Reestablissement of transzonal projections and growth of bovine oocytes in vitro

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Abstract. Transzonal projections (TZPs) that maintain bidirectional communication between oocytes and granulosa cells or cumulus cells are important structures for oocyte growth. However, whether TZPs develop between TZP-free oocytes and granulosa cells, and whether reestablished TZPs support oocyte growth, is unknown. We first examined changes in TZPs after denudation of bovine oocytes collected from early antral follicles (0.5–0.7 mm). Twenty-four hours after denudation, almost all the TZPs disappeared. We also examined the reestablishment of TZPs by coculturing TZP-free denuded oocytes (DOs) with mural granulosa cells (MGCs) collected from early antral follicles. In addition, to confirm if the reestablished TZPs were functional, the reconstructed complexes (DO+MGCs) were subjected to in vitro growth culture and found that the MGCs adhered to TZP-free DOs and TZPs were reestablished. During in vitro growth culture, DO+MGCs developed and formed antrum-like structures. After culture, the number of TZPs in DO+MGCs increased, and the oocytes grew fully and acquired meiotic competence. These results suggest that reestablished TZPs are able to support oocyte growth.

Key words: Bovine oocyte, In vitro growth, Mural granulosa cell, Transzonal projection

Studies of in vitro growth culture of mammalian oocytes have used oocyte-somatic cell complexes or whole follicles [1] because somatic cells—especially granulosa cells and cumulus cells—surrounding oocytes are essential for oocyte growth [2, 3]. In the ovary, oocytes are surrounded by granulosa cells or cumulus cells. Granulosa cells adhere directly to oocytes in the primordial follicles. As the follicles develop, the surrounding granulosa cells transition from squamous to cuboidal morphology, and the zona pellucida is formed around the oocytes. Granulosa cells extend transzonal projections (TZPs) penetrating the zona pellucida to maintain direct contact with the oocytes [2, 3]. Bidirectional communication between oocytes and granulosa cells regulates both types of cells throughout the follicular developmental stages [4, 5]. This communication is thought to be mediated through paracrine signaling and gap junctional communication at the ends of TZPs [2, 3, 5, 6]. Oocyte-derived growth factors regulate granulosa cell development and function, and nutrients and mRNA from granulosa cells or cumulus cells are transported through TZPs for oocyte growth [7–10]. Therefore, TZPs are important structures for the successful growth of oocytes in vitro.

Recently, some studies have reported the reestablishment of TZPs and reconstruction of oocyte-granulosa cell complexes. Barrett et al. [11] showed that the number of TZPs, which had decreased by cryopreservation, increased during subsequent in vitro growth culture of mouse, monkey, and human secondary follicles and that the reestablished TZPs were functional. El-Hayek et al. [12] reported that mouse granulosa cells developed new TZPs in reconstructed oocyte-granulosa cell complexes. In livestock animals, Oi et al. [13] demonstrated that porcine denuded oocytes cultured in reconstructed oocyte-granulosa cell complexes grew and acquired the ability to develop into blastocysts. However, whether TZPs develop between TZP-free oocytes and granulosa cells and whether the reestablished TZPs support oocyte growth, have never been examined.

In this study, we confirmed the disappearance of TZPs by denudation of bovine growing oocytes. Then, we examined the reestablishment of TZPs during the coculture of TZP-free denuded oocytes (DOs) with mural granulosa cells (MGCs). As the cytoskeletons of TZPs are primarily composed of F-actin [10–12, 14–16], reestablished TZPs were detected by phallolidin staining. Finally, to confirm that the reestablished TZPs were functional, the reconstructed complexes (DO+MGCs) were subjected to in vitro growth culture. The integrity of DO+MGCs and antrum formation were examined throughout the growth culture period. After culture, the number of TZPs, the diameters of oocytes, and the meiotic competence of oocytes were determined.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Collection of OCGCs, DOs, and MGCs

Oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) were collected from bovine early antral follicles as described previously [17]. Briefly, bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory. Ovaries were washed once with 0.2% (w/v) cetyltrimethylammonium bromide (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and three times with Dulbecco’s PBS containing 0.1% (w/v) polyvinyl alcohol (PBS-PVA). Ovarian cortical slices were collected using a surgical blade (No. 21; ELP, Akiyama-seisakusyo, Tokyo, Japan) and forceps. Early antral follicles (0.5–0.7 mm in diameter) were dissected from ovarian cortical slices in 25 mM HEPES-buffered medium 199 (HEPES-199;...
Dojindo Laboratories, Kumamoto, Japan) containing 0.1% (w/v) PVA, 0.85 mg/ml sodium bicarbonate (FUJIFILM Wako Pure Chemical Corporation), and 0.08 mg/ml kanamycin sulfate. The follicles were opened using a surgical blade (No. 10; Feather Safety Razor, Tokyo, Japan) and forceps to collect OCGCs containing growing oocytes. Some of the OCGCs were used for in vitro growth culture and the remainder was used to prepare denuded oocytes (DOs) and mural granulosa cells (MGCs). First, oocyte-cumulus cell complexes (OCCs) and MGCs were separated from OCGCs as described previously [17]. Subsequently, cumulus cells were removed completely from the OCCs using a narrow pipette and the DOs were collected. The diameter of oocytes in OCGCs and DOs (excluding the zona pellucida) was measured to the nearest 1 μm with an ocular micrometer (Olympus, Tokyo, Japan), and then the diameter of the oocyte was measured. OCGCs collected from antral follicles served as an in vivo fully grown control.

Disappearance and reestablishment of TZPs, and reconstruction of DO+MGC complexes

To prepare TZP-free DOs, groups of 2–10 DOs collected from at least 4 biological replicates were cultured individually in 12 µl microdrops of culture medium covered with paraffin oil in Petri dishes (Falcon No. 351007; Becton Dickinson and Co., Franklin Lakes, NJ, USA) at 38.5°C under a controlled humidified atmosphere of 5% O2, 5% CO2, and 90% N2 for at least 4 biological replicates were cultured individually in 12 µl microdrops of culture medium covered with paraffin oil in Petri dishes (Falcon No. 351007) at 38.5°C under the same atmospheric conditions for 48 h. Subsequently, the OCGCs were transferred to Millicell inserts placed in Petri dishes (Falcon No. 351008) and cultured for 12 days. The DO+MGCs and control OCGCs were cultured on Millicell inserts at 38.5°C under a controlled humidified atmosphere of 5% O2, 5% CO2, and 90% N2 for 5 days, followed by an atmosphere of 5% CO2 in air for 7 days [20]. The day on which DOs and OCGCs were collected was designated as Day 0, and half of the culture medium was replaced with fresh medium every other day after Day 6.

Antrum formation by the complexes was observed daily by identifying visible spaces surrounded by somatic cells. Complexes with cytoplasmic degenerative oocytes, detachment of somatic cells from the zona pellucida, and collapsed complexes were classified as disintegrated complexes; all others were regarded as complexes that maintained their integrity.

After culture, the diameter of the oocyte was measured as described above. Some of the oocytes were denuded mechanically to examine the number of TZPs, the thickness of the zona pellucida, or the meiotic stages. The number of TZPs was determined as described above. The thickness of the zona pellucida was measured at four locations (top, bottom, right, and left) per oocyte in the imaged DOs using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The mean of the four values was considered as the mean thickness of the zona pellucida per oocyte. To assess the meiotic stages, the oocytes were fixed with acetic acid-ethanol (1:3) and stained with 1% (w/v) aceto-orcein (FUJIFILM Wako Pure Chemical Corporation). The stages of meiotic division were assessed using Nomarski interference microscopy. The oocytes were classified based on the morphology of the chromatin and nuclear envelope [21, 22]. The stages of oocytes before meiotic resumption were classified as filamentous chromatins (FC), stringy chromatins (SC), and germinal vesicles I–IV (GV I–IV). After resumption of meiosis, the stages were classified as early diakinesis (ED), late diakinesis (LD), metaphase I (MI), anaphase I and telophase I (AI-TI), and metaphase II (MI). Oocytes showing cytoplasmic or nuclear abnormalities were regarded as degenerated oocytes.

In vitro maturation culture of complexes

The DO+MGCs and OCGCs that maintained their integrity after in vitro growth culture were further used for in vitro maturation, which was performed as previously described [17]. OCGCs collected from early antral follicles (0.5–0.7 mm) and antral follicles (4–6 mm) were also subjected to maturation and served as in vivo controls. Briefly, the complexes were cultured in 50 µl microdrops of maturation medium covered with paraffin oil at 38.5°C under a controlled
atmosphere (5% CO₂ in air) for 22 h. Each microdrop contained 4–5 complexes collected from at least 4 biological replicates. The maturation medium was TC-199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) FBS, 0.1 mg/ml sodium pyruvate (Nacalai Tesque, Kyoto, Japan), 2.2 mg/ml sodium bicarbonate, 0.08 mg/ml kanamycin sulfate, and 0.1 IU/ml human menopausal gonadotropin (Aska Pharmaceutical, Tokyo, Japan).

After 22 h, the oocytes were denuded mechanically using 0.1% (w/v) hyaluronidase and a narrow pipette. They were then fixed with acetic acid-ethanol (1:3) and stained with 1% (w/v) aceto-orcein to assess the stage of oocyte maturation.

**Statistical analysis**

The difference between mean (± SEM) diameters of *in vitro*- and *in vivo*-grown oocytes, the number of TZPs, and the thickness of the zona pellucida was analyzed by one-way ANOVA followed by the Tukey-Kramer multiple range test (Excel software with the add-in Eksuser-Toukei 2010; Social Survey Research Information, Tokyo, Japan). The number of TZPs was also analyzed using Smirnov-Grubbs’ outlier test (P < 0.05). All other experimental data were analyzed by the Chi-square test. Statistical significance was set at P < 0.05.

**Results**

**Disappearance and reestablishment of TZPs**

TZPs were observed throughout the zona pellucida of growing and fully grown oocytes, and some of the TZPs penetrated the zona pellucida to reach the oocyte surface (Fig. 1A, a, b). In the oocytes collected from early antral follicles and antral follicles, the mean number of TZPs that reached the oocyte surface was 100.4 ± 3.8, and 98.7 ± 3.6, respectively (Fig. 1B). The mean number of TZPs was fewer at 24 h than 12 h after denudation (1.8 ± 0.5 and 7.7 ± 1.1, respectively) (Fig. 1A, c, d; Fig. 1B).

Oocytes collected from early antral follicles were denuded (Fig. 2A, a0), and 24 h after denudation, the DOs were cocultured with the mass of MGCs in new microdrops (Fig. 2A, a1). After coculture for 24 h (Fig. 2A, a2), the MGCs adhered to the DOs and the culture substrate. All the DOs that had been kept on the mass of MGCs for 24 h after starting coculture were surrounded by MGCs, and complexes (DO+MGCs) were reconstructed. When the DOs tumbled down and were away from the MGCs during the coculture, the MGCs did not adhere to the DOs and the complex did not form. DO+MGCs were picked up with a pipette and transferred onto Millicell inserts (Fig. 2A, a2'). The DO+MGCs attached to the Millicell inserts and their size increased gradually (Fig. 2A, a3–6).

During coculture, the number of TZPs increased (Fig. 1A, a–e; Fig. 1B), and 72 h after coculture, the number of TZPs in some oocytes was similar to that in the oocytes *in vivo* (Fig. 1B).

**Development of reconstructed complexes and oocyte growth**

We confirmed the disappearance of the TZPs by denudation of the oocytes. We found that MGCs adhered to DOs to reconstruct complexes, following which, TZPs reestablished over time. Next, we examined the development of DO+MGCs to access whether oocytes in DO+MGCs would grow fully and acquire meiotic competence similar to OCGCs.

The typical morphologies of DO+MGCs during the growth culture are shown in Fig. 2B. After DOs (Fig. 2B, b0) were cultured for 24 h, they were cocultured with the mass of MGCs (Fig. 2B, b1). After coculture for 24 h, reconstructed complexes consisting of DOs and MGCs (Fig. 2B, b2) were transferred onto Millicell inserts. The size of the reconstructed complexes increased (Fig. 2B, b3–14) and became similar to the size of the OCGCs (Fig. 2C).

The integrity of DO+MGCs and OCGCs during culture is shown in Fig. 3A. On Day 2, 92% of the DOs were surrounded by MGCs, and 98% of the OCGCs maintained integrity. Because some of the DOs had been away from and were not surrounded by the MGCs during coculture, the integrity of DO+MGCs decreased. On Day 7, 85% of the DO+MGCs and 90% of the OCGCs maintained spherical structures containing an oocyte in the center surrounded by mural granulosa cells or cumulus cells. However, as the coculture progressed, some of the structures of DO+MGCs and OCGCs collapsed and the oocytes became denuded. On Day 14, OCGCs showed significantly higher integrity than DO+MGCs (81% and 58%, respectively).

As the complexes developed, some formed antrum-like structures (Fig. 2B, C, b6–14, c5–14). OCGCs started forming antrum-like structures on Day 4, that is, two days earlier than DO+MGCs (Fig. 3B). The percentages of complexes forming antrum-like structures increased in both the groups of OCGCs and DO+MGCs until Day 9 (84% and 66%, respectively). After Day 9, the percentage in the OCGC group reached a plateau (approximately 80%). In contrast, the percentage in the DO+MGC group decreased to 55% on Day 14.

After 14 days of growth culture, many TZPs in DO+MGCs penetrated the zona pellucida to reach the oocyte surface in a manner similar to the TZPs in OCGCs (Fig. 4A, c, d). The mean number of TZPs in DO+MGCs was 83.8 ± 2.0, which was similar to the numbers in OCGCs (89.7 ± 2.1) (Fig. 4B). The mean number of TZPs in fully grown oocytes *in vivo* collected from antral follicles was 122.1 ± 2.1. Although the mean thickness of the zona pellucida in DO+MGCs after culture was higher than that in the oocytes before culture, it was smaller than that of *in vivo* fully grown oocytes (Fig. 4C).

The mean diameters of oocytes grown in DO+MGCs and OCGCs increased significantly to 126.9 ± 0.9 µm and 128.1 ± 0.8 µm compared to oocytes before culture (96.0 ± 0.4 µm and 97.1 ± 0.4 µm, respectively) (Supplementary Table 1). Oocytes in DO+MGCs and OCGCs grew to a size similar to that of *in vivo* fully grown oocytes (125.3 ± 0.8 µm).

After culture, 90% of the oocytes in DO+MGCs and 93% of the oocytes in OCGCs reached the GV stage (Supplementary Table 2, Supplementary Fig. 1, b1, c1). Growing oocytes collected from early antral follicles were at the FC or SC stages (Supplementary Fig. 1, a1), while all *in vivo* fully grown oocytes collected from antral follicles were at the GV stage (Supplementary Fig. 1, d1). In the subsequent maturation culture, 72% of the oocytes grown in DO+MGCs and 83% of the oocytes in OCGCs reached MII (Table 1, Supplementary Fig. 1, b2, c2). The growing oocytes collected from early antral follicles remained at the FC, SC, and GV stages after 22 h of maturation culture (Supplementary Fig. 1, a2), while 85% of the fully-grown oocytes collected from antral follicles reached MII (Supplementary Fig. 1, d2).

**Discussion**

This study showed that denudation of bovine oocytes caused the disappearance of TZPs wherein the number of TZPs decreased over time after denudation. Previous studies have reported that actin-based TZPs extend from granulosa cells or cumulus cells [10–12, 14–16]; therefore, the mechanical disconnection of TZPs from cumulus cells by pipetting seems to induce actin depolymerization in the TZPs. In our experiment, almost all TZPs disappeared 24 h after denudation. Therefore, we considered the DOs cultured for 24 h to be TZP-free DOs.

Next, we showed that the coculture of TZP-free DOs with MGCs...
caused MGCs to adhere to TZP-free DOs to reconstruct complexes. MGCs surrounded oocytes in a manner similar to cumulus cells, and DO+MGCs developed into spherical structures similar to OCGCs. Within this structure, TZPs were reestablished. The newly established TZPs extended from MGCs and penetrated the zona pellucida to reach the oocyte surface. After coculture, the number of TZPs increased with the development of DO+MGCs. The number of TZPs in some oocytes after coculture for 72 h became similar to that in vivo oocytes, suggesting that MGCs first proliferate to surround oocytes and elaborate TZPs toward the oocytes. Mizumachi et al. [23] suggested that the culture medium viscosity is involved in strengthening the contact between oocytes and cumulus cells. Therefore, the addition of polyvinylpyrrolidone to our culture media may have supported the maintenance of the complex while preventing cell migration and preserving cell adhesion as TZPs developed.

Diaz et al. [24] reported that mouse MGCs and cumulus cells express different subsets of transcripts in antral follicles, which are caused by opposing gradients of follicle stimulating hormone and oocyte-derived factors. This study showed that MGCs collected from early antral follicles (0.5–0.7 mm) and fully grown oocytes collected from antral follicles (4–6 mm). DOs collected from early antral follicles cultured for 12 and 24 h. DOs cocultured with MGCs 24 h after denudation, for 24–120 h. Different letters (a–f) denote significantly different values (P < 0.05).

Fig. 1. Fluorescent staining of oocytes showing transzonal projections (TZPs) (A) and changes in the number of TZPs (B) in bovine oocytes after denudation and coculture. TZPs were observed throughout the zona pellucida of oocytes collected from early antral follicles (a) and antral follicles (b), and some TZPs penetrated the zona pellucida to reach the oocyte surface. Disappearance of TZPs over time after denudation (c, d). Gradual reestablishment of TZPs after coculture of denuded oocytes (DOs) with mural granulosa cells (MGCs) (e–i). Alexa Fluor 488 phalloidin stained F-actin green (a1–i1), and DAPI stained chromatin blue. Bright field images are merged with fluorescent staining images (a2–i2). The nucleus is often not observed in the widest cross-section of oocytes due to being out of focus. The number of visible actin-based TZPs that penetrated the zona pellucida to reach the oocyte surface was counted in the widest cross-section of the oocytes. The scale bar represents 50 µm. The number of oocytes (n) used in each group and the mean (± SEM) number of TZPs are shown at the top (B). 1) In vivo growing oocytes collected from early antral follicles (0.5–0.7 mm) and fully grown oocytes collected from antral follicles (4–6 mm). 2) DOs collected from early antral follicles cultured for 12 and 24 h. 3) DOs cocultured with MGCs 24 h after denudation, for 24–120 h. Different letters (a–f) denote significantly different values (P < 0.05).
components of filopodia (Dann1, Fscn1, and Myo10) in cumulus cells and that GDF9 probably affects the number of TZPs. Baena and Terasaki [16] proposed that granulosa cells search for oocytes through their filopodia. In their proposed model, a contact-mediated paracrine interaction with the oocyte induces granulosa cells to exhibit a cumulus cell-specific phenotype and the contacting filopodia become TZPs. Although the control mechanism of TZP extension has not been well elucidated, we speculate that MGCs that adhered to DOs
in our experiment received oocyte-derived factors, extended TZPs, and became cumulus cell-like cells.

Next, in our in vitro growth experiment, we found that DO+MGCs developed similar to OCGCs, even though the integrity of DO+MGCs during growth culture was lower than that of OCGCs. Bidirectional communication between oocytes and granulosa cells or cumulus cells through paracrine signaling and gap junctions is essential for follicle development and oocyte growth [4–6]. Oocyte-derived factors such as GDF9, BMP15, and FGF8B regulate the metabolic cooperativity with cumulus cells, including by mediating processes such as glycolysis, amino acid uptake, and cholesterol biosynthesis, which are not accomplished by the oocyte alone [7–9]. Such nutrient supplies from

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**Table 1.** Meiotic competence of in vitro-grown bovine oocytes in cocultured denuded oocytes and mural granulosa cells (DO+MGCs) and oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) after in vitro maturation

| In vitro growth (day) | Types of complexes | Number of complexes used | Number (% of oocytes at each stage) |
|-----------------------|--------------------|--------------------------|-------------------------------------|
|                       | DO+MGCs            | OCGCs                    | FC | SC | GV | ED | LD | MI | AI-TI | MII | DG |
| 0 2)                  | -                  | -                        | 30 | 23 | 14 | 0  | 0  | 0  | 0  | 0  | 0  |
| 14                    | DO+MGCs            | 18                       | 0  | 0  | 0  | 0  | 0  | 2  | 11 | 1  | 6  | 2  |
|                       | OCGCs              | 23                       | 0  | 0  | 0  | 0  | 0  | 0  | 9  | 0  | 19 | 2 |
| In vivo 3)            | -                  | -                        | 27 | 0  | 0  | 0  | 0  | 4  | 15 | 0  | 23 | 0 |

1) DO+MGCs and OCGCs collected from early antral follicles (0.5–0.7 mm) were subjected to in vitro maturation culture after in vitro growth culture. 2) Oocytes were collected from early antral follicles for culturing. 3) Fully grown oocytes were collected from antral follicles (4–6 mm). 4) FC, filamentous chromatin; SC, stringy chromatin; GV, germinal vesicle I–IV; ED, early diakinesis; LD, late diakinesis; MI, metaphase I; AI-TI, anaphase I and telophase I; MII, metaphase II; DG, degeneration.
cumulus cells were probably shut off in the TZP-free oocytes in this study. Therefore, quick reconstruction of DO+MGCs — in other words, quick reestablishment of the TZPs that realize bidirectional communication — is key for the survival of oocytes. After coculture of DOs with MGCs for 72 h, the number of TZPs was similar to that of in vivo oocytes. Thus, the DOs in disintegrated complexes may have degenerated before reestablishing a sufficient number of TZPs for oocyte survival.

Although antrum formation in DO+MGCs occurred later than in OCGCs, DO+MGCs also formed antrum-like structures. Some reports have suggested that such antrum-like structures provide an appropriate microenvironment for oocyte growth and the acquisition of meiotic competence [17–19]. Alam et al. [28] proposed that GDF9 produced by oocytes is involved in the formation of antrum-like structures. Therefore, the DOs in reconstructed complexes may have the ability to communicate with MGCs to promote the formation of antrum-like structures in a manner similar to that of OCGCs.

After culture, the mean number of TZPs in the reconstructed complexes by DO+MGCs increased until they became similar to those in OCGCs. TZPs probably develop simultaneously complexes develop. The mean thickness of the zona pellucida in DO+MGCs and OCGCs increased during culture. However, the mean thickness of the zona pellucida in both groups after growth culture was smaller than that of in vivo fully grown oocytes, as reported in in vitro grown mouse oocytes cultured with polyvinylpyrrolidone [23].

Although the mean number of TZPs in DO+MGCs was significantly lower than that in fully grown oocytes in vivo, the mean diameter of oocytes in DO+MGCs reached 120 μm or more, which is similar to that of fully grown oocytes in vivo. In addition, oocytes in DO+MGCs adequately progressed to the nuclear stage and matured to MII at a high rate after maturation culture. Considering that cumulus cells provide nutrients for oocyte growth and regulate cGMP levels for meiotic arrest through TZPs [2, 3], our results suggest that reestablished TZPs are able to support oocyte growth.

In summary, we demonstrated that the denudation of bovine oocytes causes the disappearance of TZPs and that TZPs are reestablished by coculture of TZP-free DOs with MGCs. In addition, the oocytes in integrally reconstructed complexes grow fully and acquire meiotic competence, suggesting that the reestablished TZPs are functional. Interestingly, MGCs developed TZPs in a manner similar to that of cumulus cells. Since the mechanism of differentiation from granulosa cells to cumulus cells is not well understood, further studies on the bilateral communication between oocytes and somatic cells are needed. In this study, we developed a culture system for TZP-free DOs to reconstruct complexes with MGCs for oocyte growth. This method may be useful for salvaging DOs destined for degeneration.

Conflict of interests: The authors declare that there is no conflict of interest associated with this study.

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