Cell surface changes during cleavage of newt eggs: scanning electron microscopic studies

HIROMASA OHSHIMA AND TOMOYUKI KUBOTA

Department of Biology, Kagoshima University, Korimoto, Kagoshima, 890 Japan

SUMMARY

Scanning electron microscopic studies were carried out on changes of the cell surface during the first cleavage of eggs of the newt, *Cynopus (Triturus) pyrrhogaster*. A dense population of elongated microvilli, which initially covered a double line indicating the initial furrow and stress marks running on each side of it, became located inside the pigmented area a little apart from its border with the unpigmented area when this borderline was established. This finding, together with data of carbon marking experiments, indicates that membrane growth occurs not only at the presumptive unpigmented surface, but also in a small portion of the presumptive pigmented area adjoining it. During cleavage within the vitelline membrane, a stable intercellular junction at the border of the pigmented and unpigmented surfaces was formed through the following successive processes: the appearance of individual lamellipodia on the borderline; contact of their apical filopodia with those of the opposite blastomere; contact of the lamellipodial bodies; and complete joining of the whole of the lamellipodia in contact. In cleavage of demembranated eggs, in which there is no chance of contact of lamellipodia of the two blastomeres, each lamellipodium persisted in an isolated state until the onset of the next division. In most cases, many strained or broken threads were seen across the space formed by opening of opposing blastomeres, at their peripheries. These threads were cytoplasmic in nature and showed cell contact. Similar threads were found stretched between blastomeres when the closed furrow was opened in medium containing EGTA.

INTRODUCTION

The early cleavage of amphibian eggs results from the integrated effects of several events: a contractile process related to a contractile ring (e.g., Selman & Perry, 1970; Perry, John & Thomas, 1971; Kubota, 1979); membrane growth required for increase in the surface area of dividing cells (Kalt, 1971; Bluemink & de Laat, 1973; Singal & Sanders, 1974; Denis-Donini, Baccetti & Monroy, 1976); and cell contact between the adjacent surfaces of the blastomeres (Selman & Perry, 1970; Bluemink, 1971*b*; Kalt, 1971). The appearance of an unpigmented surface in the furrowing region has been considered to be evidence for addition of new membrane in this region (Selman & Waddington, 1955; Bluemink, 1970; Selman & Perry, 1970). This view is supported by sudden increase in ionic permeability of the egg membrane at the time of appearance of the unpigmented surface (e.g., Woodward, 1968; de Laat & Bluemink, 1974; Kline, Robinson &

Nuccitelli, 1983). Amphibian eggs establish a stable intercellular junction at the boundary between the pigmented and the unpigmented surfaces during cleavage (Bluemink, 1971a; Kalt, 1971). Transmission electron microscopic studies on cleaving newt eggs by Selman & Perry (1970) have shown that this junction is formed by a close approach and the subsequent contact of the ridges of opposing blastomeres. But the three-dimensional features of this process are still uncertain.

The present studies on cleaving newt eggs by scanning electron microscopy showed the detailed sequence of junction formation at the border of the unpigmented surface and indicated that membrane growth during early cleavage occurs in the area of surface that later becomes the pigmented surface adjoining the unpigmented one. Results also showed that interblastomeric threads found in specimens by the critical point drying method are formed at the sites of cell adhesion.

MATERIALS AND METHODS

Fertilized, uncleaved eggs of the newt, *Cynops* (*Triturus*) *pyrrhogaster* were obtained by ovulation induced with chorione gonadotropine hormone, and were decapsulated surgically. The vitelline membrane was removed with watchmaker's forceps at a desired stage before or after fixation. Eggs demembranated at the onset of division were placed on a concave paraffin surface of a small vessel unless otherwise noted, and allowed to cleave there till a fixative was introduced into the vessel. Observations and operations on individual eggs were made in Steinberg solution at room temperature (17–18°C) under a dissecting microscope.

Eggs were fixed in 3 % glutaraldehyde in 0·1 M-phosphate buffer (pH 7·2) for more than 12 h at room temperature, and then washed with two or more changes of the buffer, which was repeatedly squirted onto the egg surface with a pipette. They were dehydrated in a graded series of alcohol, rinsed in *iso*-amyl acetate, and then dried by the critical point method with CO_2. Then they were coated with gold, and examined in a Hitachi S-450 scanning electron microscope (SEM).

For observation by transmission electron microscope (TEM), eggs were fixed, dehydrated, and critical point dried exactly as for SEM. Then they were returned to dehydrated alcohol, transferred to glycidyl *n*-butyl ether, and embedded in Epon. Thin sections were doubly stained with 2 % uranyl acetate and lead citrate and examined in a JEM-100C electron microscope.

Marking of the cell surface with small carbon particles was done by the method of Sawai (see Sawai & Oda, 1983). Marked eggs were photographed from above with still films every 5 min.

OBSERVATIONS

Pigmented surface

Observation of the pigmented surface was focused on the development of the microvilli (MV) and the subsequent fate. For examination of the possible influence of the absence of vitelline fluid on the configuration of the MV, eggs were removed of their vitelline membranes at the onset of cleavage, just before egg fixation, or after its completion. No difference was seen in the MV of the three groups of eggs. Therefore no consideration was paid to the time of removal of the membrane in the present work.

The early cleavage of amphibian eggs before the appearance of the unpigmented surface is divided into four stages, namely the appearance of a single stripe, double stripe, dimple, and shallow groove (Bluemink, 1970). *Cynops* eggs used in the present study showed a single and a double row of aggregated MV on the pigmented animal surface at the earliest two stages. At the dimple stage, eggs showed stress marks running from the original double stripe to each side of it (Fig. 1). Elongated and branched MV (EBMV) were seen covering the whole of the double stripe and stress marks (Figs 2, 3).

In living eggs, stress marks disappeared at the shallow groove stage, but radially arrayed, diffuse lines of EBMV could still be discerned after this stage (Fig. 4). When the unpigmented surface appeared in the groove, and then the boundary between the unpigmented and pigmented regions became more clearly defined, as will be described in detail later, the eggs developed lamellipodia at the boundary (Fig. 4). A remarkable finding was that a mass of EBMV, derived from those in the region of the stress-mark double stripe, was seen located at some distance (40 μm) from lamellipodia (Figs 4, 5, 6. See also Fig. 12). An essentially identical result was obtained from carbon marking experiments on demembranated eggs. As seen in Fig. 7, carbon particles located on the double stripe were later found inside the pigmented surface, not on its boundary.

Boundary between the pigmented and the unpigmented regions

In cleavage within the vitelline membrane, the border between the pigmented and unpigmented regions is concealed at an early cleavage stage by the junction of the blastomeres. However, in cleavage without the vitelline membrane, the unpigmented surface is exposed externally, and thus the boundary is visible from the outside throughout cleavage. Lamellipodia appeared sparsely on the settling borderline around the original animal pole (Fig. 8) and then increased in number to form a plural row (Fig. 9). With the advance of furrowing, the row of lamellipodia extended along the borderline, eventually encircling the whole egg. The latter fact was ascertained by observing inverted eggs.

Each lamellipodium had apical filopodia of various lengths, which were often in contact with adjacent lamellipodia (Fig. 9). In open furrows, each lamellipodium persisted in isolation till initiation of the next division. However, when the lamellipodia of the two forming blastomeres came in contact during cleavage within the membrane, they formed a close junction.

In most cases, formation of a firm junction began near the animal pole. This event was inferred from the appearance and extension of the interblastomeric joint not dissociated by EGTA treatment. Attempts to demonstrate them by direct observation were unsuccessful. Therefore, SEM micrographs of the early stages from contact of the apical filopodia of lamellipodia on different blastomeres (Fig. 10) to the meeting of the lamellipodial bodies (Fig. 11) were taken in the eggs occasionally seen, in which the junction started far from the animal pole and
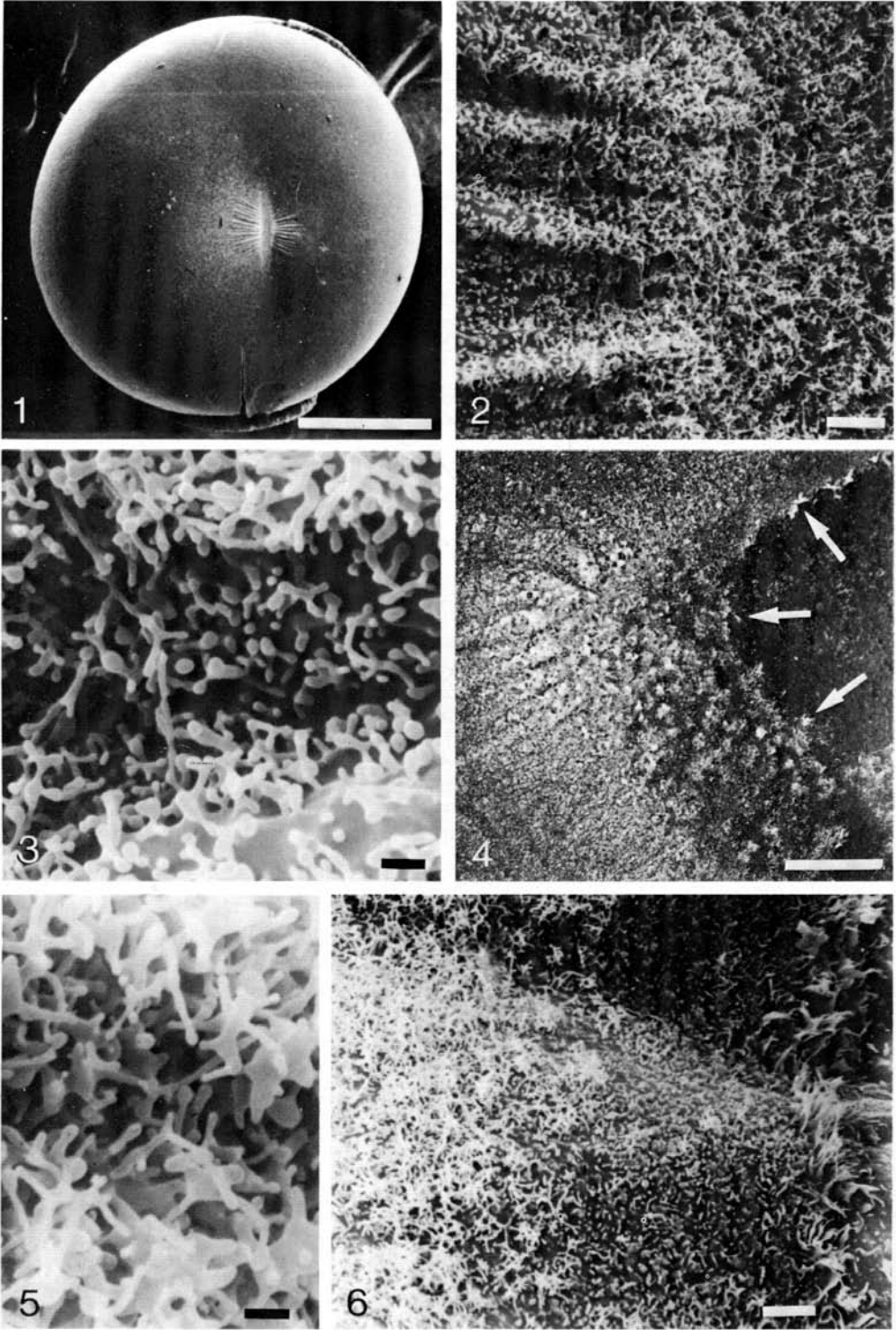
Figs 1–6.

progressed in the reverse direction to the pole. The later stage of uniting the lamellipodia could be seen by pulling apart the egg surfaces on either side of the junction (Figs 12, 13). When the furrow tip reached the vegetal pole, the junction site in the animal hemisphere still had the sparse remnants of projecting lamellipodia on its surface (Fig. 13). But subsequently the surface of this region became indistinguishable from the rest of the surface, except in the deepest position.

Opposing unpigmented surfaces

The open unpigmented surfaces of the forming blastomeres grew to meet each other in the deeper portions as the furrow deepened. In the present study, most SEM specimens prepared from such eggs showed interblastomeric threads at the bottoms of the open portions of furrows (Fig. 14), and in specimens with gaps between opposing unpigmented surfaces, the threads were seen at their peripheries (Fig. 15). Each of these threads was less than a few μm in maximal diameter, and they appeared strained or broken in the middle with tapering tips.

Examination of these SEM specimens by TEM revealed that the threads were composed of egg cytoplasm and had an intercellular junction close to, or remote from, their roots (Fig. 16), suggesting that the threads were produced around the sites of cell adhesion. For examination of the nature of these threads, we transferred cleaving eggs to 3 mM-EGTA-containing medium immediately before initiation of close junction formation. Then we removed their membrane, and placed the eggs on a flat surface to allow the furrow to open fully under the force of gravity. We fixed the eggs as soon as the bottom of the furrow was exposed. On SEM observation of such eggs, the central furrow region was seen to be traversed by long, thin threads (Fig. 17), indicating that threading occurs in the living eggs also and that it is caused by stretching of the egg surface, at sites of cell adhesion that are resistant to EGTA.

Fig. 1. A whole egg of dimple cleavage stage viewed from the pole, showing the relative size and position of the double row indicating the initial furrow, and the stress marks. Bar equals 500 μm.

Fig. 2. Enlargement of part of Fig. 1. MV are most conspicuous in the region of stress marks and the adjoining double stripe. Bar equals 5 μm.

Fig. 3. Further enlargement of part of Fig. 2, showing branched, elongated MV on a stress mark region. Bar equals 1 μm.

Fig. 4. A stage when the border between the pigmented and unpigmented regions is settling. The borderline is indicated by the row of lamellipodia on it (arrows). Note that a mass of elongated MV has already become separated from the borderline. Bar equals 50 μm.

Fig. 5. Enlargement of part of Fig. 4, showing a dense aggregate of elongated MV still remaining after regression of stress marks in the living egg. Bar equals 1 μm.

Fig. 6. Pigmented surface at an intermediate cleavage stage, showing a space between the area covered with elongated MV (left) and the row of lamellipodia (right). Bar equals 5 μm.
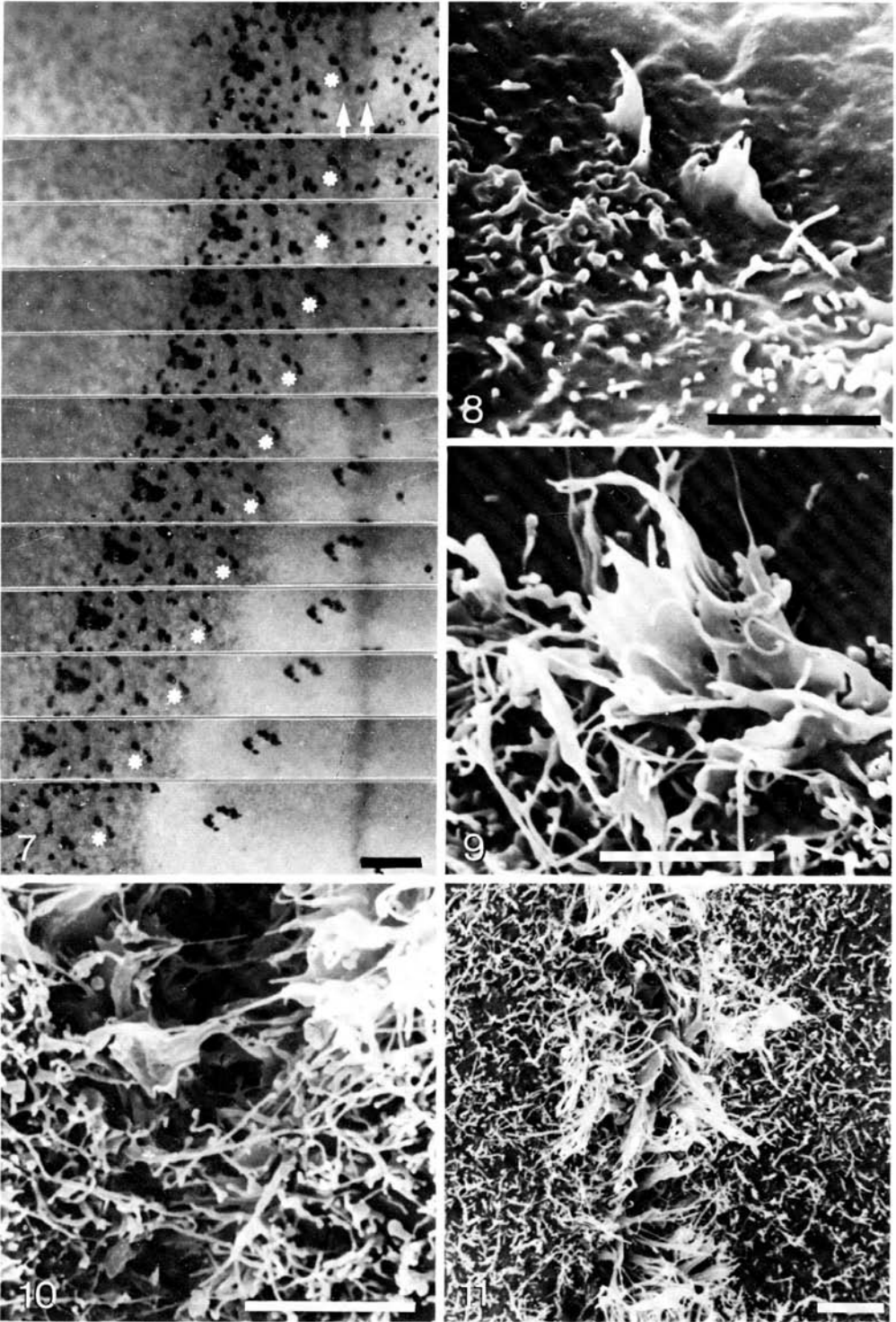
Figs 7–11.

DISCUSSION

MV on pigmented surface

In the present study it was found that EBMV first covered the whole of the double line and the stress marks, but a little later became located as a large mass inside the pigmented surface. Furthermore, carbon particles on the double line are later found inside the pigmented surface. These findings indicate the absence of topographical correspondence between the early double line and the later furrow margin. This view is also compatible with previous reports that surface markers, such as carbon particles (Sawai & Oda, 1983) or cortical pigment granules (Selman & Waddington, 1955), in the neighbourhood of the initial furrow migrate parallel to the furrow toward the pole and then turn in a direction away from the furrow.

The absence of correspondence of the double line with the furrow margin implies that extensive growth of the narrow band demarcated by the double line, besides producing the whole unpigmented surface, as elucidated already (Selman & Waddington, 1955; Bluemink & de Laat, 1973), constitutes a small part of the adjoining pigmented surface, which has previously been regarded as composed entirely of pre-existing egg membrane. In dividing *Xenopus* eggs, Kline *et al.* (1983) detected non-uniform ion currents through the pigmented membrane and with the largest magnitude in the area close to the unpigmented membrane. Since increase in ionic permeability during the first cleavage of amphibian eggs has been shown to be correlated with membrane growth, this pigmented area in *Xenopus* eggs may include newly formed membrane, as observed in the present study.

Formation of close interblastomeric junctions by lamellipodia

Close interblastomeric junctions at the boundary between the pigmented and the unpigmented surfaces are formed between MV in *Xenopus* eggs (Bluemink & de Laat, 1973; Singal & Sanders, 1974) and between ridges in *Triturus* eggs (Selman & Perry, 1970). Their process begins in the polar region in the former


Fig. 7. A series of light microscopic photographs taken every 5 min, showing that the carbon particle (asterisk) located on the double line of the initial furrow (indicated by two arrows in the top micrograph) is later found inside the pigmented surface. Bar equals 100 μm.

Figs 8–13. Lamellipodia formed on the border of the unpigmented surface and their fate.
    Fig. 8. Lamellipodia on their first appearance at the animal pole. Bar equals 5 μm.
    Fig. 9. Fully developed lamellipodia with filopodial processes at their apex. Some filopodia are seen to be in contact with adjacent lamellipodia. Bar equals 5 μm.
    Figs 10 and 11. Photographs of an egg in which joining by lamellipodia proceeded toward the animal pole. See text for explanation. Fig. 10. Early step in junction formation, showing contact of the apical processes of lamellipodia of the two blastomeres. Bar equals 5 μm. Fig. 11. Side-to-side contact of lamellipodial bodies. This process follows the contact of apical filopodia shown in Fig. 10. Bar equals 5 μm.
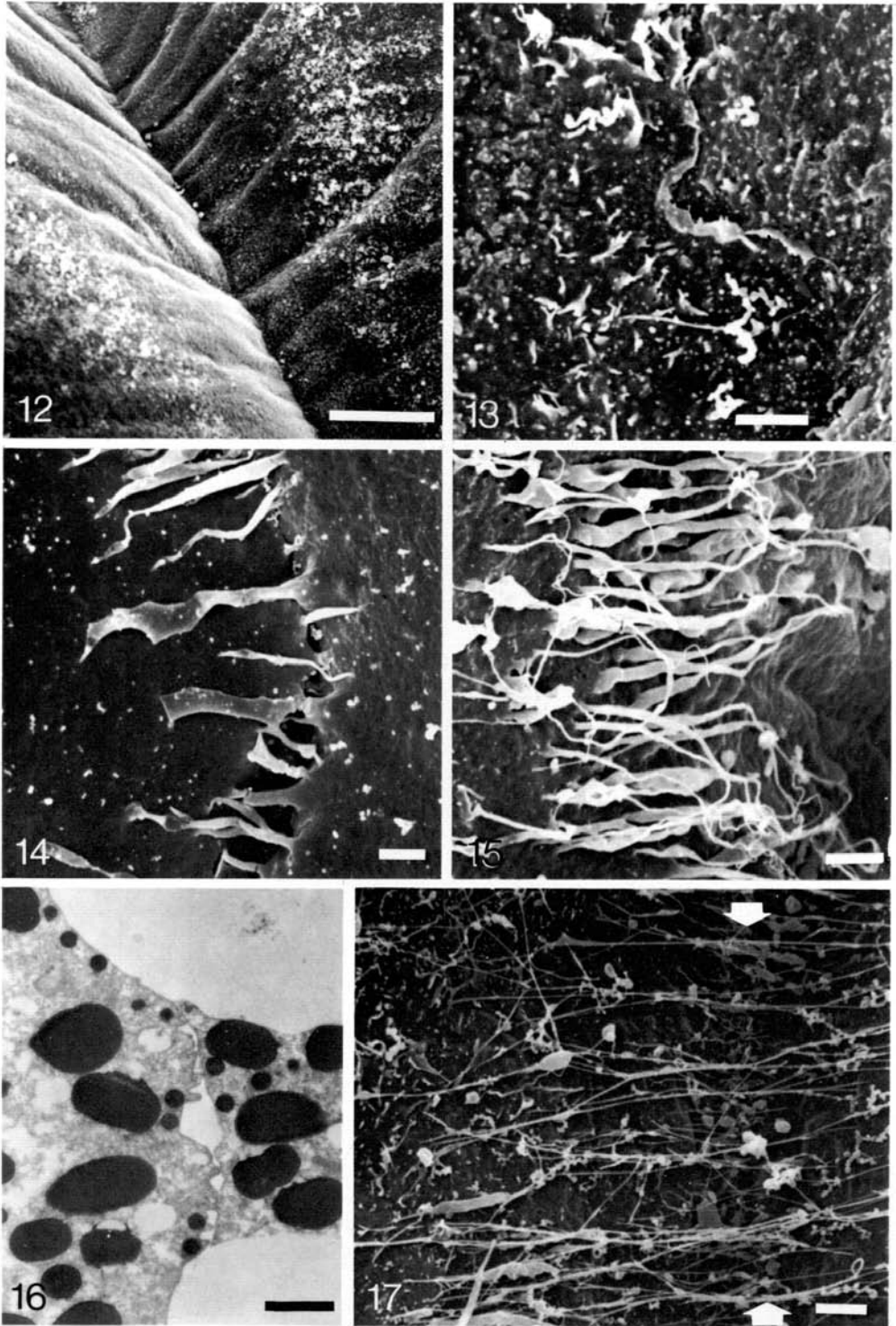
Figs 12–17.

eggs (Bluemink, 1971*a*) and apart from the pole in the latter (Selman & Perry, 1970). But there are no reports of three-dimensional studies on this process. Here the process was found to proceed by successive steps of appearance, partial contact and close adhesion of lamellipodia.

In cleavage of demembranated eggs forming separate blastomeres, the free lamellipodia of each blastomere persisted until the onset of the second cleavage in spite of partial contact with one another. On the contrary, in cleavage within the vitelline membrane, contact between lamellipodia of the two blastomeres rapidly formed close junction. What mechanism causes conversion of mere contact to adhesion? The lamellipodia of different blastomeres might possess different specificities responsible for the initiation of adhesion. However, this possibility seems unlikely, since the acquisition of such specificity generally requires some time after completion of mitosis. As observed by Selman & Perry (1970), during cleavage inside a membrane, the touching of lamellipodia was preceded by approach and contact of the opposing blastomeric surfaces in the surrounding region. Thus a more likely mechanism seems to be that antecedent contact of the unpigmented surfaces triggers adhesion of the adjoining lamellipodia.

Threads between blastomeres

In the present SEM study, fine cytoplasmic threads of less than few μm in maximum diameter were observed across the bottoms of the open furrows and, in some eggs, in the peripheral zones of the gaps between opposing blastomeres. These threads included a cell adhesion site. The threads and the gap must be artifacts, because these figures cannot be seen in TEM photographs so far published (Selman & Perry, 1970; Bluemink, 1971*b*; Kalt, 1971). Thus it is concluded that the threads-bearing surfaces were opposed in living eggs, and shrinkage of the blastomeres during critical point drying, instead of breaking the point of adhesion, drew out the egg surface around this site into a thread. Fine

Fig. 12. A close junction at the stage when the furrow tip has reached the vegetal pole. Note also that there is an elongated MV-free surface close to the junction (compare with Fig. 6). Bar equals 50 μm.

Fig. 13. Enlargement of the junction of Fig. 12. A few remnants of lamellipodia are seen projecting from the surface. Bar equals 5 μm.

Fig. 14. The bottom of the open part of a furrow, showing threads across the bottom. Bar equals 5 μm.

Fig. 15. Broken threads seen in the periphery of an interblastomeric gap (right). The left area is the open unpigmented surface. Bar equals 5 μm.

Fig. 16. A TEM micrograph of a specimen, showing the presence of cellular adhesion close to the root of a thread. The thread contains yolk granules (large dark areas) and pigment vesicles (small dark areas). Bar equals 1 μm.

Fig. 17. Thin threads traversing a furrow which was opened in EGTA-containing medium before fixation. Arrows in the photograph indicate the position of the furrow bottom before opening. Specimen prepared without squirting of buffer, to preserve thin threads: See Materials and Methods. Bar equals 5 μm.

interblastomeric threads were also drawn out experimentally in EGTA-containing medium, indicating the presence of strong and EGTA-resistant adhesion in cleavage of living eggs.

In the case of cultured cells, three types of contact have been distinguished: focal adhesion (FA: 10–20 nm gap), close contact (30–50 nm gap), often surrounding FA, and extracellular matrix contact (> 100 nm gap). The strongest of them is FA (Chen, 1981), which is a plaque-like site of about 1–2 μm in diameter (see Abercrombie & Dunn, 1975) that is EGTA-resistant (Culp, 1976) and is often found at the periphery of the cell (Chen & Singer, 1982). FA's are formed more than 2 min after contact of cells, and threads are pulled out on separation of contacted cells (Heaysman & Pegrum, 1973). In *Xenopus* eggs, junctions with a gap of 16 or 20 nm have been observed between blastomeres (Bluemink, 1971*b*; Singal & Sanders, 1974).

The above data strongly suggest that the adhesion found in the present study corresponds to FA or FA plus close contact, which was present before egg fixation. Further studies, especially TEM observation of specimens prepared by the routine method is necessary to identify the adhesion described as FA and to determine its exact distribution.

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