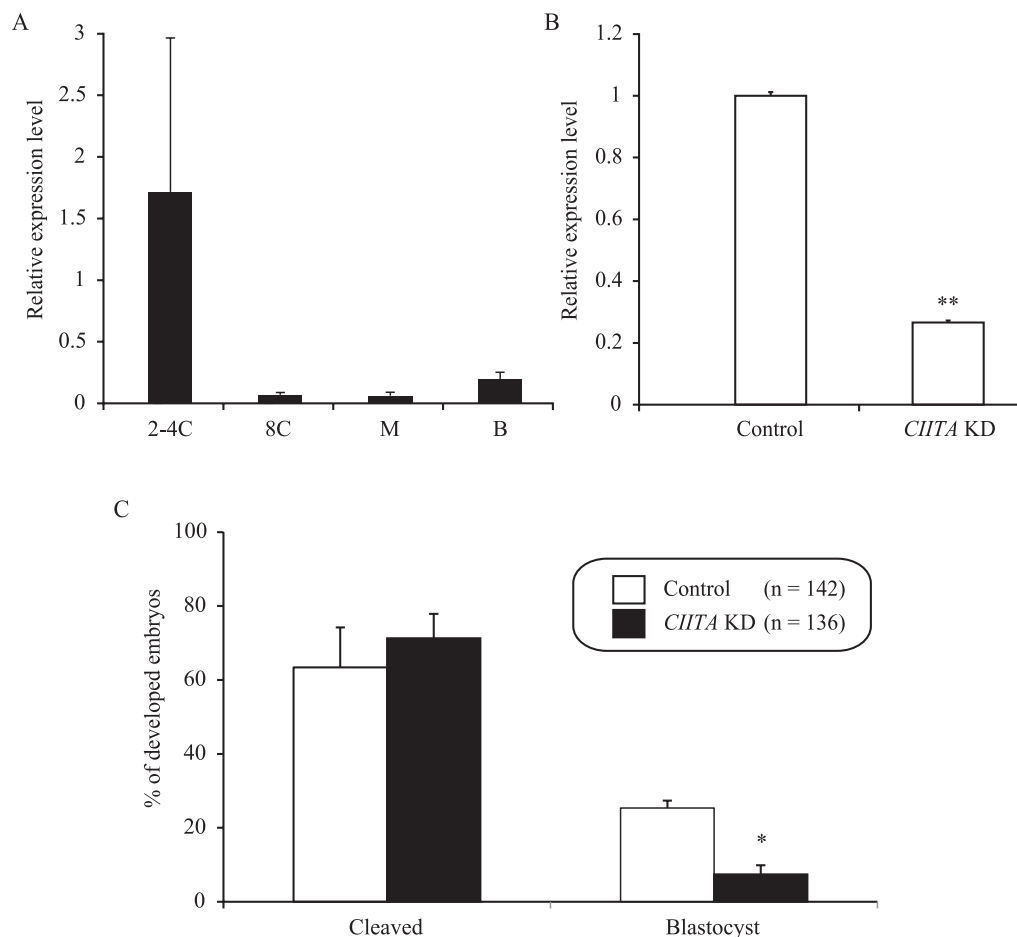


Fig. 4. The critical role of *CIITA* mRNA expression for bovine preimplantation development in vitro. (A) Transcriptional dynamics of *CIITA* mRNA during preimplantation development. The results are represented as the mean $\pm$ s.e.m. (error bars) of three replicate experiments. (B) RNAi by microinjection of *CIITA*-shRNA expression vector was introduced to downregulate *CIITA* mRNA expression. qPCR was used to detect the mRNA level of *CIITA*. The results are shown as the mean $\pm$ s.e.m. (error bars) of four replicate experiments, ** $P < 0.01$. KD: knockdown. (C) Effect of *CIITA* knockdown on subsequent embryonic development into the blastocyst stage. Values are represented as means $\pm$ s.e.m. (indicated by error bars). Three independent experiments were replicated. Approximately 40 to 50 embryos were used for *CIITA* knockdown and control. The asterisk indicates $P < 0.05$.

manner between developmentally normal in-vivo derived blastocysts and developmentally abnormal SCNT blastocysts possess critical roles for preimplantation development into the blastocyst stage. *CIITA* is known as the master regulator of MHC class II gene transcription and is expressed in antigen-presenting cells such as dendritic cells, macrophages, and B cells⁵. We first investigated the *CIITA* mRNA expression dynamics during preimplantation development (Fig. 4A). The *CIITA* mRNA expression was highest at the 2- to 4-cell stage during preimplantation development. To examine the role of *CIITA* in early bovine

embryogenesis, we further performed an RNA interference experiment by microinjecting *CIITA* shRNA expression vectors into in vitro-derived zygotes. The relative expression level of *CIITA* was significantly decreased in the zygotes injected with *CIITA* shRNA ($P < 0.01$) (Fig. 4B). The *CIITA*-knockdown embryos showed a lower rate of blastocyst formation compared to controls ($7.4 \pm 2.5\%$ vs $25.4 \pm 2.0\%$; $P < 0.05$) (Fig. 4C). Taken together, these results clearly show the importance of *CIITA* expression within ICM for embryo survival during preimplantation bovine embryo development.

Discussion

The blastocyst stage is a critical period for fate specification of blastomeres and is the time when the first cell segregation event into ICM and TE lineages occurs. We previously determined the genes expressed in a lineage-differentially manner in the ICM and TE by mechanically separating the ICM and TE sides of in vitro-derived blastocysts. However, because only in vitro-derived embryos were analyzed, we were unable to determine the effects of embryo production procedures on the transcriptomic profiles of cells undergoing lineage specification at the blastocyst stage¹⁴. In vitro culture and/or nuclear reprogramming after somatic cell nuclear transfer into enucleated ooplasm might affect the transcriptional machinery involved in establishing lineage specification at the blastocyst stage.

In the conventional assessments from other groups, transcriptional differences between in vivo- and in vitro-derived whole blastocysts including both ICM and TE cells have been reported^{4,7,11}. Our comparison of whole embryos was consistent with these results (Fig. 2B). However, our PCA analysis results revealed that both the ICM and TE cells from in vitro-derived embryos had the similar expression patterns to those from in vivo-derived embryos, as well as ontological comparison using FatiGO. In vitro-derived embryos have been used for experimental materials instead of in vivo-derived embryos in cattle, because it is difficult to prepare a number of in vivo-derived embryos from large animals such cattle. Our results provide the clear demonstration that transcriptomic profile of in vitro-derived embryos is similar to that of in vivo-derived embryos from the aspect of cell differentiation at the blastocyst stage.

In previous studies performed using whole SCNT-generated blastocysts, the gene expression profiles of SCNT and fertilized bovine embryos differed markedly^{2,18,20,21}. Moreover, unusual GO terms, including those involved in cellular catabolism, were associated with the expression

profiles of SCNT blastocysts, but not with those of in vivo- and in vitro-derived blastocysts²⁰. In the present study, we found that GO terms associated with catabolism were characteristic of the ICM-side cells of SCNT embryos. Therefore, the atypical expression profile of whole SCNT blastocysts might be attributable to disrupted expression in ICM cells.

Next, in order to determine developmentally critical genes unaffected by embryo production procedures, we analyzed ICM- and TE-predominant gene expression in all the embryo types performing IPA¹⁰. We focused on *CIITA*, only a transcriptional regulator that was consistently detected in ICM-side samples of three embryo types (Fig. 3A–C), because we had addressed the site-specific *CIITA* expression in ICM of in vitro-derived blastocyst¹⁴ and *CIITA* was one of transactivators to regulate the other gene expression and cell differentiation¹. Interestingly, in mouse and human trophoblast cells, *CIITA* expression is silenced, and therefore, MHC class II genes are also repressed^{8,12,13,22}. Thus, silencing of *CIITA* transcription was speculated to protect fetuses possessing paternally derived MHC class II genes from allogeneic rejection reactions by the mother. However, it has not been addressed whether *CIITA* expression plays a direct role in preimplantation development in the bovine species. When the bovine embryos were injected with *CIITA* shRNA expression vectors, the blastocyst formation rate was significantly lower. *CIITA* is known to regulate an “on/off” for MHC class II gene transcription through its interaction with several octamer-binding proteins, such as Oct1 and Oct2, that regulate the transcription of other genes as transcription factor⁶. Ectopic expression of *CIITA*, and perhaps MHC class II genes, in the ICM might play an important role in differentiating cells into the ICM and TE lineages in bovine preimplantation embryos.

Our findings raise the possibility that immune function-related genes are associated with preimplantation bovine development. Using a comparative global expression profiling approach

for ICM and TE, a subset of genes that are differentially expressed in a broad range of mammalian species, excluding mice, has been identified^{9,14,17}. Cross-species gene expression comparisons of ICM and TE transcriptomes could provide deeper insight into the factors required for induction and maintenance of cell pluripotency in mammals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.14943/jjvr.63.4.159>

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