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Original Article

Reestablishment of transzonal projections and growth of bovine oocytes *in vitro*

Mihoko FUSHII[1)], Rie YAMADA[1)], Jibak LEE[1)] and Takashi MIYANO[1)]

[1)]*Laboratory of Developmental Biotechnology, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan*

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

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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 phalloidin 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;

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Correspondence: M Fushii (e-mail: 189a216a@stu.kobe-u.ac.jp)

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) attached to an inverted microscope. Oocytes with diameters 90–105 µm were selected for further analysis.

To collect OCGCs containing fully grown oocytes, OCGCs from antral follicles (4–6 mm in diameter) were aspirated with follicular fluid using a syringe and a needle (18 ga; Terumo, Tokyo, Japan), and then the diameter of the oocytes 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% O_2, 5% CO_2, and 90% N_2 for up to 24 h. The basic culture medium used was α-minimum essential medium (αMEM, Cat. No. 11900-024; Invitrogen, Tokyo, Japan) supplemented with 5% (v/v) fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 50 µg/ml ascorbic acid 2-glucoside (Hayashibara Biochemical Laboratories, Okayama, Japan), 55 µg/ml cysteine, 0.05 µM dexamethasone, 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (molecular weight 360,000), 2.2 mg/ml sodium bicarbonate, and 0.08 mg/ml kanamycin sulfate [18]. Based on a previous report [19], the culture medium was supplemented with 10 ng/ml 17β-estradiol and 10 ng/ml androstenedione (Tokyo Chemical Industry, Tokyo, Japan). The disappearance of TZPs was assessed after culturing for 12 and 24 h.

To reconstruct oocyte-granulosa cell complexes, the masses of MGCs with a size larger than that of the DOs were collected from early antral follicles. The collected MGCs were cocultured with DOs individually cultured for 24 h in 12 µl microdrops of culture medium in Petri dishes (Falcon No. 351007). After coculture for 24 h, the reconstructed complexes in which MGCs adhered to DOs (DO+MGCs), were transferred to Millicell inserts (cell culture inserts 0.4 µm, 30 mm diameter; Merck Millipore, Darmstadt, Germany) placed in Petri dishes (Falcon No. 351008; Becton Dickinson and Co.). The reestablishment of TZPs was examined after coculture for 24, 48, 72, 96, and 120 h.

Fluorescence microscopy

To identify the TZPs rich in actin filaments, fluorescence microscopy was performed as described previously [19] with some modifications. Briefly, the oocytes surrounded by cumulus cells or MGCs were denuded mechanically. The denuded oocytes were washed twice in PBS-PVA and fixed in 4% paraformaldehyde in PBS-PVA for 60 min. After being washed twice in PBS-PVA, the fixed oocytes were stored in PBS-PVA containing 1 mg/ml bovine serum albumin (PBS-PVA-BSA) at 4°C overnight. The oocytes were then incubated with Alexa Fluor 488 phalloidin (1:80 in PBS-PVA-BSA; A12379; Molecular Probes, Invitrogen, Carlsbad, CA, USA) at 38.5°C under controlled atmosphere (5% CO_2 in air) for 90 min. They were then washed three times for 15 min each in PBS-PVA-BSA and mounted on glass slides with ProLong Gold antifade reagent with DAPI (P36931; Molecular Probes). The TZPs were visualized under a confocal laser scanning microscope (FV1000-KDM; Olympus, Tokyo, Japan). 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.

In vitro growth culture of complexes

The DO+MGCs were transferred to Millicell inserts placed in Petri dishes as described above and cultured for 12 days. To ensure uniform conditions, groups of 4–10 OCGCs 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) 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% O_2, 5% CO_2, and 90% N_2 for 5 days, followed by an atmosphere of 5% CO_2 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 oocytes 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 chromatin (FC), stringy chromatin (SC), and germinal vesicle 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 (MII). 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% CO_2 in air) for 22 h. Each microdrop contained 4–5 complexes collected from at least 4 biological replicates. The maturation medium was TCM-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 Ekuseru-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, e–i; 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

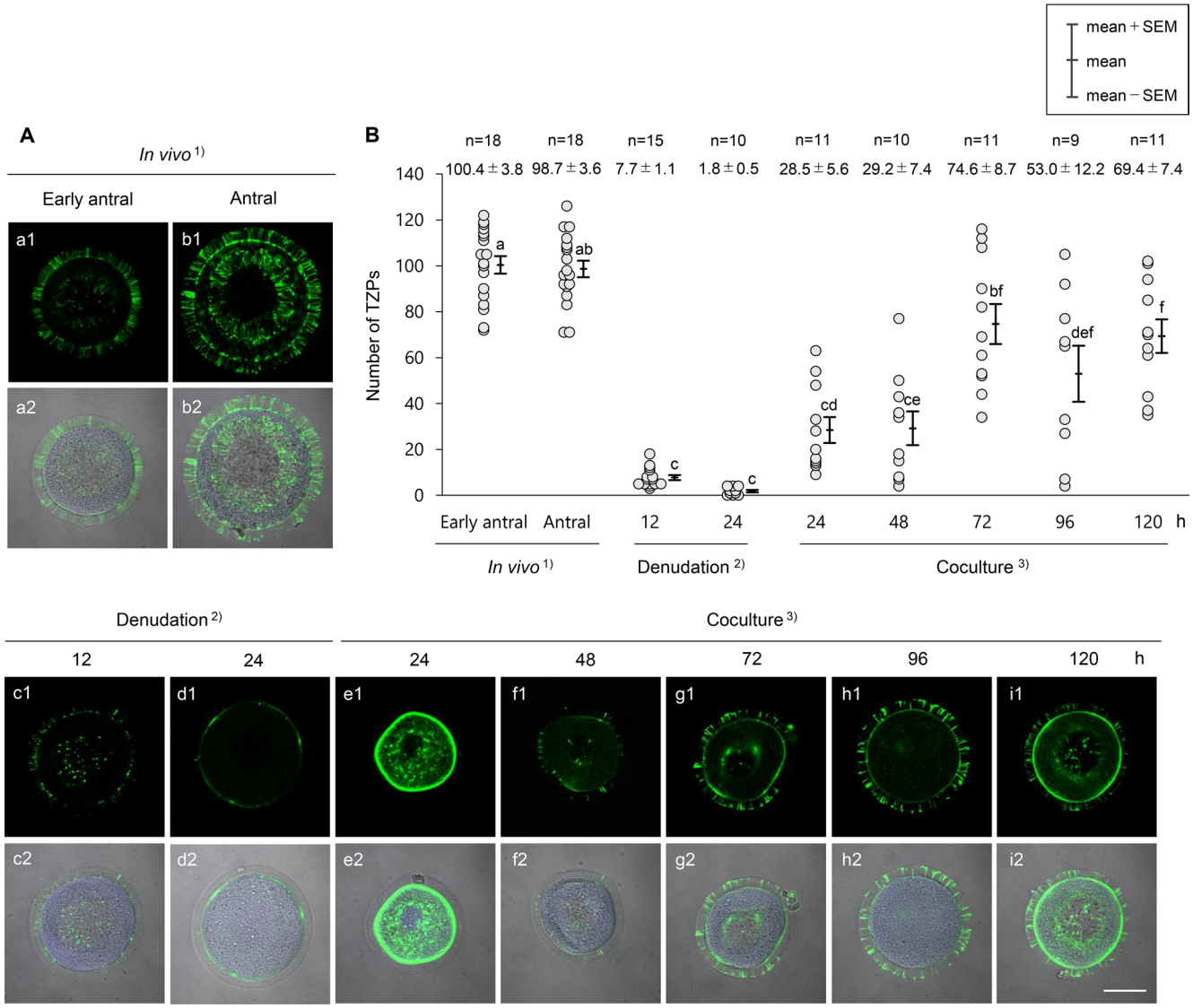

Fig. 1. Fluorescent staining of oocytes showing transzonal projections (TZPs) (A) and changes in the number of TZPs (B) in bovine oocytes after denudation and coculture. TZPs were observed throughout the zona pellucida of oocytes collected from early antral follicles (a) and antral follicles (b), and 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 ($P < 0.05$).

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 *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 have the ability to develop TZPs similar to cumulus cells. Some reports suggest that growth differentiation factor 9 (GDF9) produced by oocytes is involved in the formation of TZPs and their extension in mice [12, 25]. Along with BMP15, GDF9 is known as a key regulator of follicular development and oocyte growth, in various mammalian species [8, 26, 27]. El-Hayek *et al*. [12] suggested that GDF9 controls the mRNA levels of the general

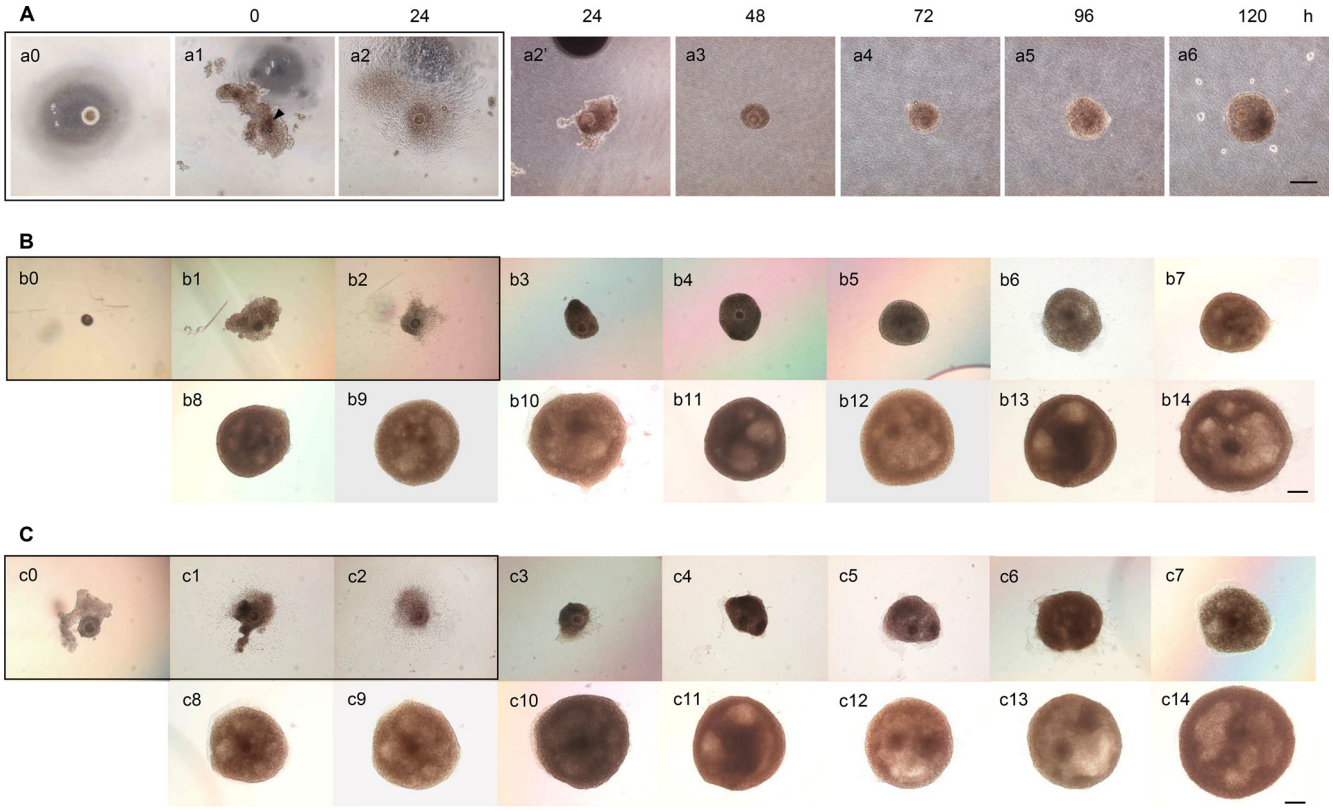

Fig. 2. Typical morphologies of bovine denuded oocytes (DOs) and DO and mural granulosa cells (DO+MGCs) during coculture up to 120 h (A) and during *in vitro* growth culture (B), and oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) during *in vitro* growth culture (C). DOs collected from early antral follicles (0.5–0.7 mm) cultured in microdrops of culture medium (a0, b0). DOs cocultured with MGCs 24 h after denudation (Day 1) (a1, b1). After coculture for 24 h (Day 2) (a2, b2), DO+MGCs transferred to Millicell inserts (a2') and cultured until 120 h (a3–a6) or an additional 12 days (b3–b14). The images framed in black indicate DOs, DO+MGCs, and OCGCs cultured in microdrops. The arrowhead indicates a DO. The scale bar represents 200 µm.

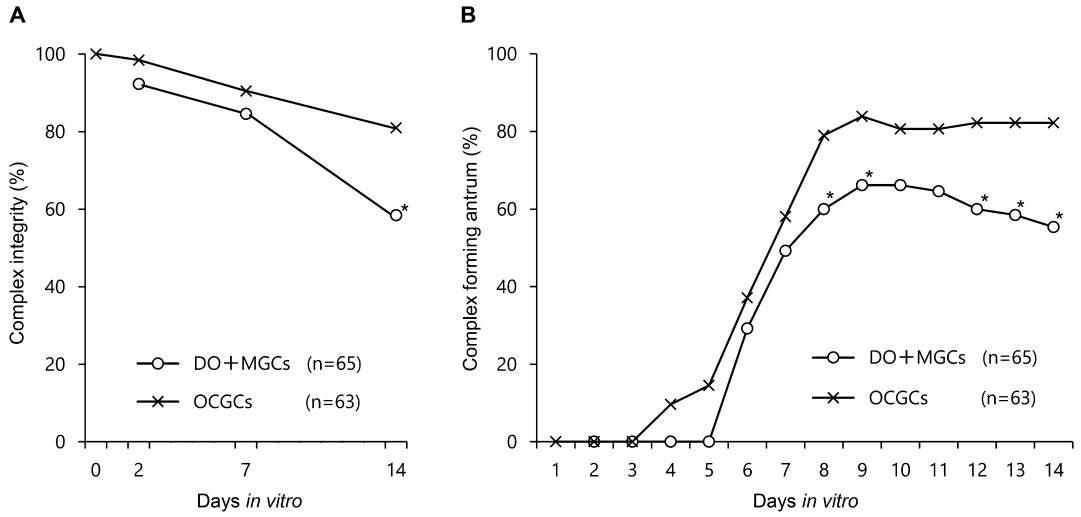

Fig. 3. Integrity of bovine cocultured denuded oocytes and mural granulosa cells (DO+MGCs) and oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) (A) and formation of antrum-like structures by DO+MGCs and OCGCs (B) during *in vitro* growth culture. DO+MGCs were examined after starting the coculture. The numbers of complexes (n) used in each group are shown on the right (A and B). * The asterisk denotes values significantly different from those of OCGCs ($P < 0.05$).

components of filopodia (*Damm1*, *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

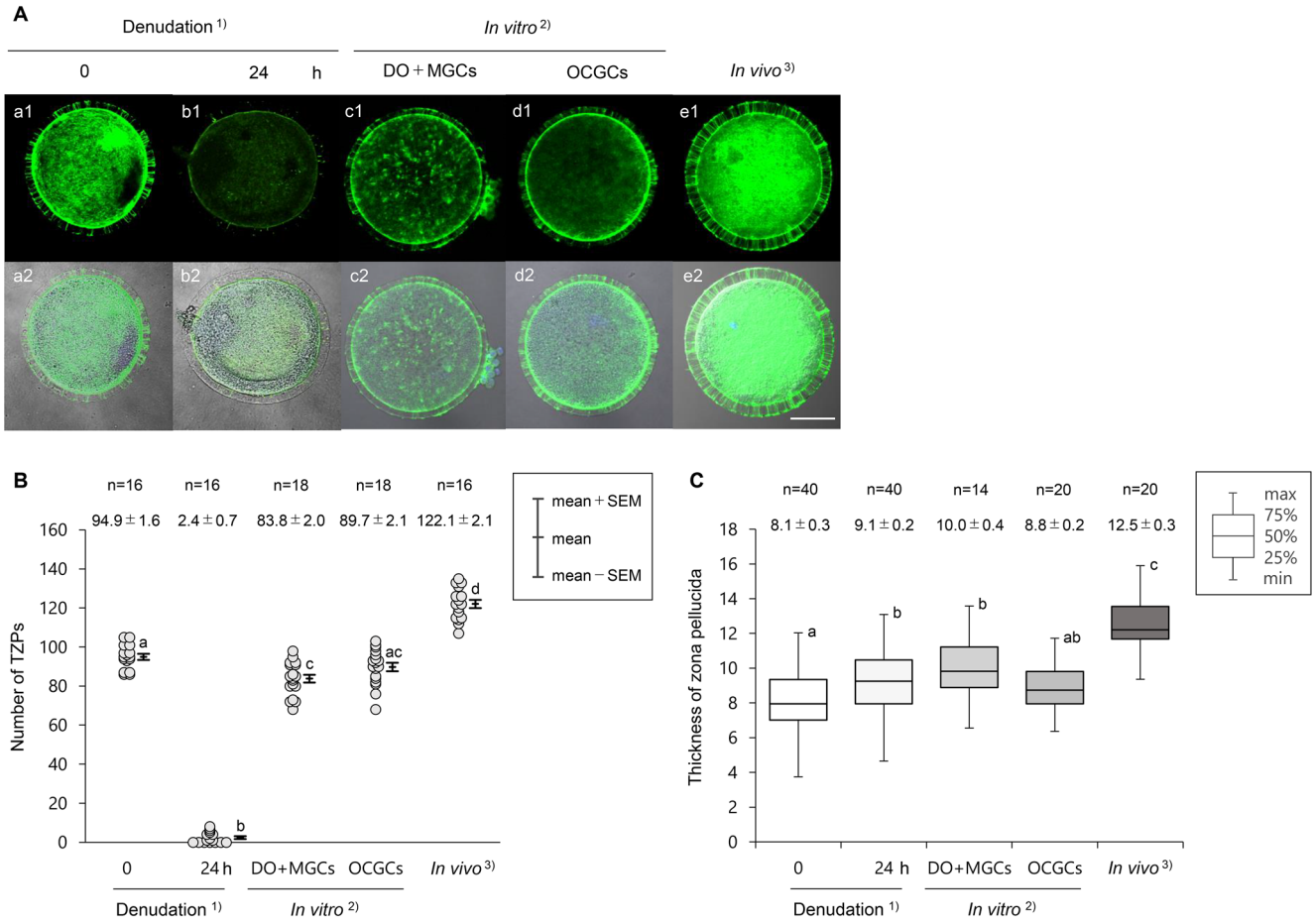

Fig. 4. Fluorescent staining of oocytes showing transzonal projections (TZPs) (A), and changes in the number of TZPs (B) and thickness of the zona pellucida (C) of bovine oocytes after *in vitro* growth culture. Oocytes collected from early antral follicles (0.5–0.7 mm) showing many TZPs (a). Disappearance of TZPs 24 h after denudation (b). TZPs penetrating the zona pellucida to reach the oocyte surface in cocultured denuded oocytes and mural granulosa cells (DO+MGCs) (c), as in oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) cultured *in vitro* for 14 days (d) and *in vivo* fully grown oocytes (e). Alexa Fluor 488 phalloidin stained F-actin green (a1–e1), and DAPI stained chromatin blue. Bright field images merged with fluorescence staining images (a2–e2). The scale bar represents 50 µm. The numbers of oocytes (n) used in each group, and the mean (± SEM) number of TZPs or the mean (± SEM) thickness of the zona pellucida are shown at the top. [1)] DOs collected from early antral follicles cultured for 24 h. [2)] DOs cultured with MGCs 24 h after denudation, for 13 days and OCGCs cultured for 14 days. [3)] *In vivo* fully grown oocytes collected from antral follicles (4–6 mm). Different letters (a–d) denote significantly different values ($P < 0.05$).

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 [1)]	Number of oocytes used	Number (%) of oocytes at each stage [4)]								
			FC	SC	GV	ED	LD	MI	AI-TI	MII	DG
0 [2)]	-	30	3 (10)	23 (77)	4 (13)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
14	DO+MGCs	18	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (11)	1 (6)	13 (72)	2 (11)
	OCGCs	23	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (9)	0 (0)	19 (83)	2 (9)
In vivo [3)]	-	27	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (15)	0 (0)	23 (85)	0 (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.

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

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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