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Author(s)	Kohri, Nanami; Akizawa, Hiroki; Iisaka, Sakie; Bai, Hanako; Takahashi, Masashi; Kawahara, Manabu
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The role of RHOA signaling in trophectoderm cell-fate decision in cattle

Nanami Kohri[†], Hiroki Akizawa[†], Sakie Iisaka, Hanako Bai, Masashi Takahashi, and
Manabu Kawahara*

Laboratory of Animal Breeding and Reproduction, Research Faculty of Agriculture,
Hokkaido University, Kita-ku, Kita 9, Nishi 9, Sapporo 060-8589, Japan

E-mail addresses: nana7mi3@shinshi-u.ac.jp (N. Kohri), akizwa-h@anim.agr.hokudai.ac.jp (H. Akizawa), s_iisaka@anim.agr.hokudai.ac.jp (S. Iisaka),
hbai@anim.agr.hokudai.ac.jp (H. Bai), [mmasashi@anim.agr.hokudai.ac.jp](mailto:mamasashi@anim.agr.hokudai.ac.jp) (M.
Takahashi), k-hara@anim.agr.hokudai.ac.jp (M. Kawahara)

*Correspondence:

Dr. Manabu Kawahara, PhD

Laboratory of Animal Breeding and Reproduction, Graduate School of Agriculture,
Hokkaido University, Sapporo 060-8589, Japan

Tel. & Fax: +81 11 706 2541

E-mail address: k-hara@anim.agr.hokudai.ac.jp

[†]These authors equally contributed to this study.

Abstract

Mammalian blastocysts are composed of two distinct cell lineages, namely the inner cell mass (ICM) and trophectoderm (TE). TE cells that give rise to the embryonic placenta are marked by an exclusive expression of the key determinant transcription factor, CDX2. Although Hippo signaling pathway is known to be responsible for this TE-specific expression of CDX2, the upstream regulator of this pathway in mammalian embryos is still controversial. In the present study, the involvement of the small molecular G protein, RHOA, in TE cell-fate decision in cattle was investigated. Inhibition of RHOA by the specific inhibitor, C3 transferase (C3), severely impaired the blastocyst formation. Further, C3 treatment significantly decreased the number of blastomeres with nuclearized YAP1, the prominent effector of Hippo pathway. An artificial isolation of ICM cells from blastocysts followed by the continuing culture to regenerate TE cells was conducted and showed that TE re-emergence from the isolated ICM is governed by Hippo pathway and suppressed by C3 treatment like that observed in developing embryos. Finally, the long-term exposure to C3 suggests the presence of alternative regulators of CDX2 expression other than RHOA signaling because there were still CDX2-positive cells after C3 treatment. These results demonstrated that

RHOA signaling plays a significant role in TE cell-fate decision by regulating Hippo pathway in cattle.

Keywords

Trophectoderm, Blastocyst, Bovine, Hippo signaling pathway, RHOA activity, differentiation

Abbreviations

TE, trophectoderm; ICM, inner cell mass; YAP1, yes-associated protein 1; TEAD4, TEA domain transcription factor 4; CDX2, caudal type homeobox 2; OCT4, POU class 5 homeobox 1 (POU5F1); RHOA, ras homolog family member A; ROCK, Rho associated coiled-coil containing protein kinase; C3, C3 transferase; iICM, isolated ICM; IVF, *in vitro* fertilization; D, days after IVF

1. Introduction

The first cell-fate decision in mammalian embryo is the segregation into two cell lineages, namely the trophectoderm (TE) and inner cell mass (ICM)[1]. These become clear at the blastocyst stage when TE surrounds the outer margin of blastocoel and ICM is positioned at the polar side of the embryo. TE and ICM are the progenitors of extraembryonic tissues and the embryo proper, respectively[1]. Thus, the proper spatiotemporal differentiation of TE and ICM is essential for normal development, which is fine-tuned by complicated mechanical and molecular cues[2]. Although there are some exceptions, the blastomeres positioned at the relatively outer part of the embryo at the morula stage tend to differentiate into the TE and the inner blastomeres differentiate into the ICM toward the blastocyst stage[3]. This relationship between the spatial distribution and cell-fate decision in each blastomere is widely recognized as the ‘inside-outside’ model[3].

This inside-outside model is explained by the Hippo signaling pathway, which is a broadly conserved phosphorylation cascade among vertebrates[4]. Hippo pathway is turned off in the outermost cells in the murine morula embryo, resulting in dephosphorylation and subsequent nuclearization of YAP1[5]. Nuclearized YAP1 interacts with a transcription factor, TEAD4, to induce TE regulator expression,

including that of *CDX2*[6]. In contrast, YAP1 is phosphorylated in the inner cells and prevented from localization to the nuclei, thereby inducing the expression of pluripotency-related factors, such as *OCT4*[5]. These initial inputs in each blastomere at the morula stage are enhanced, and consequently, contrasting expression patterns of *CDX2* and *OCT4* are accomplished in the TE and ICM, respectively[7]. The signal transduction composed of YAP1-TEAD4-*CDX2* in the Hippo pathway is evidenced to regulate TE differentiation in preimplantation embryos in various species including cattle[5,8]. However, the upstream regulators of Hippo pathway that convert the positional information to molecular signaling remain to be elucidated.

One of the candidate enzymes for the upstream regulation of the Hippo pathway is ras homolog family member A (RHOA)[9]. RHOA activity is site-specific and is more pronounced in the TE than that in the ICM in mouse blastocysts[9]. The association between RHOA activity and TE specification has been studied using specific inhibitors and catalytically deficient dominant negatives, which indicated that RHOA suppresses the phosphorylation of Amot and restricts the interaction between Amot and Nf2 to switch off the Hippo pathway in the outer cells [9]. Rho associated coiled-coil containing protein kinase (ROCK) is the major target of RHOA in the context of cell lineage differentiation during preimplantation development[10]. Once ROCK is

activated by RHOA, YAP1 translocation into the nuclei is stimulated in the TE cells of mouse blastocyst[10]. On the contrary, activated ROCK inhibits YAP1 nuclearization in bovine embryos, which was demonstrated by the supplementation of ROCK inhibitor resulting in the increase of cells with nuclearized YAP1 [11]. This discrepancy between the mouse and bovine blastocysts may be explained by differences in the species-specific biological properties, such as actin cytoskeleton. Therefore, a more precise understanding of how the RHOA activity regulates YAP1 nucleocytoplasmic shuttling in bovine embryos will be useful to uncover the bovine-specific cell lineage segregation toward the blastocyst stage.

Hence, we investigated the role of RHOA signaling in the specification of TE cell lineage in bovine embryo. We used the synthetic small molecule C3 transferase (C3), which specifically inhibits RHOA signaling [12,13] as well as isolated ICM (iICM) as the model of fate conversion from ICM to TE [8], so as to study the role of RHOA signaling in TE cell-fate decision in cattle.

2. Materials and Methods

2.1. Ethical approval

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

2.2. Bovine *in vitro* fertilization and embryo culture

Bovine oocyte retrieval, *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF), and subsequent *in vitro* embryo culture (IVC) were performed as described previously [14]. Briefly, cumulus-oocyte complexes (COCs) collected from slaughterhouse-derived ovaries were matured by culturing in TCM-199 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 38.5 °C in a humidified atmosphere of 5% CO₂ for 22-24 h. IVM oocytes were transferred to Brackett and Oliphant (B.O.) medium [15] 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 the B.O. medium, and the spermatozoa were added to the COCs at a final concentration of 5 × 10⁶ cells/mL. After 18 h of incubation, the presumptive IVF zygotes were denuded by pipetting and cultured in mSOFai medium [14] at 38.5 °C in a humidified atmosphere of 5% CO₂ and 5% O₂. The start of insemination was regarded as day 0 (D0) after IVC.

2.3. Isolation of ICM from bovine blastocysts

Isolation of the ICM from bovine blastocysts was performed as described previously [8]. Briefly, at D6.5, the zona pellucida of the early blastocyst stage embryos was removed by using 0.05% (w/v) proteinase K (Wako Pure Chemical Industries, Ltd.). To denature only the TE cells, zona-free blastocysts were carefully treated with phosphate buffered saline (PBS) containing 0.2% (v/v) Triton X-100 (Wako Pure Chemical Industries, Ltd.) and 0.2% (v/w) polyvinyl alcohol (PVA) (Sigma-Aldrich, St. Louis, MO, USA) at 20-22 °C for approximately 10 seconds. The embryos were then washed in PBS containing 0.9 mM CaCl₂, 0.33 mM MgCl₂, 10 mg/mL glucose (Wako Pure Chemical Industries, Ltd.), 3.6 µg/ mL sodium pyruvate (Wako Pure Chemical Industries, Ltd.), and 40 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich) for 15 min at 37 °C. Subsequently, to collect ICMs, denatured TE cells were detached by gentle pipetting. The iICMs were individually cultured in droplets of 20 µL of mSOFai medium at 38.5 °C in a humidified atmosphere of 5% CO₂ and 5% O₂.

2.4. Embryo treatment with RHO inhibitor

Embryo treatment with RHO inhibitor was performed according to a previous study [9] with a slight modification. The stock solution containing 0.1 mg/mL global RHO

inhibitor C3 transferase (C3) (Cytoskeleton, Colorado, USA) was stored at -20°C until use. Embryos at the early morula stage at D5.0 (intact) and iICMs derived from early blastocysts at D6.5 were cultured in droplets of mSOFai containing 0.01, 0.02, or 0.1 $\mu\text{g}/\text{mL}$ C3 for 12 h in a humidified atmosphere of 5% CO_2 and 5% O_2 . The mSOFai medium supplemented with the identical amount of distilled water to that of stock solution was used as a vehicle control. After the C3 treatment of embryos, the intact embryos and iICMs were fixed and used for subsequent examinations.

2.5. Immunostaining

Immunofluorescence staining was performed as described previously [8]. Briefly, zona pellucida-removed intact embryos or iICMs were fixed with 4% (w/v) paraformaldehyde (PFA; Wako Pure Chemical Industries, Ltd.) in PBS for 60 min, and then permeabilized for 60 min with 0.2% (v/v) Triton X-100 in PBS. Subsequently, the embryos and iICMs were blocked with PBS containing 20% (v/v) Blocking One (Nacalai Tesque, Inc.) and 0.01% (v/v) Tween 20 (Wako Pure Chemical Industries, Ltd.) for 45 min followed by incubation with primary antibodies overnight. The primary antibodies used were as follows: anti-CDX2 (ab76541, rabbit monoclonal, 1:300, Abcam, Cambridge, UK) and anti-YAP1 (H00010413-M01, mouse monoclonal, 1:100,

Novus biologicals, Littleton, CO, USA). The reaction temperatures for the primary antibodies were 37 °C for CDX2 and 20-22°C for YAP1, respectively. After five washes in 0.1% (v/v) Triton X-100 and 0.3% (w/v) BSA (Sigma-Aldrich) in PBS for 10 min each, the embryos were incubated with secondary antibodies at 20-22°C for 30 min. The secondary antibodies used were as follows: Alexa Fluor 488 goat anti-mouse IgG (A11001, polyclonal, 1:400, Invitrogen, Tokyo, Japan) and Alexa Fluor 555 goat anti-rabbit IgG (A21428, polyclonal, 1:400, Invitrogen). All the secondary antibodies were diluted in PBS containing 5% (v/v) Blocking One and 0.01% (v/v) Tween 20. After the reaction with secondary antibody, embryos were washed and submitted for DNA counterstaining with 25 µg/ mL Hoechst 33342 (Sigma-Aldrich) at 20-22 °C for 5 min. Fluorescence signals were visualized using a LAS X with DMI8 fluorescence microscope (Leica, Tokyo, Japan) with UV filters (480 nm for Alexa 488, 560 nm for Alexa 555, and 350 nm for Hoechst 33342). The proportion of the marker protein (CDX2 or YAP1)-positive blastomeres to all the blastomeres within an embryo was calculated by manual counting of the blastomeres from the immunofluorescence images. For each protein, blastomeres in which nuclear fluorescence signals were obviously stronger than those within the cytoplasm were regarded as positive.

2.6. Statistical analysis

Statistical significance was analyzed using Student's t-test. The data are presented as mean $\pm$ SEM. The percentage data were subjected to an arcsin transformation before analysis. R software (Comprehensive R Archive Network, <https://cran.r-project.org/>) was used for statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of inhibition of RHOA signaling on YAP1 localization in TE cells

We investigated the effect of inhibition of RHOA signaling on the bovine embryonic development by the supplementation of a RHOA-specific inhibitor, C3 transferase (C3), into the culture medium from D5.0 to D5.5 after *in vitro* fertilization (Fig. 1A). The C3 treatment severely impaired the blastocyst formation at any concentration of C3 (Table 1). The lowest concentration of C3 (0.01 $\mu\text{g}/\text{mL}$) did not affect the compaction at D5.5 but the higher concentrations (0.02 and 0.1 $\mu\text{g}/\text{mL}$) kept the blastomeres round and inhibited whole-embryo compaction (Fig. 1B). Next, the YAP1 localization was determined by performing immunofluorescent staining. In agreement with a previous report[16], the embryos in the vehicle control group underwent compaction at D5.5, of which the outermost blastomeres exhibited intense signals of YAP1 within the nuclei (Fig. 1B). Meanwhile, the C3 treatment drastically reduced the rate of nuclear YAP1-positive (YAP1+) blastomeres to the total cell number at any concentration ($P<0.05$, Fig 1B and C). These results suggest that RHOA signaling plays roles in regulating YAP1 nucleocytoplasmic shuttling in the presumptive TE cells at the morula stage.

3.2. Involvement of Hippo signaling in TE regeneration and mediation of RHOA

signaling in the regulation of YAP1 localization

The ICM isolation technique was used as a validation model of TE regeneration to gain detailed insights into the role of Hippo signaling in the mediation of RHOA signaling in YAP1 nucleocytoplasmic shuttling. We previously demonstrated that bovine ICM cells at the early blastocyst stage could transform into the TE to form a functional placenta after removal of the original TE cells[8]. However, whether the process of TE regeneration is similar to that of TE development in developing embryos remained to be elucidated. Therefore, we first performed immunostaining of YAP1 and CDX2 at different time points after the ICM isolation. Both YAP1⁺ and CDX2⁺ blastomeres were absent in the iICMs immediately after the isolation (Fig.2A), which was consistent with our previous observations [8]. Intriguingly, YAP1 started to localize into the nuclei as early as 0.5 h after the isolation (Fig. 2A). The number of YAP1⁺ cells gradually increased during culture, and subsequently, CDX2 began to be expressed at 6h after isolation (Fig. 2A). These continuous expression patterns of YAP1 and CDX2 suggested that cell-fate conversion to TE in the outer blastomeres of iICM was regulated by Hippo pathway as observed at the morula-to-blastocyst transition in developing intact embryos[16]. The effect of RHOA signaling inhibition on TE regeneration from iICM was then evaluated by supplementing 0.01 ug/ mL C3 into the culture medium (Fig. 2B). As shown in Fig.

2C, the C3 treatment significantly reduced the proportions of YAP1- and CDX2-positive blastomeres to total cells in iICMs ($P < 0.05$), respectively. Taken together, these results demonstrated that RHOA signaling is involved not only in the TE cell-fate decision in the developing embryos but also in the conversion from the ICM to TE.

3.3. Effect of long-term inhibition of RHOA on YAP1 and CDX2 expression in developing embryos

Finally, the effect of long-term exposure to C3 was evaluated in intact embryos. The 24 h-treatment of embryos with C3 from D5.0 to D6.0 significantly reduced the rate of both YAP1+ and CDX2+ cells compared to that in the vehicle control (Fig. 3A and B). However, the decrement width of 24 h-treatment group was smaller than that observed for the 12h-treatment group; namely, 30% of CDX2+ cells were observed (Fig. 3A and B). Furthermore, the proportion of YAP1- and CDX2+ blastomeres was rather increased after the 24h treatment, suggesting that alternative mechanism(s) other than RHOA signaling are involved in the maintenance of CDX2 expression (Fig. 3B). Thus, these results indicate that although RHOA signaling regulates the TE cell-fate decision by mediating CDX2 expression, alternative machineries other than RHOA signaling are also associated with the maintenance of CDX2 expression.

Discussion

Trophoblast specification during mammalian blastocyst formation is a stepwise process. First, the spatial allocation of each blastomere within an embryo becomes clear with an increase of the cell number by repeating cleavages. Then, each blastomere reinforces one of the two types of developmental programs: ICM and TE according to the relative position within an embryo. Consequently, inner and outer blastomeres express key determinant factors, such as OCT4 in the ICM and CDX2 in the TE, exclusively. Hippo signaling pathway plays essential roles in the transformation of the positional information into intracellular signaling[5], but the upstream regulator of this pathway is still controversial. Here, we demonstrated that RHOA signaling regulates the subcellular distribution of YAP1, which is a key mediator of Hippo pathway, to specify TE cell-fate in bovine embryos.

The inhibition of RHOA signaling using C3 drastically decreased the rate of nuclearized YAP1 in TE cells (Fig.1). A previous study showed that C3 supplementation inhibited YAP1 nuclearization in TE cells in mouse blastocysts by inactivating ROCK signaling [9]. While the direct inhibition of ROCK signaling by the specific inhibitor, Y27632, also decreased the number of YAP1+ cells in murine embryos, Y27632 treatment

conversely increased YAP1+ cells in bovine blastocysts[10,11]. Thus, the decrease of YAP1+ cells by C3 treatment in the present study was presumably independent of ROCK inactivation. Further, C3 treatment severely impaired blastocyst formation even at a low concentration (0.01 $\mu\text{g/mL}$), which cannot be explained by only YAP1 perturbation because the adverse effect of YAP1 knockdown on the blastocyst formation was shown to be relatively small[16]. Rather, RHOA inhibition on C3 treatment might affect cell properties required for continuing development, such as cell-cell adhesion, because the frequency of the embryos showing compaction was decreased in a C3 concentration-dependent manner (Table 1). Taken together, although the direct target(s) remains to be elucidated, RHOA signaling definitely plays an important role in determining TE cell-fate through the regulation of YAP1 intracellular distribution in bovine embryos.

Bovine ICM cells at the early blastocyst stage were shown to possess the capacity to regenerate functional TE, but the mechanistic similarity between such TE regeneration and general TE differentiation in developing embryos remained to be determined [8]. Our time-series observations revealed that YAP1 started to localize into the nuclei at 0.5 h after TE removal (Fig. 2A). This initiation of YAP1 nuclear localization preceded CDX2 expression that began at 6 h after TE removal, indicating that the induction of TE developmental program in those cells was facilitated by turning off the Hippo pathway

(Fig. 2A). Furthermore, the perturbation of YAP1 nuclear translocation by C3 treatment emphasized the overlap of the upstream regulator of YAP1 localization between regenerated TE from iICM and general differentiated TE in the developing embryos (Fig. 2B and C). Consequently, these results demonstrate high similarity in the molecular machineries between TE re-emergence from iICMs and TE differentiation in developing embryos, suggesting that the experimental system using TE regeneration from iICMs would provide a valuable platform to explore the inside-outside model.

There were still CDX2⁺ cells in the blastocysts even after 24h C3 treatment (Fig.3). This indicated the existence of an alternative signaling pathway(s) that was responsible for the initiation and/or maintenance of CDX2 expression other than RHOA activity in the TE cells. CDX2, which is known to have self-activating property[17], perhaps stimulated the self-expression. Alternatively, other signaling pathways, such as the Notch pathway, which was shown to be able to induce CDX2 expression independent of Hippo pathway, might also be involved[18]. Future studies are needed to elucidate the upstream regulator of CDX2 expression other than RHOA and YAP1 in iICMs so as to uncover the overall mechanisms of TE cell development in blastocysts.

In summary, we demonstrated the involvement of RHOA signaling in the TE cell-fate decision using both developing embryos and iICMs. RHOA signaling stimulates the

expression of the key TE regulator, CDX2, through nuclearizing YAP1. Together with the results obtained using iICM[8], we propose that RHOA activity and the relative position of blastomere within an embryo coordinately determine the TE cell-fate decision in bovine embryo.

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Table 1. Effect of C3 treatment on the blastocyst formation in cattle

Concentration of C3 transferase [$\mu\text{g/ mL}$]	No. of D5.0 embryos tested	No. of D5.5 embryos developed to (% $\pm$ S.E.M)	
		Compacted morula	Early blastocyst
0	35	11 (31.4 $\pm$ 11.1)	14 (40.0 $\pm$ 12.4)
0.01	36	14 (38.9 $\pm$ 15.9)	2 (5.56 $\pm$ 5.13) [†]
0.02	35	7 (20.0 $\pm$ 1.85)	0 [†]
0.1	35	1 (2.86 $\pm$ 3.33)	0 [†]

N.A.: Not applicable; S.E.M.: Standard error of the mean; †: $P < 0.1$ as compared to vehicle controls

Figure legends

Figure 1. The effect of C3 transferase on the transition from morula to blastocyst

A Schematic representation of C3 transferase treatment. Embryos at D5.0 after *in vitro* fertilization were cultured in medium supplemented with C3 transferase for 12 h.

B Representative (upper) light micrographs and (lower) immunofluorescent images of YAP1 of D5.5 embryos treated with different concentrations of C3 transferase for 12h.

DNA was stained with Hoechst 33324. Scale bar = 100 μ m

C The proportion of YAP1 positive cells to total cells in D5.5 embryos treated with different concentrations of C3 transferase for 12h. Data are represented as means $\pm$ S.E.M.

* $P < 0.05$.

Figure 2. C3 transferase treatment in the isolated ICMs

A, B Representative immunofluorescent images of YAP1 and CDX2. (A) Whole embryo at D6.5 and isolated inner cell masses (iICMs) followed by *in vitro* culture. (B) iICMs from D6.5 after the cultivation with or without 0.01 μ g/ mL of C3 transferase for 12 h.

DNA was stained with Hoechst 33342. Scale bar = 100 μ m

C The proportion of YAP1- or CDX2-positive blastomere to total cells in D6.5 iICMs after the cultivation with or without 0.01 μ g/ ml of C3 transferase for 12 h. Data are represented as means $\pm$ S.E.M. Asterisks represent the significant differences (Student's t

test, $P < 0.05$).

Figure 3. The effect of long-term exposure to C3 on TE properties

A Representative immunofluorescent images of YAP1 and CDX2. Whole embryos at D5.0 were treated with 0.01 $\mu\text{g}/\text{mL}$ of C3 for 24h. DNA was stained with Hoechst 33342.

Scale bar = 100 μm

B The proportion of YAP1-positive (YAP1 (+)), CDX2-positive (CDX2 (+)), double positive and double negative blastomere to total cells.

A **B** **C**