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Ultrastructure of the Follicular Oocyte Surface in *Rana dybowskii*

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Rana ovarian follicles consist of oocyte, vitelline envelope, granulosa cells, and theca/epithelial layer. Using scanning electron microscopy, the surface structure of each follicular component was investigated. Changes in oocyte surface during oocyte maturation were also examined. Theca/epithelial layer was almost transparent and some blood vessels and granulosa cells were observed underneath in intact follicle. The number of granulosa cells was estimated to be 6700-7200 per oocyte. The granulosa cells partially overlapped each other and their microvilli penetrated the vitelline membrane via holes present in the vitelline envelope and seemed to be linked to oocyte microvilli. After removal of the vitelline envelope by microforcep, oocyte microvilli were observed on the surface of the devitellined oocyte. The oocyte microvilli formed partial clusters on the surface of white spot area which appears just before germinal vesicle breakdown (GVBD), whereas they were evenly distributed in other areas. The microvilli became shorter and less dense with oocyte maturation. The lengths of oocyte microvilli in the immature and mature oocyte were 1.5 μm and 0.6 μm, respectively. The present study suggests a fundamental structural change occurring on the oocyte surface during maturation.

In amphibian ovaries, the follicular oocyte is enveloped by several layers of somatic cells, such as theca/epithelial layer (THEP), granulosa cell layer, and vitelline envelope which fill the gap between the oocyte and granulosa cells (Anderson and Yatvin, 1970; Dumont, 1972). Formation of the vitelline envelope begins during early follicular development (Matsuyama et al., 1991). Direct cytoplasmic connections between the oocyte and granulosa cells are maintained with the microvilli that perforate through vitelline membrane and this specialized membrane junction is known as nexuses or gap junctions (Browne et al., 1979; Browne and Werner, 1984). The cytoplasmic connections allow transfer of various signals and nutrient molecules from the granulosa cells to the oocyte during the early stages of follicular maturation (Browne and Werner, 1984; York et al., 1993). It is well established that amphibian ovarian follicle cells (granulosa cells) produce and release progesterone in response to pituitary gonadotropins (Fortune et al., 1975), and binding of progesterone on the oocyte surface initiates the oocyte maturation process (Liu and Patino, 1993). Thus, the junctional communication between granulosa cell and oocyte is initiated in the early growing period and is maintained until fully grown (Browne and Werner, 1984; Matsuyama et al., 1991). When full grown oocytes undergo oocyte maturation and ovulate in response to gonadotropin surge *in vivo*, the connection between the granulosa cells and oocytes are destroyed, and the oocytes are released into oviduct (Schuetz, 1985).

However, morphology of oocyte surface in ovarian follicle and its connection with granulosa cells have been poorly understood in amphibians. The present study, therefore, aims to inrest-gate three-dimensional structure of the oocyte surface, its association with granulosa cells, oocyte surface with the vitelline envelope, and changes in microvilli during oocyte maturation.

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Materials and Methods

Collection of animals

Hibernating female frogs (*R. dybowskii*) were collected during November and December from the streams in the Chonnam area of South Korea and kept in a state of artificial hibernation. The animals were maintained as described previously (Bandyopadhyay et al., 1998).

Oocyte preparation and Culture

Ovaries from adult female frogs were surgically

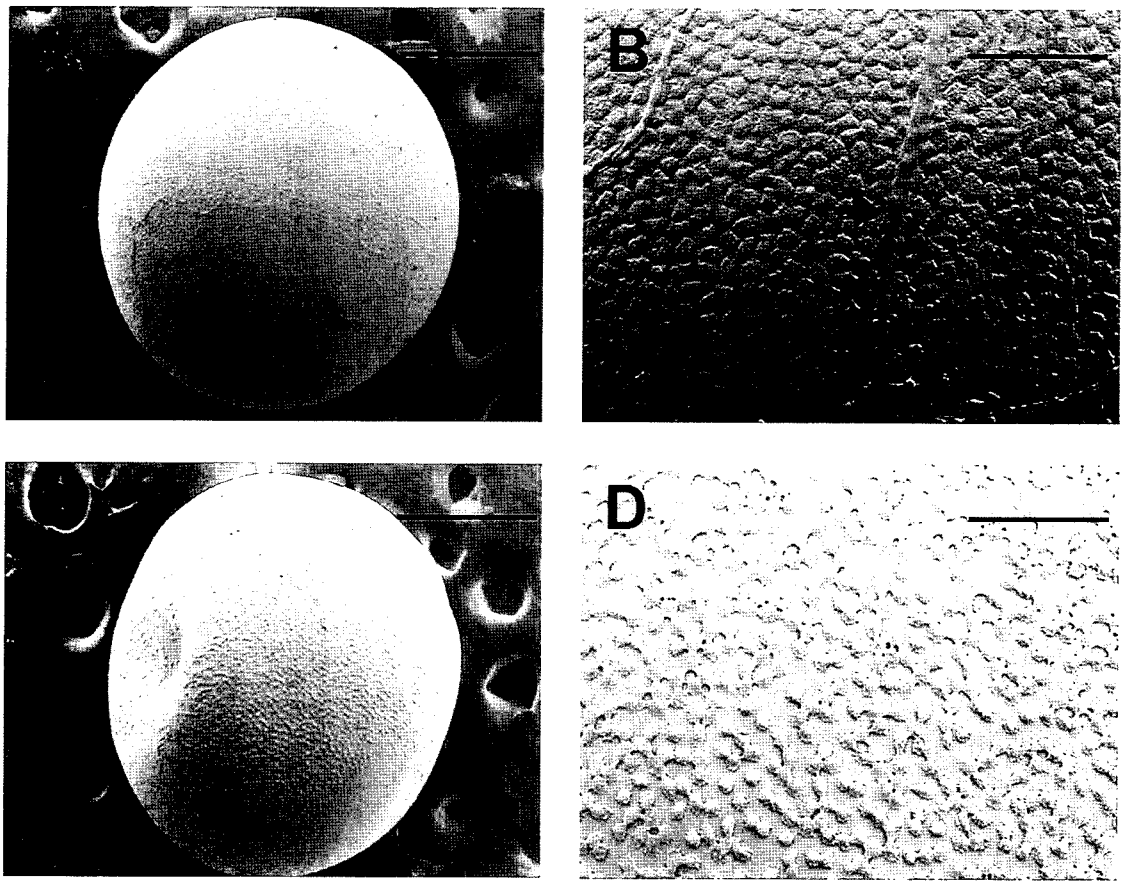

Fig. 1. Scanning electron microscopy showing surface of intact follicle and granulosa cell enclosed oocyte (GCEO) in *Rana dybowskii*. A-B, Surface of intact follicle. The arrow in B indicates blood vessel. C-D, Surface of GCEO which removed theca/epitherial layer by microdissection. Scale bars= 120 μm (B, D) and 480 μm (A, C).

removed and immediately placed in Amphibian Ringers medium (AR). Fully grown oocytes were manually defolliculated and incubated with calcium free AR to remove granulosa cells as described earlier (Kwon and Lee, 1991). From the denuded oocytes, the outer vitelline layer were peeled off with a microforcep and devitellined oocytes were obtained. To observe vertical section of oocyte surface, intact follicle, granulosa cell enclosed oocytes, and devitellined oocyte were divided with micro forcep under stereomicroscope. To get mature oocytes, intact follicles were incubated in the absence or presence of progesterone for 24 h in a shaking incubator at 20℃.

Fixation for scanning electron microscopy (SEM)

Oocytes were fixed overnight in AR containing 2.5% glutaraldehyde and later dehydrated in a graded ethanol series (50%, 70%, 80%, 90%, 95%) for 1 h and in 100% ethanol twice for 1 h each. After drying, the oocytes were coated with gold particles and observed by a scanning electron microscope (Hitachi, S4700, Japan) at Korea Basic Science Institute, Kwangju branch.

Results

Surface structure of Rana oocyte

Initially, the outer surface of intact *Rana* follicles was observed by scanning electron microscopy (SEM). It consisted of theca/ epithelial layer (THEP) with some blood vessels. The THEP layer tightly surrounded the granulosa cell enclosed oocytes (GCEO) with vitelline envelope (Fig. 1). As seen in Fig. 1A, some blood vessels and somatic cells were observed vaguely at low magnification (×50). The THEP layer was almost transparent. Thus, the dot-like cells were considered to be the granulosa cells. At higher magnification (×200), several branches of blood vessels passing through the patches of granulosa cell were clearly observed (Fig. 1B). Based on the observation on each oocyte surface and cell counting, the number of granulosa cells was estimated to be 330-350 cells/mm^2 and the total number about 6,700-7,200 per oocyte. In order to observe the surface of GCEO, THEP was removed by defolliculation. As seen in Fig. 1C and Fig. 1D, the dot-like shape of granulosa cells was clearly observed. They had flat shape and were attached tightly to oocyte.

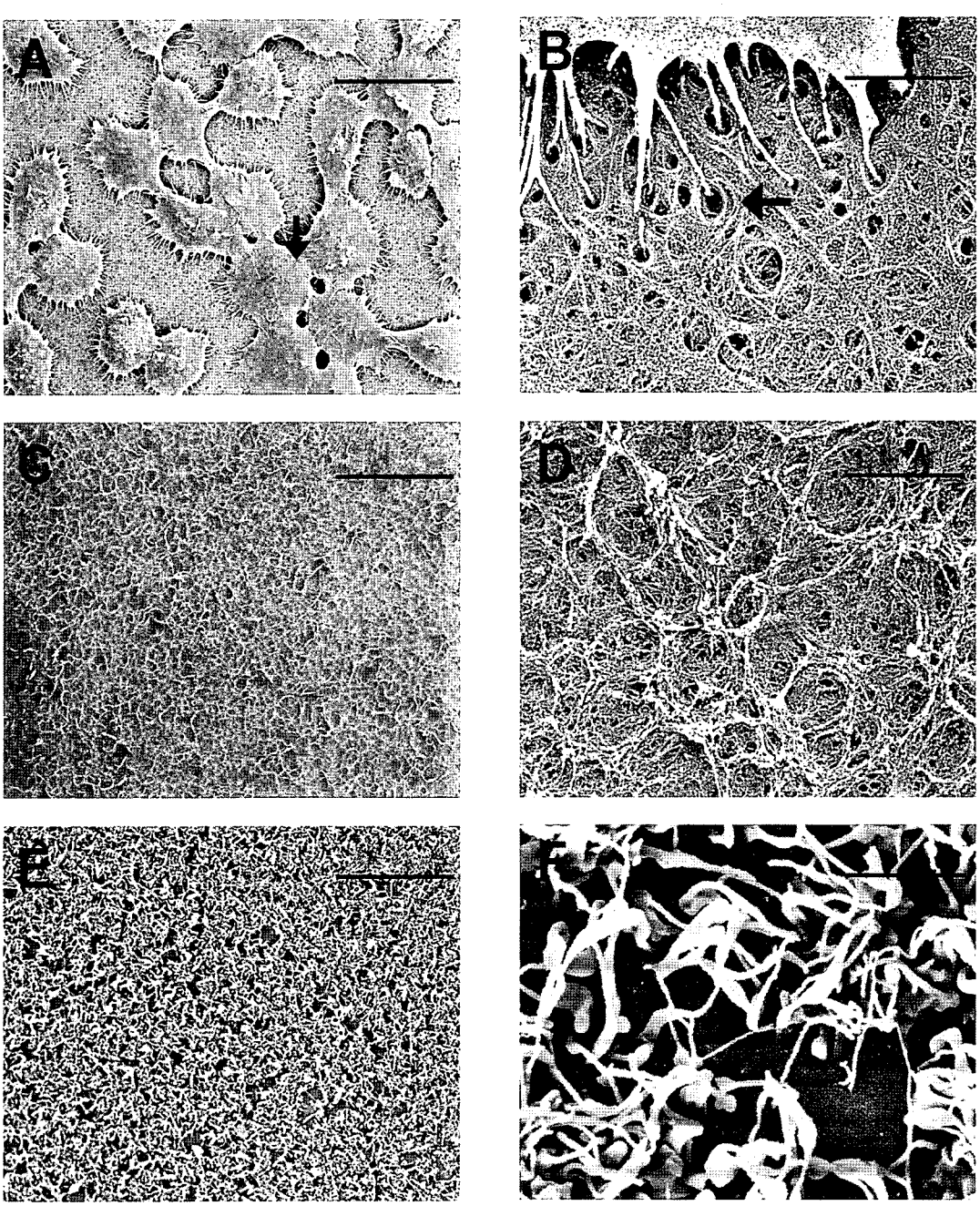

Fig. 2. Scanning electron microscopy showing surface of GCEO, denuded and devitellined oocyte in *Rana dybowskii*. A-B, Low and high magnification of GCEO. The arrow in A indicates that granulosa cells were overlapped with each other and attached to oocyte surface. The arrow in B indicates the possible heterocellular association between granulosa cell and oocyte through pore in vitelline envelope. C-D, Low and high magnification of denuded oocytes showing the surface of vitelline membrane. E-F, Low and high magnification of devitellined oocyte showing the microvilli from oocyte plasma membrane. Scale bars=2.4 μm (B, D, F) and 24 μm (A, C, E).

To observe the surface of vitelline envelope of the oocyte, the granulosa cells were removed by shaking in calcium free AR, and the surface of the denuded oocyte was examined. The outer surface of vitelline envelope was usually smooth at low magnification (< ×200), but a sponge like mesh structure was observed at high magnification (> ×1,000) (Fig. 2C, D). It is of particular interest that the spike-like cytoplasmic processes of granulosa cells went into the hole in mesh like vitelline envelope. Some processes had branches and each branch went into a separate hole in the vitelline membrane (Fig. 2B). When the vitelline envelope was removed manually by microforceps, the oocyte microvilli were clearly observed on the surface of devitellined

oocyte (Fig. 2E, F). At low magnification, some thread-like structures were observed (×1,000), but at high magnification (×10,000), a cluster of microvilli was clearly observed.

In order to observe the side view of follicle walls and its connection to oocyte, the vertical section of follicle, defolliculated oocyte, and devitellined oocytes were carried out. As seen in Fig. 3A, the oocyte was tightly surrounded by relatively thin theca/epitherial layer. When the THEP was removed, an oval shaped granulosa cell and thick vitelline membrane were clearly observed. There was clear perivitelline space, and microvilli of oocyte filled the space (Fig. 3B). When the vitelline membrane was removed, microvilli of oocyte were observed clearly (Fig. 3C).

At high magnification, it was observed that granulosa cells overlapped each other and each cell had a number of thread-like spikes attached to the egg surface. Evidently, the spike exhibited microvilli of the cell. At highest magnification, it was also observed that granulosa cell microvilli penetrated the vitelline membrane through holes in the vitelline envelope (Fig. 2A, B).

Structural changes in oocyte surface during oocyte maturation

At early stages of oocyte maturation, the germinal vesicle moves to surface of the animal pole where it gives rise to a white spot. Interestingly, microvilli on the white spot area appear to make clusters rather than being distributed evenly as in other areas (Fig. 4). At high magnification, the cluster was clearly observed (Fig. 4B). Even in other areas of the oocyte surface, microvilli seemed to be branched and formed a cluster to some extent (Fig. 4C). Clear views of microvilli were obtained when a vertical section of the devitellined oocytes was observed (Fig. 5). The devitellined oocytes obtained from intact follicle (immature) had longer villi than those obtained from cultured follicle (matured). Thus, the microvilli seemed to become shorter and less dense during maturation (Fig. 5). The lengths of the microvilli were 1.5 μm and 0.6 μm in the immature and mature oocytes, respectively. Thus it seems that intercellular association between granulosa cells and oocyte is disconnected during oocyte maturation and causes the microvilli to be shorter.

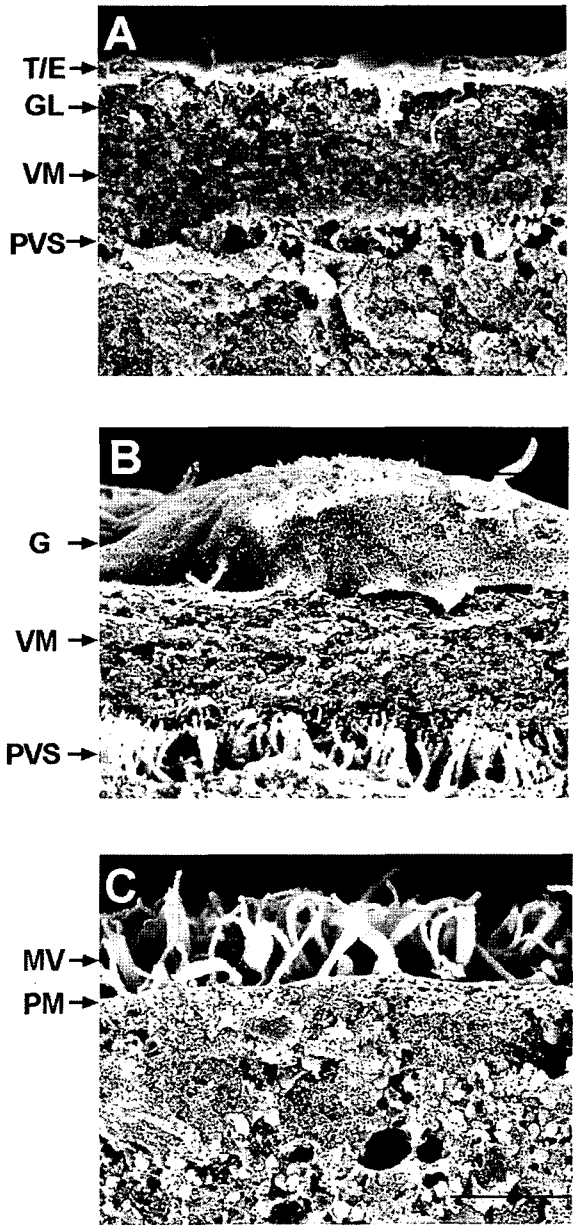

Fig. 3. Scanning electron microscopy showing vertical view of intact follicle, defolliculated, and devitellined oocytes. A, Intact follicle, × 10,000, B, GCEO showing a oval granulosa cell in outside surface. × 10,000, C, Devitellined oocyte showing the numerous microvilli in the oocyte surface. T/E, Theca/epithelial layer; GL, granulosa cell layer; G, granulosa cell; VM, vitelline membrane; PVS, perivitelline space; MV, microvilli; PM, plasma membrane. Scale bars=2.4 μm.

Discussion

In this study, using SEM, we observed the three-dimensional surface structure of *Rana* oocyte, which was enveloped by several layers of somatic cells: (theca/epithelial and granulosa cell layers) and the vitelline envelope with perivitelline space. A theca/epithelial layer tightly surrounded the oocyte granulosa cells. After removing the THEP layer, we identified a single layer of granulosa cells attached to oocyte surface tightly. From a vertical view, we also confirmed that the granulosa cells were separated from the oocyte by a vitelline envelope and extended large cytoplasmic processes through the envelope to the oolemma. These processes seemed to make a contact with microvilli on the surface of the oocyte. These follicular processes have been termed "macrovill" to distinguish them from the shorter but structurally similar microvilli (Wishchnitzer, 1996). During development, intercellular association between the granulosa cell

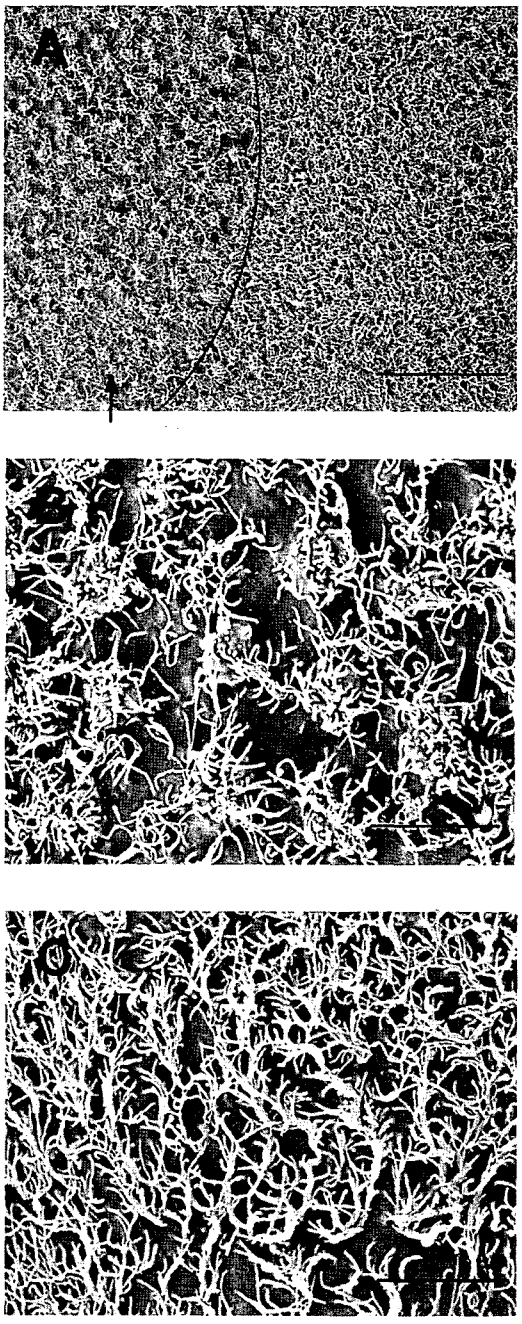

Fig. 4. Scanning electron microscopy showing surface of devitellined mature oocyte. A, White spot area is observed at upper left side of pannel. B, Higher magnification of white spot area (arrow) showing clustered microvilli. C, Higher magnification of other area showing less clustering of microvilli. Scale bars=4.8 μm (B, C) and 24 μm (A).

macrovilli and the shorter oocyte microvilli become separated by thickening of vitelline envelope (Browne and Werner, 1984). In fish, amphibian and mammal, direct cytoplasmic connections between the oocyte and granulosa cells are established early in oogenesis (Anderson and Albertini, 1976; Browne and Werner, 1984; Matsuyama et al., 1991) and contribute to the

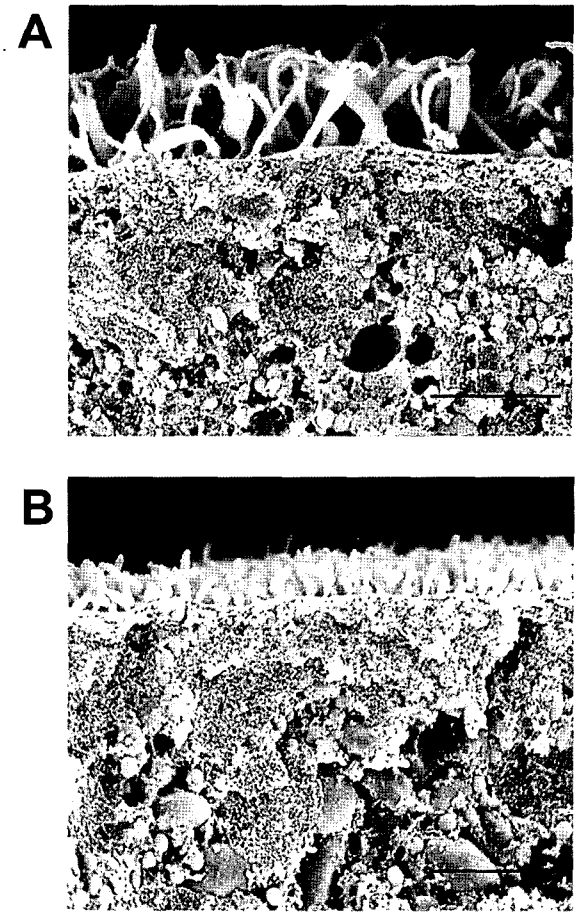

Fig. 5. Scanning electron microscopy showing the change of microvilli during progesterone-induced oocyte maturation. Intact follicles were cultured for 24 h in the presence of progesterone and oocyte microvilli were observed after removal of vitelline membrane. Immature oocyte was obtained from follicles not cultured and follicle walls and vitelline membrane was removed. The length of microvilli on the surface of matured oocyte (B) was shorter than that of immature oocyte (A) and the microvilli seemed to be broken. Scale bars=2.4 μm.

growth of oocytes. During breeding season, pituitary gonadotropin stimulates granulosa cells to produce and release progesterone, which acts at the surface of the oocyte to promote maturation (Fortune et al., 1975; Patino and Purkiss, 1993). It is known that intercellular associations between granulosa cells and oocytes in *Rana* ovarian follicles disappear following progesterone or hCG-induced oocyte maturation and ovulation (Schuetz, 1985). The oocyte microvilli became shorter and less dense during oocyte maturation of *Rana* (Fig. 6). This result supports the notion that the intercellular association between granulosa cell and oocyte was disconnected during oocyte maturation in response to progesterone.

Germinal vesicle breakdown (GVBD) during the progesterone-induced maturation of amphibian oocytes results in reorganization of microtubule array (Gard, 1992). As the oocyte microvilli formed clusters the germinal vesicle disappears during oocyte maturation

in *Rana dybowskii* (Fig. 4), it is presumed that the cluster formation is correlated with the reorganization of microtubules in the animal hemisphere. However, we do not have any evidence about this correlation yet and a further study is needed to explain this interacting phenomenon. Previously, we have shown that immobilized progesterone could induce maturation of denuded oocytes in *Rana* (Bandyopadhyay, et al., 1998). As the denuded oocytes are covered with vitelline membrane, it is evident that large molecules such as progesterone conjugated with bovine serum albumin (P-BSA) can penetrate the vitelline membrane and reach the oolemma where progesterone binding sites are believed to be present. In this study, we successfully removed the vitelline membrane from the oocyte surface without damaging the plasma membrane and microvilli, and obtained devitellined oocytes (Fig. 3C). Thus, the devitellined oocyte could be a useful tool to visualize progesterone binding sites in oocyte membrane utilizing progesterone conjugated with BSA and fluorescent chemicals, and confocal microscopy.

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