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Morphological Studies on Cell-Binding in Parthenogenetic Mouse Embryos during the Course of Blastocyst Formation

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Abstract: *The formation of junctional complexes and the location of actin and cytokeratin were morphologically examined in parthenogenetic mouse embryos during the course of blastocyst formation. Although there was no junctional complex in untransformed 8- and 16-cell embryos, zonula occludens, predesmosomes and gap junctions appeared between each pair of flattened blastomeres of transformed 8- and 16-cell embryos. In these transformed embryos, gap junctions were observed between every two round blastomeres as well as between a flattened blastomere and a round one. Desmosomes also appeared between some pairs of blastomeres in morulae. In blastocysts, zonula occludens, zonula adherens, desmosomes, predesmosomes and gap junctions existed between each pair of trophoblast cells, and predesmosomes and gap junctions existed between every two inner-cell-mass cells and between a trophoblast cell and an inner-cell-mass cell. The kinds and locations of junctional complexes observed in parthenogenetic embryos and the developmental stages of their appearance did not differ from those of fertilized embryos. The location of actin and cytokeratin in parthenogenetic embryos was also similar to that in fertilized embryos, whereas in parthenogenetic embryos at the morula and blastocyst stages, there existed a small number of blastomeres or cells devoid of these proteins. From these results, it was inferred that the cell-binding in parthenogenetic embryos is comparable to that of fertilized embryos.*

Key words: *Parthenogenetic mouse embryo, Blastocyst formation, Junctional complex, Cytoskeletal protein, Cell-binding.*

Parthenogenesis is the phenomenon in which an ovum begins to develop by activation without sperm penetration. Artificial induction of this phenomenon is thought to be useful in clarifying the mechanisms of ovum activation and to investigate the role of sperm in embryo development [1].

Although parthenogenetic embryos are prepared in many mammals by various methods, all attempts to obtain newborns from such embryos have failed [2, 3]. Furthermore, parthenogenetic embryos are also known to have a low potential of development *in vitro* [4, 5]. Slower cell division [1, 5, 6], low metabolic activities [7–11], ultrastructural disorders [1, 12–14] and the lack of imprinting of paternal genomes [15–17] have been reported to explain the low developmental potency of parthenogenetic embryos and the failure to obtain newborns.

On the other hand, it has been reported that the chromosomal aberration from the normal number is frequently observed in parthenogenetic embryos [4, 6, 18, 19]. Such an abnormality is also thought to be a factor involving the low developmental potency of parthenogenetic embryos [4, 6]. From an experiment with neural crest cells treated by ethanol in the salamander [20], the following mechanism is thought to be a cause of abnormality in chromosome numbers of parthenogenetic embryos [18, 19]: some change occurs in actin filaments in the peripheral cytoplasm of the parthenogenetic ova due to the ethanol treatment for activation; this change results in incomplete formation of spindles during the 2nd meiotic division; and then non-disjunction of chromosomes occurs due to such incomplete spindle formation. Since actin, which is a cytoskeletal protein, is known as a constituent of *zonula adherens* [21], it is assumed that parthenogenetic embryos induced by the ethanol treatment have not only an abnormality in chromosome numbers, but also a different state of junctional complexes from those of fertilized embryos. Ethanol treatment has been widely performed to prepare par-

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thenogenetic embryos in recent years [7, 9–11, 14, 16, 18, 19], but there have been no reports with regard to the formation of junctional complexes in those embryos.

The present report therefore deals with electron microscopic observation of junctional complexes and with histochemical detection of actin and cytokeratin in parthenogenetic mouse embryos during the course of blastocyst formation, and discusses the binding of blastomeres or cells in parthenogenetic embryos.

Materials and Methods

Animals

Two hundred and fifty mature female mice of the ICR strain were used in the present study. The mice were kept and fed in a room at 24°C, and were superovulated with 5 i.u. PMSG (Serotropin, Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan), and with 5 i.u. hCG (Gonatropin, Teikoku Hormone Manufacturing Co. Ltd.) injected 48 hrs later. About one third of superovulated female mice were mated with ICR males of proven fertility.

Preparation of embryos

To induce parthenogenesis, unfertilized ova were recovered from superovulated female mice at 14 hrs after the hCG injection and were immersed in M2 medium [22] containing 100 μM EDTA-2Na (EDTA-M2 medium) and 7% ethanol for 7 min at room temperature, and then in EDTA-M2 medium containing cytochalasin B (Sigma Chemical Co., MO, USA) at 5 μg/ml for 6 hrs at 37°C. The treated ova were immersed in EDTA-M2 medium containing 0.1% hyaluronidase (Sigma Chemical Co.) to remove the cumulus cells, and then were cultured in EDTA-M2 medium for 22 hrs in a CO_2 incubator (5% CO_2 in air) at 37°C. After being rinsed 3 times with M16 medium [23], 2-cell embryos developed from the treated ova were further cultured in M16 medium. As controls, sperm-penetrated ova were collected from superovulated and mated female mice at 14 hrs after the hCG injection, and cultured in EDTA-M2 medium for 28 hrs, and then successively cultured in M16 medium.

The parthenogenetic and fertilized embryos observed were at the stages of untransformed 8- and 16-cell, transformed 8- and 16-cell, morula and blastocyst.

Observation of junctional complexes

About one third of the parthenogenetic embryos and of the fertilized embryos portioned for electron microscopic observation were fixed in a 0.1 M cacodylate buffer solution (pH 7.4) containing 4% glutaraldehyde and 2% paraformaldehyde for 3 hrs at 4°C. Rinsed 3 times in a 0.1 M cacodylate buffer solution (pH 7.4) overnight, they were post-fixed in a 0.1 M cacodylate buffer solution (pH 7.4) containing 1% osmium tetroxide for 1 hr at 4°C. The fixed embryos were dehydrated through an acetone series, and then embedded in Quetol 812. Some of the embedded samples were stained with toluidine blue (pH 7.0) after being sectioned, and then observed under a light microscope. The other samples were cut with an ultramicrotome, stained with uranium acetate and lead nitrate, and then photographed under a CM-200 electron microscope (Philips Electron Optics, Eindhoven, Netherlands).

Observation of cytoskeletal proteins

For the detection of actin, about half of the remaining parthenogenetic and fertilized embryos were fixed in a phosphate-buffered saline (PBS, pH 7.4) [24]containing 3.7% formaldehyde for 30 min at room temperature. Rinsed in a PBS, they were immersed in a PBS containing 0.25% Tween-20 (Bio-Rad Laboratories, Richmond, USA) for 5 min at room temperature. Again rinsed with a PBS, the embryos were immersed in 100 μl PBS containing 16.5 ng NBD-phallacidin (Molecular Probes Inc., Junction City, USA) for 20 min at room temperature. As for the controls, a few embryos were immersed in a PBS devoid of phallacidin.

For the detection of cytokeratin, the remaining parthenogenetic and fertilized embryos whose zonae pellucidae were removed with pronase (Sigma Chemical Co.) were fixed in a PBS containing 2% paraformaldehyde for 30 min at room temperature. After being rinsed in a PBS containing 100 mM glycine (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 0.3% bovine serum albumin (BSA, Sigma Chemical Co.) (BSA-PBS), they were immersed in a BSA-PBS containing 0.25% Tween-20 for 5 min at room temperature. Again rinsed in a BSA-PBS, they were immersed in rabbit anti-keratin serum (Transformation Research Inc., Framingham, USA) that reacts with cytokeratin polypeptides (molecular weights; 50,000, 54,000 and 57,000) for 60 min at room temperature (the primary antibody treatment), this primary antibody being diluted 50 times with a PBS. The embryos treated with the antiserum were rinsed in a PBS, and immersed in FITC-conjugated goat anti-rabbit IgG (Cappel, West Chester, USA), which was diluted 64 times with a PBS, for 60 min at room temperature (the secondary antibody treatment). As for the controls, a few embryos were treated with the secondary antibody previously treated with nor-

mal rabbit serum, or simply treated with the secondary antibody.

Each of the embryos prepared for the observation of actin and cytokeratin was placed in the center of four vaseline spots on a slide. A cover slip was then carefully placed on the vaseline spots and pressed gently to anchor the embryo in between the cover slip and the slide. Observation was carried out under a reflected-light fluorescence microscope (Nikon Corporation, Tokyo, Japan).

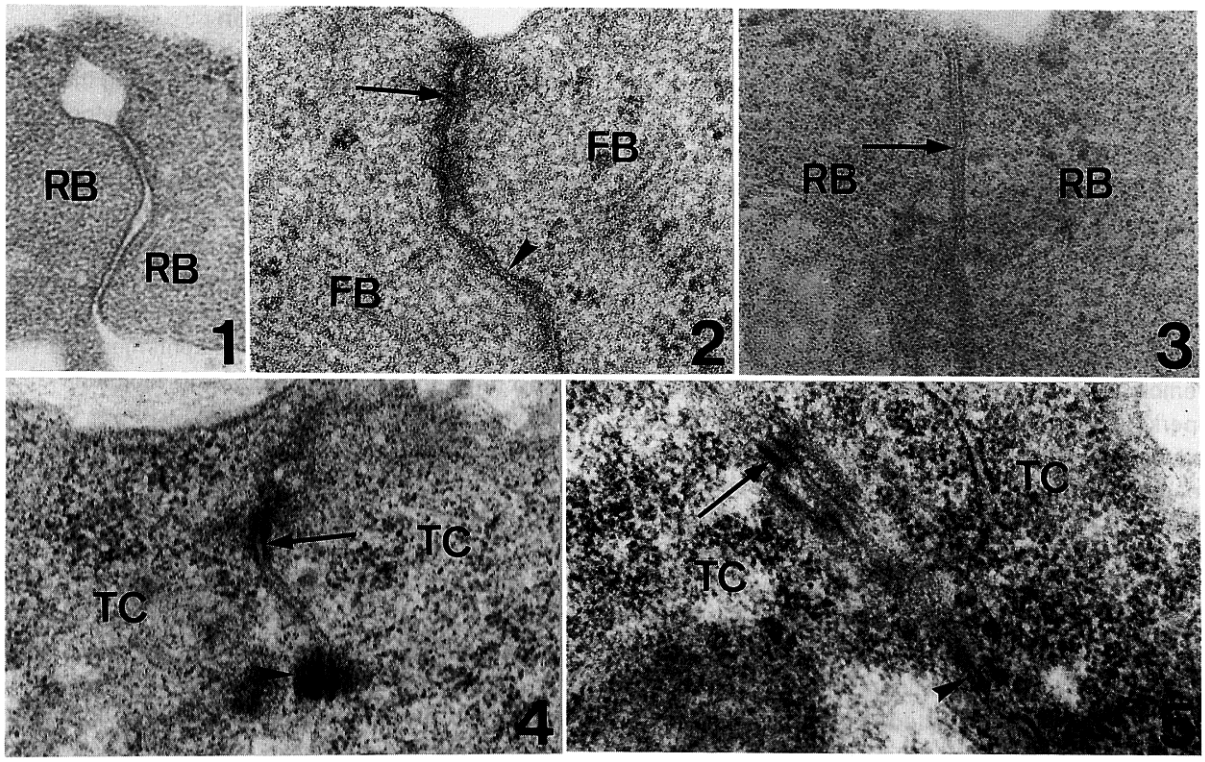
Results

Morphology of embryo

The shape of blastomeres in untransformed embryos of both parthenogenetic and fertilized groups was round, but in the transformed embryos and morulae, that of outer blastomeres was flat. The morulae in each group had compacted. The blastocysts were composed of round inner-cell-mass cells and flat trophoblast cells.

Junctional complex

Parthenogenetic embryos: Although no junctional complex was present between any pair of blastomeres of untransformed 8- and 16-cell embryos (Fig. 1), *zonula occludens* always existed between each pair of flattened blastomeres of transformed 8- and 16-cell embryos, frequently accompanied by predesmosomes and/or gap junctions inside such the *zonula occludens* (Fig. 2). In these transformed embryos, gap junctions were found between every two round blastomeres as well as between a flattened blastomere and a round one. In morulae, *zonula occludens* were always observed between each pair of flattened blastomeres, frequently



All the photographs are of parthenogenetic mouse embryos taken under an electron microscope.

Fig. 1. An untransformed 8-cell embryo. No junctional complex is seen between two round blastomeres (RB). × 47,000.

Fig. 2. A transformed 16-cell embryo. A *zonula occludens* (arrow) and a gap junction (arrowhead) are seen between two flattened blastomeres (FB). × 60,000.

Fig. 3. A morula. A gap junction (arrow) is seen between two round blastomeres (RB). × 70,000.

Fig. 4. A blastocyst. A *zonula adherens* (arrow) and a desmosome (arrowhead) are seen between two trophoblast cells (TC). × 40,000.

Fig. 5. A blastocyst. A *zonula adherens* (arrow) and a gap junction (arrowhead) are seen between two trophoblast cells (TC). × 43,000.

accompanied by desmosomes, predesmosomes and/or gap junctions inside the *zonula occludens*. In morulae, predesmosomes and gap junctions were frequently observed between every pair of round blastomeres (Fig. 3) and between a flattened blastomere and a round one. In blastocysts, *zonula occludens* always existed between each pair of trophoblast cells, often accompanied by *zonula adherens*, desmosomes, predesmosomes and/or gap junctions inside the *zonula occludens* (Figs. 4, 5), and predesmosomes and/or gap junctions existed between every pair of inner-cell-mass cells and between a trophoblast cell and an inner-cell-mass cell.

Fertilized embryos: No junctional complex was present in 8- and 16-cell embryos which consisted of round blastomeres only. In embryos from the transformed 8- and 16-cell stage to the blastocyst stage, the kinds and locations of junctional complexes were the same as seen in parthenogenetic embryos.

Actin

Immersed in a phallacidin solution, all the parthenogenetic and fertilized embryos showed fluorescence in the cytoplasm (Figs. 6–8), but not when immersed in a solution devoid of phallacidin (Fig. 9).This finding indicates that the fluorescence shows the presence of actin.

Parthenogenetic embryos: The fluorescence showing the presence of actin was seen to be bright in the peripheral cytoplasm of all the blastomeres of untransformed 8- and 16-cell embryos (Fig. 6). The fluorescence was the same in transformed 8- and 16-cell embryos and morulae, but it was much brighter in the peripheral cytoplasm where two blastomeres adhered to each other. In blastocysts, the fluorescence appeared in both the trophoblast cells and inner-cell-mass cells, and was especially bright in peripheral areas where two trophoblast cells adhered to each other (Fig. 7). In such morulae and blastocysts, however, there existed a small number of blastomeres or cells devoid of the fluorescence (Fig. 7).

Fertilized embryos: The fluorescence showing the presence of actin was detected in the cytoplasm of all the blastomeres or cells of fertilized embryos at every stage (Fig. 8), and the pattern of distribution of fluorescence was similar to that in parthenogenetic embryos.

Cytokeratin

Treated with rabbit anti-keratin serum and then with FITC-conjugated goat anti-rabbit IgG, fluorescence appeared in the cytoplasm of all the parthenogenetic and fertilized embryos (Figs. 10–12), but none appeared when treated with normal rabbit serum, or when treated with the secondary antibody only (Fig. 13). This finding indicates that the fluorescence shows the presence of cytokeratin.

Parthenogenetic embryos: The fluorescence showing the presence of cytokeratin was granular in the blastomeres of untransformed 8- and 16-cell embryos (Fig. 10), but was filamentous in flattened and round blastomeres of transformed 8- and 16-cell embryos (Fig. 11) and morulae, and in trophoblast cells and inner-cell-mass cells of blastocysts (Fig. 12). In morulae and blastocysts, however, there existed a small number of blastomeres or cells devoid of the fluorescence (Fig. 12).

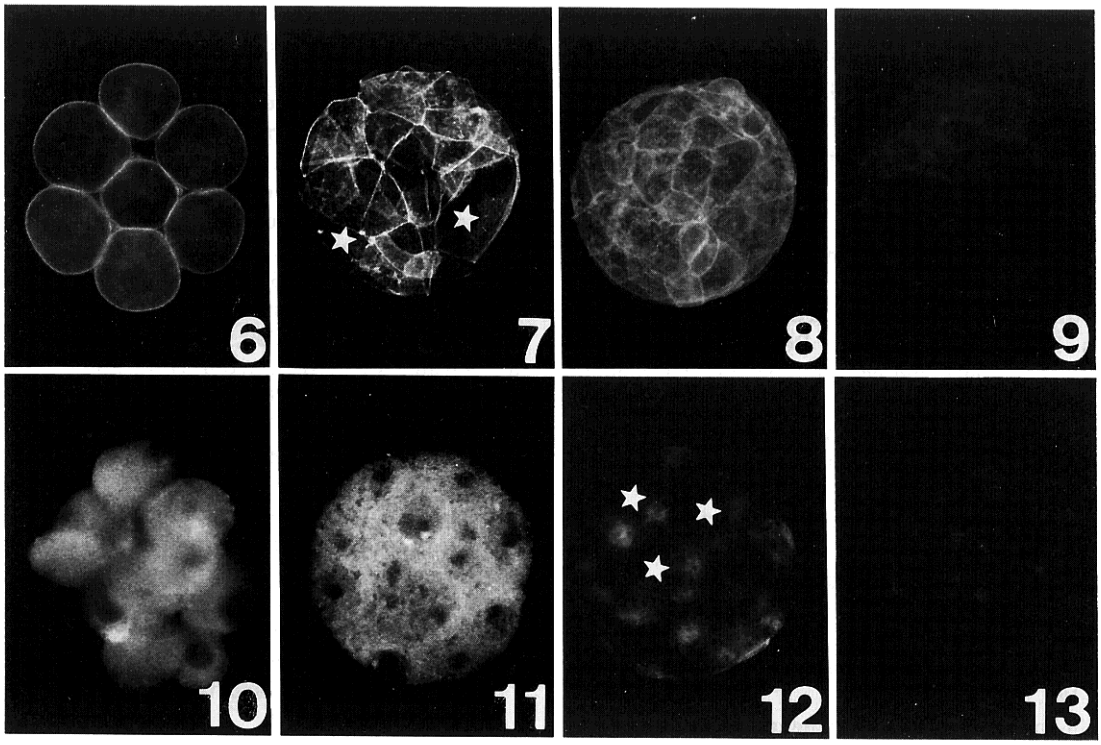
Fertilized embryos: The fluorescence showing the presence of cytokeratin was detected in the cytoplasm of all the blastomeres or cells of fertilized embryos at every stage, and the pattern of distribution of fluorescence was similar to that in parthenogenetic embryos.

Discussion

Since there have been reports suggesting the occurrence of change in actin filaments in the cytoplasm of ethanol-treated ova [18, 19], it is inferred that the state of cell-binding in parthenogenetic embryos prepared with ethanol treatment may differ from that of fertilized embryos. But the formation of junctional complexes and the location of cytoskeletal proteins in such parthenogenetic embryos have not yet been examined.

In the present study it was first confirmed that there are no differences in the kinds and locations of the junctional complexes formed or in the stages of development in their appearance between parthenogenetic and fertilized embryos. Even in parthenogenetic embryos, it was clarified that junctional complexes are formed normally.

Actin and cytokeratin generally known as cytoskeletal proteins are the constituents, the former of *zonula adherens* and the latter of desmosomes [21]. The results of the present study revealed that the pattern of distribution of these proteins in parthenogenetic embryos is comparable to that of fertilized embryos. The results on cytoskeletal proteins supported the results of electron microscopic observations in which the state of junctional complexes in parthenogenetic and fertilized embryos did not differ. It was confirmed from the results of electron microscopic and histochemical observations that the state of cell-binding in parthenogenetic embryos is comparable to that of fertilized embryos. In addition, the pattern of distribution of cytoskeletal proteins in fertilized embryos observed in



All the photographs are of whole mount mouse embryos taken at a magnification of × 250 under a reflected-light fluoresceing microscope.

Fig. 6. A parthenogenetic untransformed 8-cell embryo treated with phallacidin. The fluorescence showing the presence of actin is seen in the cytoplasm of each blastomere, and is especially bright in the peripheral cytoplasm.

Fig. 7. A parthenogenetic blastocyst treated with phallacidin. The fluorescence showing the presence of actin is seen in the cytoplasm of both trophoblast and inner-cell-mass cells, and is especially bright in the peripheral cytoplasm where two trophoblast cells are adhering to each other. There are a small number of cells (stars) devoid of the fluorescence.

Fig. 8. A fertilized blastocyst treated with phallacidin. The fluorescence showing the presence of actin is seen in the cytoplasm of all trophoblast and inner-cell-mass cells, and is especially bright in the peripheral cytoplasm where two trophoblast cells are adhering to each other.

Fig. 9. A fertilized blastocyst treated with PBS devoid of phallacidin (control). No fluorescence is seen in the cytoplasm of any cell.

Fig. 10. A parthenogenetic untransformed 16-cell embryo treated with rabbit anti-keratin serum and FITC-conjugated goat anti-rabbit IgG. The granular fluorescence showing the presence of cytokeratin is seen in the cytoplasm of all blastomeres.

Fig. 11. A parthenogenetic transformed 16-cell embryo treated with rabbit anti-keratin serum and FITC-conjugated goat anti-rabbit IgG. The filamentous fluorescence showing the presence of cytokeratin is seen in the cytoplasm of all flattened and round blastomeres.

Fig. 12. A parthenogenetic blastocyst treated with rabbit anti-keratin serum and FITC-conjugated goat anti-rabbit IgG. The filamentous fluorescence showing the presence of cytokeratin is seen in the cytoplasm of trophoblast and inner-cell-mass cells. There are a small number of cells (stars) devoid of the fluorescence.

Fig. 13. A fertilized morula treated only with FITC-conjugated goat anti-rabbit IgG (control). No fluorescence is seen in the cytoplasm of any blastomere.

the present study was consistent with that reported by Yotsutani *et al.* [25].

It has been reported that the number of cells in parthenogenetic mouse blastocysts is significantly fewer than that in fertilized blastocysts [1, 5, 6]. In the present study, actin and cytokeratin were observed in all blastomeres and cells of fertilized embryos at every stage, whereas small numbers of blastomeres and cells lacked

these proteins in parthenogenetic embryos at the morula and blastocyst stages. As blastomeres and cells that lacked these proteins are thought to have been dead, the presence of such cells is thought to be a reason for the small number of cells in parthenogenetic blastocysts.

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