Role of Cell Contact in Compaction during the Third Cell Cycle of the Mouse Embryo

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Abstract: *The necessity of cell contact during the third cell cycle for the formation of compacted mouse embryos at the 8-cell stage was examined. Early 4-cell embryos (0–2 h post division) were disaggregated into 4 blastomeres, and then reaggregated at the early, mid or late stage of the third cell cycle. The reaggregated embryos were cultured upto the 16-cell stage, and the features of compaction were observed. Embryos reaggregated at the mid or late stage of the third cell cycle, had their compaction retarded by 2 h as compared with control embryos. In embryos reaggregated at the mid or late stage, the establishment of gap junctional communication was also retarded. We concluded that cell contact from the early to the mid stage of the third cell cycle is necessary to regulate the events during compaction, especially for the establishment of the gap junction.*

Key words: *Mouse embryo, Cell contact, Third cell cycle, Compaction, Gap junction.*

Compaction at the 8-cell stage of the mouse embryo, is the first morphological change which occurs during the preimplantation development. The morphological changes include: the flattening of blastomeres upon each other to maximize cell contact which results in obscure intercellular boundaries [1], polarization of cell surface microvilli [2–5], and establishment of cell-to-cell communication between blastomeres via gap junctions [6–10]. Compaction is necessary for the cell diversification and blastocyst formation [11].

The development of the mouse embryo to the early 2-cell stage is controlled by the maternal genome [12, 13], and the embryonic genome starts to function at the mid 2-cell stage [12]. Transcriptions necessary for compaction are completed by the mid 4-cell stage [14–16]. Recently, it has been reported that cell adhesion glycoproteins, E-cadherin, and gap junction forming protein, connexin43, which are important proteins for compaction, have already been synthesized by the 4-cell stage [17–20].

We have investigated here the role of cell contact during the third cell cycle in the formation of compacted mouse embryos at the 8-cell stage. To intercept the cell contact for some period of the third cell cycle, early 4-cell embryos were disaggregated into 4 blastomeres, and then these blastomeres were reaggregated at the early, mid, or late stage of the third cell cycle. We have whether there are time lags for the end points of compaction between those reaggregated embryos. Our results indicate (1) that interception of the cell contact from the early stage to the mid stage of the third cell cycle led to the establishment of compaction being retarded, and (2) that this retardation of compaction coincided with that of the formation of gap junctions.

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Materials and Methods

Embryo collection and culture in vitro: B6C3F1 female mice (6–10 weeks old) were superovulated by intraperitoneal injection of 8 IU pregnant mare's serum gonadotrophin (PMSG, Sankyou, Japan) followed 48 h later by 8 IU human chorionic gonadotrophin (hCG, Teikokuzouki, Japan). The female mice were placed with ICR male mice overnight and mating was confirmed by a vaginal plug the following morning. Pregnant mice were slaughtered by cervical dislocation, and late 2-cell embryos were collected by flushing the oviducts with phosphate-buffered medium containing 3 mg/ml bovine serum albumin (BSA: Fraction V, Sigma) (PBI + BSA) [21] 44–46 h post-hCG. After washing several times, the embryos were cultured in fresh drops of BWW containing 5 mg/ml BSA (BWW + BSA) [22] and 0.04 mg/ml EDTA at 37.3°C in an atmosphere of 5% CO_2 in air.

Zona pellucida removal and embryo disaggregation: Embryos cultured individually were observed every 2 h for evidence of division to 4-cell. The developmental stage of these newly formed 4-cell stage embryos was defined at 0 h postdivision to 4-cell, picked up and ma-

nipulated immediately as follows. The embryos were placed in prewarmed Ca^{2+}, Mg^{2+}-free PBI (PBI^-) at 37°C for 8 min in order to loosen the cell adhesion between blastomeres. They were then exposed to prewarmed 0.5% pronase (Calbiochem-Behring Corp.) in PBI^- at 37°C for 5–10 min to remove the zona pellucida. After washing several times in fresh PBI^-, zona free embryos were disaggregated into 4 blastomeres by pipetting gently with a flame-polished micro pipette.

Embryo reaggregation: Four blastomeres derived from an embryo were reaggregated at three different stages (early, mid, and late stage) of the third cell cycle. For embryos reaggregated at the early stage, 4 blastomeres were aggregated just after disaggregation. For embryos reaggregated at the mid stage, 4 blastomeres were cultured individually for 4 h in separate drops, and then aggregated. For embryos reaggregated at the late stage, 4 blastomeres were cultured for 8 h in different drops, and then aggregated. Aggregation was completed by exposure to 5 μg/ml phytohemagglutinin P (Difco Laborotories). These embryos were cultured individually in drops for a further 12 h, with the features of compaction observed every 2 h. To confirm whether manipuleted embryos develop normally, blastocyst formation was observed at 96 and 120 h post hCG. As a control experiment, embryos from which the zona pellucida were removed, were cultured without disaggregation of the blastomeres and their development was observed.

Criteria for judgement of compaction: Compaction was assessed visually by phase contrast microscope (IMT-2, Olympus, Japan). Embryos in which cell contacts were maximized, cell boundaries were obscure, and the outline of the embryo was smooth, were judged to be compacted (Fig. 1B).

Assessment of polarized microvilli: To assess the polarization of microvilli in the reaggregated embryos, observation by scanning electron microscopy was performed. Embryos were fixed for 1 h with 3% glutaraldehyde and 0.5% paraformaldehyde in Hanks' balanced salt solution (Hanks' BSS) according to the method described by Koyama *et al.* [23]. They were then placed on coverslips coated with poly-L-lysine (Sigma). Specimens were fixed for 1 h with 1% osumium tetroxide in Hanks' BSS. They were then stained with 1% tannic acid aqueous solution for 2 h and further stained with 1% osumium tetroxide aqueous solution for 1 h. They were dehydrated through a graded alcohol series. Samples were critical-point dried using liquid CO_2, mounted, and coated with gold. Each embryo was then observed with a Hitachi S-800 scanning electron microscope at 5 kV, to acurately examine the distribution of the microvilli on the surface of all blastomeres.

Immunofluorescence assay for location of gap junction: The primary antibody used to visualize the gap junction in the whole and 2/4 embryos, was a mouse monoclonal antibody against connexin43 (Zymed Laboratories, Inc., San Francisco, CA), a synthetic peptide corresponding to a 19 amino acids chain found in the rat connexin43 sequence, and used at a dilution of 1:200. The secondary antibody was FITC-conjugated goat anti-

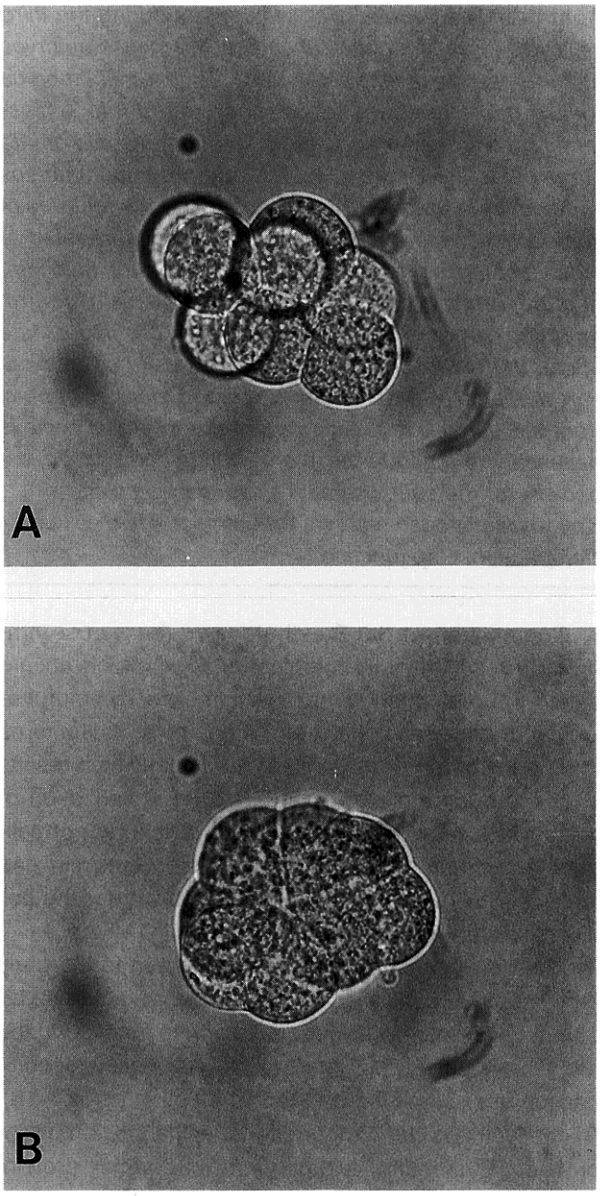

Fig. 1. Photographs of mouse reaggregated embryos. (A) A non-compacted late 8-cell embryo. (B) A fully compacted late 8-cell embryo. × 400.

mouse IgG (Tago, Inc., Burlingame, CA), and was used at a dilution of 1:180. Embryos were fixed for 45 min with 4% paraformaldehyde in PBS, and washed three times with 0.05% Tween-20 in PBS (PBS-Tween). The nonspecific binding sites were blocked in PBS containing 3% BSA at 37°C for at least 30 min and then the specimens were incubated at 37°C for 90 min or at 4°C overnight, in the primary antibody solution. After washing three times in PBS-Tween to remove the unbound primary antibody, the embryos were labelled with the secondary antibody at 37°C for 30 min. Unbound antibody was removed by washing at least three times in PBS-Tween, and the embryos were mounted in glycerol. Labelled embryos were viewed with a Bio-Rad MRC 500 confocal laser scanning microscope.

2/4 embryos for counting the number of cell contacts and gap junctional plaque: The rate of gap junctional formation in cell contact regions was investigated. For ease of counting the number of cell contact regions, 2/4 embryos were used. The 4-cell embryos which disaggregated into pairs of blastomeres are referred to as natural 2/4 embryos. The embryos which disaggregated into 4 blastomeres and reaggregated at the three different stages as mentioned above, are reffered to as reaggregated 2/4 embryos.

Statistical analysis of data: The data were analyzed by chi-square to determine differences in the rate of fully compacted embryos, and the rate of embryos which developed to blastocyst.

Results

Comparison between control embryos and reaggregated embryos on developing compacted state: Forty nine control embryos, 50 embryos reaggregated at the early stage, 49 embryos reaggregated at the mid stage, and 48 embryos reaggregated at the late stage, were observed the end point for complete compaction, and the formation of blastocyst. There was no difference among the 4 groups in the rates of embryos developed to blastocysts stage (Table 1). In the control embryos, the rate of fully compacted embryos was 14.3%, 42.9%, and 69.4%, at 12, 14, and 16 h post division to 4-cell, respectively. By 18 h post division to 4-cell, almost all embryos had completed compaction (93.9% at 18 h and 95.9% at 20 h post division to 4-cell) (Table 2). In the embryos reaggregated at the early stage, the rate of fully compacted embryos was lower than that of the control (22.0%: 42.9%; $P<0.05$) at 14 h post division to 4-cell. In the embryos aggregated at the mid or late stage, the rates were significantly lower than those of the control embryos from 14 h to 18 h post division to 4-cell (Table 2). These results suggest that the end points for complete compaction in embryos reaggregated at

Table 1. Effects of disaggregation and reaggregation at the 4-cell stage on development *in vitro*

Stage at aggregation	No. of embryos	No. of blastocysts (%)	No. of abnormal (%)	No. of degeneration (%)
Control	49	45 (91.8)	3 (6.1)	1 (2.1)
Early	50	42 (84.0)	2 (4.0)	6 (12.0)
Mid	49	44 (89.8)	1 (2.0)	4 (8.2)
Late	48	45 (93.8)	3 (6.2)	0 (0.0)

Differences not significant.

Table 2. Effect of disaggregation and reaggregation 4-cell stage on the compaction *in vitro*

Stage at aggregation	No. of embryos	No. of compacted embryos (%) post division to 4-cell (h)				
		12	14	16	18	20
Control	49	7 (14.3)	21 (42.9)	34 (69.4)	46 (93.9)	47 (95.9)
Early	50	2 (4.0)	11 (22.0)*	33 (66.0)	43 (86.0)	47 (94.0)
Mid	49	3 (6.1)	9 (18.4)**	22 (44.9)**	38 (77.6)**	43 (87.8)
Late	48	2 (4.2)	5 (10.4)**	22 (45.8)**	37 (77.1)**	46 (95.8)

* Singnificantly different with control ($P<0.05$). ** Significantly different with control ($P<0.01$).

the mid or late stage were 2 h later than those of the control embryos.

Comparison of the suface polarity: The degree of surface polarity was determined by the distribution of microvilli on the surface of all blastomeres of an embryo, and embryos were classified into three grades as below: "non-polarized" embryos showed all blastomeres with a uniform distribution of microvilli (Fig. 2A), "partly polarized" embryos showed some blastomeres with a uniform distribution of microvilli and the others with a polarized distribution of microvilli on the opposite side of the cell-to-cell contact region (Fig. 2B), and "fully polarized" embryos showed all blastomeres with a polarized distribution of microvilli on the opposite side of the cell-to-cell contact region (Fig. 2C). At 14 h post division to 4-cell, a large number of the embryos were classified as "non-polarized" or "partly polarized" and few embryos were classified as "fully polarized". At 18 h post division to 4-cell, all embryos were graded as "partly polarized" or "fully polarized", but no embryos were classified as "non-polarized" at all. This trend was not different among control and reaggregated embryos (Table 3). Some embryos had unflattened blastomeres which had polarized distribution of microvilli.

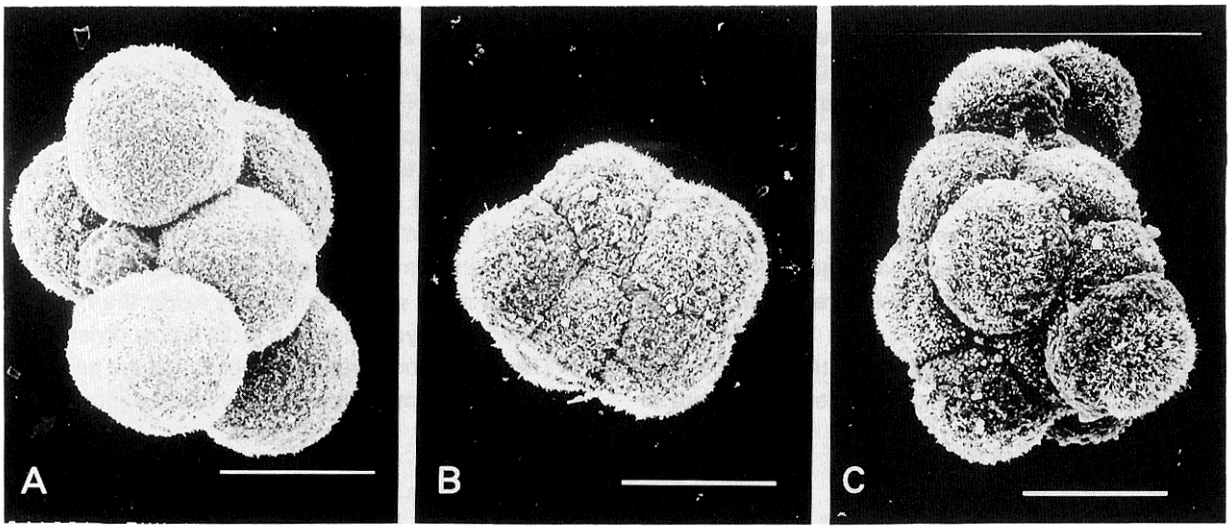

Fig. 2. Scanning electron micrographs of mouse embryo. (A) A "non-polarized" embryo. (B) A "partly polarized" embryo. Some blastomeres had polarized distribution of microvilli. (C) A "fully polarized" embryo. Bar=25 μm.

Table 3. Degree of suface polarity in blastomeres of reaggregated embryos

Hours post division to 4-cell (h)	Stage at aggregation	No. of embryos	Polarized distribution of microvili*		
			non	partly	fully
14	Control	23	16	6	1
	Early	17	11	4	2
	Mid	17	9	7	1
	Late	19	12	7	0
16	Control	21	1	9	11
	Early	18	5	10	3
	Mid	15	2	6	7
	Late	19	4	7	8
18	Control	11	0	3	8
	Early	7	0	3	4
	Mid	8	0	1	7
	Late	7	0	3	4

*Classification of polarized distribution of microvilli is described in the text.

Comparison of development of gap junctions: A mouse monoclonal antibody against connexin43 was used to detect the gap junction. In a preliminary experiment, this antibody was tested with embryos at the 4-cell stage, and the early (non-compacted) and late (compacted) 8-cell stage to assess the efficiency of this probe. Connexin43 was detected in plasma membranes as plaque-like intercellular foci suggestive of gap junctions at the late 8-cell stage, but was not detected either at the 4-cell stage or at the early non-compacted 8-cell stage (data not shown).

First, to make clear to what degree the cell-to-cell communication via the gap junction was established, we examined the number of cell contacts at which the plaque of connexin43 was observed by immunofluorescence in natural or reaggregated 2/4 embryos at 12 h to 18 h post division to 4-cell (Fig. 3). In all of the blastomeres but 4 cases, no plaque of connexin43 was detected in the plasma membranes of cell contacts at 12h (Table 4). In natural 2/4 embryos, the plaque of connexin43 began to be detected at 14 h, and existed in almost all cell contacts at 18 h, while in the 2/4 embryos reaggregated at the mid and late stages of the third cell cycle, the plaque of connexin43 was detected in only 50% of cell contacts (Table 4).

Next, we observed the plaque of connexin43 in the control embryos and embryos reaggregated at the mid or late stage at 12 h to 18 h post division to 4-cell. In the control embryos, the plaque of connexin43 was detected at 14 h post division to 4-cell, but not detected in the reaggregated embryos (Fig. 4A, B). In the reaggregated embryos, the plaque of connexin43 began to be detected at 16 h. The stage of reaggregation had no effect on the timing of plaque formation in those embryos reaggregated at the mid and late stage of the third cell cycle nor in the 2/4 embryos.

Discussion

In mouse embryos, intercellular contact is relatively little until the 8-cell stage when compaction is completed [1]. In the 4-cell embryos, 2 blastomeres are connected via a midbody channel, intercellular bridges maintained by midbodies remaining from the previous cleavage division [8, 24, 25]. However, it is not clear whether the midbody channel is important for mouse embryonic development. In this experiment, disaggregation of embryos and disruption of the midbody did not affect the development of reaggregated embryos, nor the time of the end point for compaction, if the blastomeres were reaggregated immediately after disaggregation. Longer disruption of the intercellular contact, however, retarded the time of the compaction end point (Table 2). Therefore, disruption of midbody channels at 4-cell stage was thought to have no effect on the formation of compaction nor on the development of the embryo, but intercellular contact might play an important role in the determination of the timing of compaction.

Compacted embryos have polarized microvilli on the surface of the cells [2–5]. To assess the degree of cell

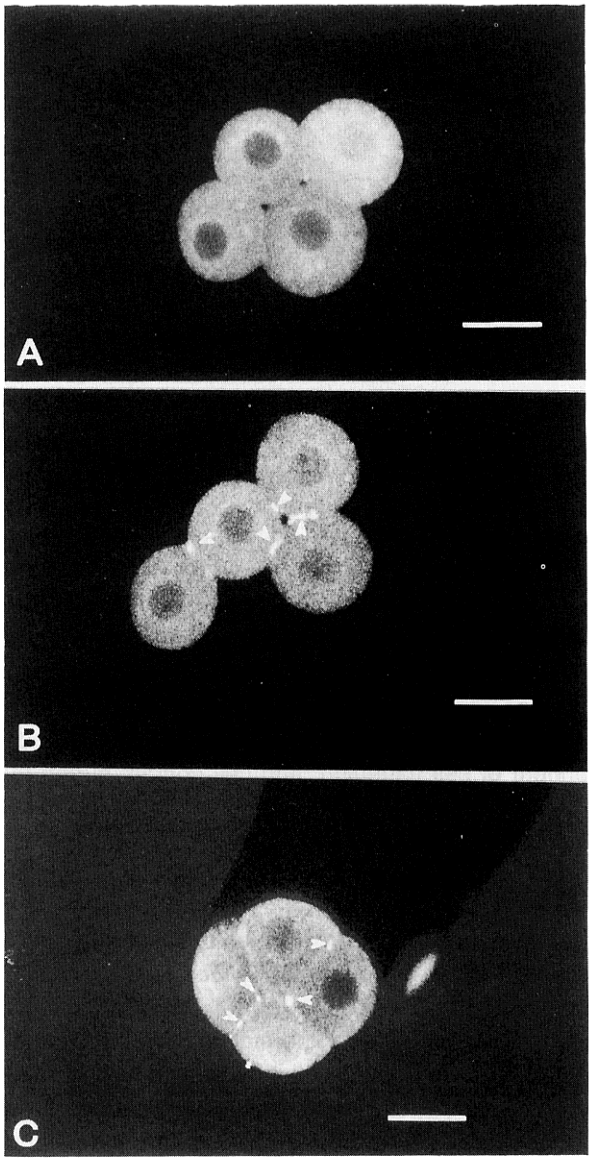

Fig. 3. The plaque of connexin43 by immunofluorescence in natural or reaggregated 2/4 embryo. Total no. of cell contacts was (A) 5, (B) 4, (C) 5, and no. of cell contacts where the plaque of Cx43 was detected was (A) 0, (B) 4, (C) 3. Bar=25 μm.

Table 4. The number of cell contacts and the plaque of connexin43 in natural or reaggregated 2/4 embryos of various age

Hours post dibision to 4-cell (h)	Stage at aggregation[a]	No. of embryos	Total no. of cellcontact (A)	No. of cell contacts where the plaque of Cx43 was detected (B)	B/A (%)
12	Natural	7	29	3	10.3
	Early	13	60	0	0.0
	Mid	10	48	4	2.1
	Late	13	55	0	0.0
14	Natural	14	63	17	27.0
	Early	18	76	32	42.1
	Mid	13	56	3	5.4
	Late	16	73	4	5.5
16	Natural	11	54	28	51.9
	Early	16	76	36	47.4
	Mid	15	65	21	32.3
	Late	25	114	33	28.9
18	Natural	8	36	35	97.2
	Early	15	79	53	67.1
	Mid	14	76	37	48.7
	Late	12	83	36	43.3

[a] "Natural" means 2/4 embryos disaggregated into pairs of 4-cell blastomeres.

polarization, FITC-ConA and SEM have been used. Ziomek and Johnson [2] observed that cell surface polarization detected by using FITC-ConA occurred earlier than compaction. They also concluded that cell surface polarization was induced by contact with other cells. In our experiment, there was no difference in the pattern of cell surface polarization between the embryos which were not disaggregated and the embryos disaggregated and reaggregated during the third cell cycle (Table 3). Consequently, cell surface polarization might be independent of cell flattening, and cell contact during the 4-cell stage might not be so crucial as at the 8-cell stage for cell surface polarization.

Cell-to-cell communication at compaction is done via the gap junction instead of the midbody channel. Gap junctional communication starts to function prior to the establishment of compaction [7]. Gap junctions are independent of cell flattening [9, 26], but functional gap junctions are required for the maintenance of the compacted state and the development to blastocyst [27, 28]. In our experiment, the retardation of gap junctional formation coincided with the delay of the end point of compaction. This tendency was exhibited in reaggregated embryos in which cell contacts were intercepted from the early to the mid or the late stage of the third cell cycle. It is thought that the cell contacts during this period are necessary for the gap junction assembly, but further investigation is needed to confirm this hypothesis.

In mouse embryos, the general activation of the embryonic genome takes place at the 2-cell stage [2]. At the same time, the transcription of the connexin43 gene is initiated [29]. The mRNA for gap junction protein connexin43 is first detected at the 4-cell stage by northern blot analysis [18, 30]. It was shown that protein synthesis of connexin43 begins as early as at the 4-cell stage by western blot analysis and by immunofluorescence assay using confocal laser scanning microscopy [18, 20]. McLachlin and Kidder [15] showed that transcription of the gap junction gene is completed between the late 2-cell stage and early 4-cell stage, and the synthesis of gap junctional protein is completed by the mid 4-cell stage, in an experiment using α-amanitin and cycloheximide, known inhibitors of transcription and protein synthesis.

The nascent connexin43 of the cytoplasm is trafficked to the intercellular membrane and transferred into the plasma membrane [20, 31]. In somatic cells, phosphorylation of nascent connexin43 is necessary for assembly into gap junctional plaques [32]. In mouse embryos, trafficking of nascent connexin occurred as late as at the early uncompacted 8-cell stage [20]. How-

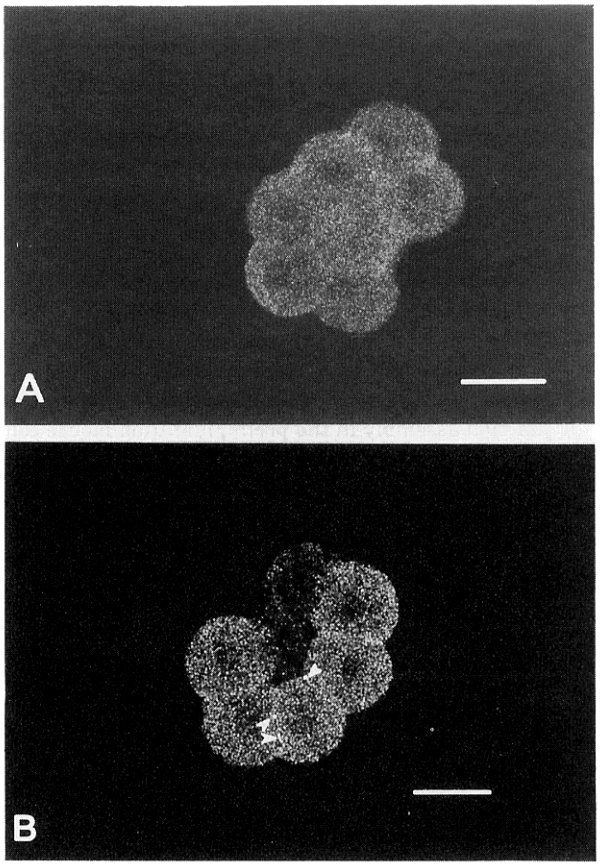

Fig. 4. Pattern of Cx43 stained by immunofluorescence. (A) No plaque of Cx43 was detected. (B) The plaque of Cx43 was detected (arrowheads). Bar=25 μm.

ever, the mechanism of trafficking of nascent connexin43 is not well known.

The role of cell contacts during the development of preimplantation embryos has been researched by disaggregation and aggregation of embryos. Synthesis of some proteins in embryo depends on cell contacts. For example, the activity of 5'-nucleotidase revealed at the cell contacts was in accordance with embryo aggregation [33]. Izquierdo and Ebensperger [34] showed that this enzyme activity was detected from the late 4-cell stage onwards. Surprisingly, the enzyme activity was detected even in 2-cell embryos, if two embryos were aggregated and cultured longer. The enzyme activity was detected at artifical contacts between two embryos as well as at natural contacts in embryos.

In this experiment, the establishment of intercellular communication via the gap junction was retarded when cell contacts were intercepted from the early to the mid or late stage of the third cell cycle (Table 4). Because the feature of connexin43 in the aggregated embryos was not investigated, it was unclear whether interception of the cell contacts caused delays in synthesis, trafficking, or phosphorylation of this protein. However, timing of the establishment of gap junctional communication was not different between embryos aggregated at the mid stage and the late stage. This result indicated that the formation of the gap junction was delayed when the cell contact was intercepted for a period during the 4-cell stage. Therefore, it is thought that cell contacts at the 4-cell stage, especially from the early to the mid stage, are necessary for establishment of the gap junction. Alternatively, signals of cell contact may be transmitted at this period, so that the period of cell contacts may be too short to transmit the signaling in embryos aggregated at the mid and late stage of the third cell cycle. It is necessary to investigate whether signals of the cell contacts stimulate the trafficking of the nascent connexin43 or the assembly into the plaque of connexin43.

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マウスコンパクション胚における第３細胞周期の割球間接触の必要性

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マウス４細胞期胚の割球間接触のコンパクションにおける必要性を，初期４細胞期胚を割球分離し，0，4，8時間後に再集合した胚を用いて検討した．その結果，4および8時間後に再集合した胚でコンパクションに遅れが見られた．そこで，コンパクションの遅れの原因を調べるために，走査型電子顕微鏡による微絨毛の観察と，ギャップ結合構成タンパク質であるコネキシン43（Cx43）抗体を用いたギャップ結合の検出を行ったところ，4および8時間後に再集合した胚でギャップ結合形成に遅延がみられた．これらのことから，コンパクションにはギャップ結合の形成が関与していると考えられた．また，ギャップ結合を形成するためのコネキシン43の細胞内輸送の開始時期は，４細胞期胚の割球間接触の有無により影響を受けることが考えられた．

キーワード：マウス胚，割球間接触，第３細胞周期，コンパクション，ギャップ結合．