Cell contact induces multiple types of electrical excitability from ascidian two-cell embryos that are cleavage arrested and contain all cell fate determinants

Motoko Tanaka-Kunishima, Kunitaro Takahashi, and Fumiyuki Watanabe

Department of Medical Physiology, Meiji Pharmaceutical University, Tokyo, Japan

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Tanaka-Kunishima M, Takahashi K, Watanabe F. Cell contact induces multiple types of electrical excitability from ascidian two-cell embryos that are cleavage arrested and contain all cell fate determinants. Am J Physiol Regul Integr Comp Physiol 293: R1976–R1996, 2007. First published July 25, 2007; doi:10.1152/ajpregu.00835.2006.—Ascidian early embryonic cells undergo cell differentiation without cell cleavage, thus enabling mixture of cell fate determinants in single cells, which will not be possible in mammalian systems. Either cell in a two-cell embryo (2C cell) has multiple fates and develops into any cell types in a tadpole. To find the condition for controlled induction of a specific cell type, cleavage-arrested cell triplets were prepared in various combinations. They were 2C cells in contact with a pair of anterior neuroectoderm cells from eight-cell embryos (2C-aa triplet), with a pair of presumptive notochordal neural cells (2C-AA triplet), with a pair of presumptive posterior epidermal cells (2C-bb triplet), and with a pair of presumptive muscle cells (2C-BB triplet). The fate of the 2C cell was electrophysiologically identified. When two-cell embryos had been fertilized 3 h later than eight-cell embryos and triplets were formed, the 2C cells became either anterior-neuronal, posterior-neuronal or muscle cells, depending on the cell type of the contacting cell pair. When two-cell embryos had been fertilized earlier than eight-cell embryos, most 2C cells became epidermal. When two- and eight-cell embryos had been simultaneously fertilized, the 2C cells became any one of three cell types described above or the epidermal cell type. Differentiation of the ascidian 2C cell into major cell types was reproducibly induced by selecting the type of contacting cell pair and the developmental time difference between the contacting cell pair and 2C cell. We discuss similarities between cleavage-arrested 2C cells and vertebrate embryonic stem cells and propose the ascidian 2C cell as a simple model for toti-potent stem cells.

ascidian cleavage-arrested blastomeres; induced differentiation; ion channels; induction by cell contact; cell fate induction

Recent progress in stem cell biology has brought us a hope to regenerate major postmitotic tissues in human adults, including neural and cardiac tissues. However, we have also come to realize that most embryonic stem cells cannot be controlled to produce a specific cell type in the amount required for treatment of injured individuals (56). One of the reasons for this difficulty is a lack of physiological analyses on the differentiation process of embryonic stem cells. Another reason is the complexity of the differentiation process, such as neurogenesis, in vertebrate embryos. The neural default theory of amphibian embryos in which the organizer produces molecular inducers, noggin, and chordin, cannot explain neurogenesis in birds and mammals (48), which requires another major inducer, basic fibroblast growth factor (bFGF) (7). Furthermore, for mammalian neurogenesis, a contribution of wingless homologues, such as Wnt proteins (18), is crucial. In view of this complexity, it is useful to consider vertebrate embryogenesis in the light of phylogeny. In such efforts, we might be able to find the essential molecular cues for differentiation in mammals to reconstruct neural or other cell types.

Although the adult form of ascidians (protochordate) is sessile living, the tadpole larva has a simplified body organization characterized by its tubular nervous system and notochord homologous to those of vertebrates (44). Thus, the ascidian embryo is known to be one of phylogenetic ancestor models of vertebrate embryos (19). On the other hand, the cell lineage studies of early ascidian embryos by Conklin (3) at the beginning of 20th century have revealed a classical example of the mosaic egg, which shows autonomous differentiation presumably by segregation of cytoplasmic factors (3). Whittaker (55) has further provided the evidence of cytoplasmic factors in the ascidian embryo by showing that cytochemical and micro-morphological characteristics of striated muscle fibers in an ascidian tadpole are expressed in presumptive muscular cells when cleavage of the embryo is arrested at the 8- to 64-cell stages. This finding has been further confirmed by examining other differentiation characteristics, such as distinct forms of electrical excitability in the individual cleavage-arrested cells due to characteristic expression of Na+, K+, and Ca2+ channels in their membrane (49, 50). Therefore, by arresting cleavage, the condition in which multiple cell fate determinants are contained in single cells was artificially achieved in ascidian embryos that usually undergo mosaic development by segregation of maternal determinants into stereotypically cleaving blastomeres (3, 27). This condition provides a unique experimental system to study the process of selection of a specific cell type in a cleavage-arrested blastomere in the presence of multiple cell fate determinants.

Previously, we proposed a cleavage-arrested cell pair composed of a neuroectodermal cell (a4.2) and neuronotochordal cell (A4.1) from an ascidian eight-cell embryo as the simplest neural induction system (35). And, recently in ascidian embryos bFGF was identified as an endogenous neural inducer as it has been shown in chick and mammalian embryos (1, 48). The a4.2 cell has both neural and epidermal fates, and, if it is contacted and induced by the presumptive notochordal A4.1 cell in the cleavage-arrested pair, expresses neuronal ion chan-

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nels and shows neuronal excitability that can be verified electrophysiologically or becomes the default epidermal cell (32, 33, 35). We also reported previously that the cleavage-arrested anterior quadrant, A3, which is derived from a four-cell-stage embryo and is the parent cell of a4.2 and A4.1 cells in an eight-cell embryo, is multifated and can differentiate into any of three cell types (anterior neural, posterior neural, and epidermal cells) when it is contacted by various types of blastomeres from a cleavage-arrested eight-cell embryo or when either bFGF or subtilisin was applied (51).

In the present experiments, we aimed to use the cleavage-arrested embryonic cell from a Halocynthia two-cell embryo (2C cell) as a model for the toti-potent embryonic stem cell. We have successfully induced the 2C cell into all major cell types in an ascidian tadpole larva, namely, epidermal, anterior neural, posterior neural, and muscular types. Since the 2C cell is naturally fated to become a half body, and thus its totipotency is similar to that of a fertilized egg, we expected the 2C cell to respond to various inductive stimuli and become any type of differentiated cells in a controllable way. The inductive stimulus tested was the cell contact with one of four cell types from a cleavage-arrested Halocynthia eight-cell embryo, in which we have demonstrated that major differentiation commitment to either epidermal or neural or notochordal or muscular type is initiated (32, 33, 51). This situation is similar to that of mammalian embryonic stem cells, differentiation of which is most successfully controlled when they were transplanted into early embryonic tissues (56). In this simple cleavage-arrested ascidian embryo system, we might be able to elucidate the molecular mechanism of the contact-induced differentiation process and apply the knowledge to the stem cell research.

MATERIALS AND METHODS

Materials. Artificial seawater Jamarin U (Jamarin Laboratory, Osaka, Japan) was used for culturing ascidian embryos and for experiments. The major ionic composition of Jamarin U is (in mM): 409 Na\(^{+}\), 9 K\(^{+}\), 10 Ca\(^{2+}\), 47 Mg\(^{2+}\), 482.3 Cl\(^{-}\), 23.7 SO\(_4^{2-}\), and 3 HCO\(_3^{-}\). Cytochalasin B, used to arrest cleavage, was purchased from Aldrich (St. Louis, MO) and was prepared as a 2-mg/ml stock solution in dimethyl sulfoxide. We added it as a one-thousandth part to seawater to arrest cell cleavage, and as a ten-thousandth part to maintain the cleavage-arrested condition. Cytochalasin B arrests cleavage of cells by preventing cytokinesis, but does not inhibit nuclear division (46). Cytochalasin B treatment allows expression of certain cell differentiation markers, such as tissue-specific enzymes and membrane excitability (12, 55).

Nomenclature of embryonic cells. Ascidian embryonic cells were named following the designation system first adopted by Conklin (4). Cells of the animal hemisphere are designated by lower-case letters, either "a" or "b", and those in the vegetal hemisphere by capital letters, either "A" or "B". Before animal and vegetal hemispheres are separated, the half embryo is designated as "AB2" at the two-cell stage, and, at the four-cell stage, when one embryo is composed of two pairs of different cells, anterior and posterior cells are named A3 and B3, respectively. "A" and "a" mean anterior cells, and "B" and "b" mean posterior cells. Suffix numbers illustrate the generation and position within a quadrant of a hemisphere.

Preparation of cell pairs from eight-cell embryos, 2C cells from two-cell embryos, and cell triplets. Embryos of Halocynthia aurantium were used in this study. We selected healthy large adult individuals to obtain embryos for the experiments. Adults were acquired at Wakkanai in northern Japan and maintained in an aquarium with circulating seawater at 3°C. The animals were immobilized by chilling with seawater at 4°C. Their esophageal ganglia, which are parts of the central nervous system in adult ascidia, were gently crushed with forceps. Then, oocytes were obtained by dissecting out gonads and were kept in seawater at 9°C for 3–10 h for maturation. Eggs were fertilized with sperm obtained from another individual and reared at 9 ± 0.5°C in filtered Jamarin seawater. Under these conditions, embryos reached the two-cell stage at 3 h and the eight-cell stage at 5 h after fertilization. Subsequently, the development at 9.0°C proceeded through blastula between 32- and 76-cell stages at about 10 developmental hours (DHs); the gastrula, which was formed after the 110-cell stage, at 10–20 DHs; the neurula around 20–30 DHs; the tail bud at 30 DHs; and hatching at 60 DHs. At the late two-cell or early eight-cell stage, embryos were transferred into filtered seawater containing cytochalasin B (2 mg/l; Aldrich) to arrest further cleavage and to obtain the cells separated from a two- or eight-cell embryo. The follicular envelope and chorion were removed manually with a pair of sharp tungsten needles, and single cells or cell pairs were separated with the use of a fine glass needle. According to Conklin (4), two-cell embryos include two equivalent embryonic cells [two AB2s (2C cells)], and eight-cell embryos consist of four different types of cell pairs, a pair of two anterior-animal cells (a4.2), a pair of two posterior-animal cells (b4.2), a pair of two anterior-vegetal cells (A4.1), and a pair of two posterior-vegetal cells (B4.1). Each pair has distinct developmental fate, as listed in Table 1. Eight-cell embryos showed an anteriorly inclined animal-vegetal axis and symmetry at the midplane. Thus, each type of the cell pair was easily identified by its size, color, and relative position in the embryo.

The 2C cells were prepared by separating two-cell embryos at the midplane with a fine glass needle. Here, we selected those two-cell embryos that showed symmetrical first cleavage at the normal developmental time of 3 h at 9°C, since, from our experience of culturing intact Halocynthia embryos in mass, the embryo that showed normal first cleavage would hatch at the regular time and become a normal tadpole. All four types of cell pairs and a4.2-A4.1 cell pairs were separated from eight-cell embryos with a fine glass needle. After preparation, 2C cells from two-cell embryos and cell pairs from eight-cell embryos were kept in filtered seawater containing 2.0 mg/l cytochalasin B for 1 h. They were then transferred into seawater containing 0.2 mg/l cytochalasin B and placed on 1.0% agarose-coated microwells as described previously (51). Various sets of three cells (triplets) were then prepared by manipulating a pair of a4.2 cells (2C-aa triplet) or a pair of A4.1 cells (2C-AA triplet) or a pair of b4.2 cells (2C-bb triplet) or a pair of B4.1 cells (2C-BB triplet) in contact with a 2C cell in a microwell by using a fine glass needle at 6–8 DHs of the 2C cell, and then cultured at 9.0 ± 0.5°C. In addition, a pair of a4.2 and A4.1 cells (aA pair) was incubated as a control for neural differentiation, and a cell pair of a4.2 and a4.2 (aa pair) as a control for epidermal differentiation.

We varied developmental time difference between the 2C cell from a two-cell embryo and the cell pair from an eight-cell embryo, and the timing of manipulated contact between the 2C cell and the cell pair to obtain controlled differentiation of the 2C cell.

Microscopy and imaging. The prepared cell pairs or triplets were cultured in the bath paved with transparent agar containing 0.08 mg/l cytochalasin B and continuously superfused with circulating Jamarin seawater containing 0.2 mg/l cytochalasin B. The bath was set on the stage of an epifluorescence microscope (Olympus IMT-70) to avoid further cell transfer and to make them always ready for observation and recording during development. We monitored the cell conditions and the epidermal tunica with a cooled charge-coupled device camera (model c3140; Hamamatsu Photonics) attached to the microscope throughout experiments. Images were stored on magnetooptical discs. Inspection and analysis of images were later performed with NIH Image or Scion Image software.

Determination of differentiation types. We confirmed the final differentiation types of the cells by using electrophysiological tech-
DILLERENTIATION OF PLURIPOTENT ASCIDIAN BLASTOMERE

Table 1. Developmental fates of Halocynthia early blastomere cells of 2-cell-, 4-cell-, and 8-cell-stage embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>Name</th>
<th>Location in Embryo</th>
<th>Developmental Fate (by cell lineage study)*</th>
<th>Autonomous Development (Cleavage-Crested and Isolated Condition)?</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell</td>
<td>AB</td>
<td>Half side</td>
<td>Whole body</td>
<td>Epidermal</td>
</tr>
<tr>
<td>4-cell</td>
<td>A3</td>
<td>Anterior quadrant</td>
<td>Parent cell of a4.2 and A4.1</td>
<td>Nonexcitable or posterior neural</td>
</tr>
<tr>
<td>4-cell</td>
<td>B3</td>
<td>Posterior quadrant</td>
<td>Parent cell of b4.2 and B4.1</td>
<td>Epidermal or infrequently muscular</td>
</tr>
<tr>
<td>8-cell</td>
<td>a4.2</td>
<td>Anterior animal</td>
<td>Ectoderm</td>
<td>Epidermal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anterior neural</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Palps, brain vesicle, ocellus pigment cell</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Epidermis, epidermal sensory neurons</td>
<td></td>
</tr>
<tr>
<td>8-cell</td>
<td>A4.1</td>
<td>Anterior vegetal</td>
<td>Ectoderm</td>
<td>Nonexcitable or posterior neural</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Posterior neural</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Motor neurons, caudal neural tube</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Photoreceptors</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mesoderm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Notochord, muscle, TLC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Endoderm</td>
<td>Epidermal</td>
</tr>
<tr>
<td>8-cell</td>
<td>b4.2</td>
<td>Posterior animal</td>
<td>Ectoderm</td>
<td>Epidermal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Epidermis, epidermal sensory neurons</td>
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<td></td>
<td></td>
<td></td>
<td>Caudal neural tube</td>
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<td></td>
<td></td>
<td></td>
<td>Mesoderm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Muscle</td>
<td></td>
</tr>
<tr>
<td>8-cell</td>
<td>B4.1</td>
<td>Posterior vegetal</td>
<td>Mesoderm</td>
<td>Nonexcitable or muscular</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mesenchyme, notochord, TVC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Muscle</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Endoderm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Endodermal strand, trunk endoderm</td>
<td></td>
</tr>
</tbody>
</table>

TLC, trunk lateral cells; TVC, trunk ventral cells. *Cell lineage or segregation of developmental fates as reported by Nishida H. and his group (9, 10, 26, 52). †Autonomous development when they are isolated under the cleavage-arrested condition as reported in Refs. 32 and 51.

Characterization of epidermally differentiated cells could be carried out by their electrical properties, namely inward rectifier K+ and Ca2+ channel currents that were recorded under voltage clamp to −120 or +20 mV(12). However, in fully differentiated epidermal cells, the transparent tunic coat on the cell surface prevented the successful electrical recording. Therefore, in most cases we identified the epidermal type by observing the tunic coat on the cell after 50 DHs (11). Muscle cells were identified with the presence of Ca2+ channel current and developed muscle-type delayed rectifier K+ current, and the electrical measurement was possible in the most of the muscullarly differentiated cells (32, 47). There was also supportive identification with histochemical staining of acetylcholine esterase as described in muscle-type cells in cleavage-arrested four-cell, eight-cell, and sixteen-cell embryos. The coincidence of the staining of acetylcholine esterase and electrophysiological identification of muscular differentiation was previously established (32).

**Estimation of gap junctional communication.** To estimate the electrical interference of the recorded 2C cell from the contact cells within a triplet, we always measured the conductance of gap junction under voltage clamp by analyzing the exponentially-rising component of capacitative current in response to rising ramp potential changes or residual exponentially decaying current (pseudocapacitative transient) in response to a step potential change or after the end of falling ramp potential change (see current traces in Figs. 3, 5, 6, and 8 and Ref. 51; Fig. 4, Bb and Bc). These currents are to charge the neighboring cell membrane capacity from the voltage-clamped cell through gap junctional communication (GJC). The ramp potential change had a gradient of 2.0 mV/ms and was applied from a holding potential of −75 mV for 10 ms, keeping the test potential changes within ±20 mV until the end of the slope, as described previously (51). Care was taken not to evoke potential-dependent conductances. The time constants of the residual current ranged from 2 to 5 ms in the majority of cases. In the case of larger time constants less residual currents were observed. If necessary, the electrical connectivity of GJC was quantitatively estimated from the time constant in response to the ramp potential change or the residual current after the voltage step, and the upper limit of the interference by neighboring cell electrical activity was estimated.

Under our experimental conditions, the pseudocapacitative transient at the beginning of potential step due to gap junctional coupling superimposed linearly on the initial part of ion channel currents. Therefore, to show the initial part of ion channel currents accurately, the current traces at the beginning of potential steps were corrected by subtracting the pseudocapacitative current with the assumption that the peak amplitude was proportional to that of the potential step, and...
the corrected traces are illustrated in addition to their original traces in all figures for current traces under voltage clamp. The elemental gap junctional transient was estimated for each cell at the lowest potential step of 10 mV from the resting level where no regenerative current was found (see Fig. 3A3, red trace). The elemental gap junctional transient is mostly a single exponential decay, and its peak is at the beginning of the potential step. For example, the corrected traces of Fig. 3A1 are illustrated in Fig. 3A2.

RESULTS

Timing requirement for 2C cell induction. To find default differentiation of 2C cells, 25 2C cell pairs, in which two 2C cells were in contact, and 11 single 2C cells were cleavage arrested and cultured. Their differentiation types were examined after 50 DHs. The results were illustrated in Table 2A. Twenty-two 2C cell pairs out of 25 and eight single 2C cells out of 11 became epidermal cells with tunic coats. Three 2C cell pairs out of 25 and two single 2C cells out of 11 showed an electrical activity of the immature epidermal type with relatively thin tunic coats, revealing egg-type Na⁺ channel currents. One exceptional case out of 11 single 2C cells showed the posterior neuronal-type response with an A-type current. This exception revealed the latent ability of the 2C cell to become a cell type other than the default epidermal type. The posterior neural type characterized by the A-type K⁺ current was defined in our previous paper (51).

Our previous triplet induction experiments (51), using an anterior quadrant cell from a four-cell Halocynthia embryo and a cell pair from an eight-cell embryo, demonstrated that success or failure of induction depends on the type of contacting cells and the relative developmental stage of contacted cells.

To examine the effect of developmental stage difference between 2C cell and eight-cell embryos on the fate of 2C cells,

Table 2. Autonomous differentiation of cleavage-arrested half 2-cell embryo and nonepidermal differentiation of 2C-cell triplets

<table>
<thead>
<tr>
<th>Differentiation Type</th>
<th>Isolated 2C</th>
<th>Contacted 2C/2C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonepidermal type</td>
<td>Post-N-type</td>
<td>1</td>
</tr>
<tr>
<td>Epidermal type</td>
<td>Egg Na channel</td>
<td>2</td>
</tr>
<tr>
<td>Epidermal type</td>
<td>Tunic coat</td>
<td>8</td>
</tr>
<tr>
<td>Sum</td>
<td>11</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 3. Differentiation type of 2C cells in triplets and difference of developmental hours

<table>
<thead>
<tr>
<th>Epi or Non-Epi</th>
<th>Differentiation Type</th>
<th>3-h Advance</th>
<th>Coincident</th>
<th>3-h Later</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epi</td>
<td>Anterior-N-type</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Non-Epi</td>
<td>Posterior-N-type</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Epi</td>
<td>Nonexcitable-type</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Epi</td>
<td>Nonepidermal</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Undetermined</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Values are observed number of 2C cells; n = total triplets examined for each type. 3-h Advance or later means that embryos from which 2C cells were prepared fertilized 3 h earlier or later than 8-cell embryos from which the cell pairs were prepared, respectively. Epi, epidermal, N-type, neural-type.

we first varied DHs of the 2C cell from two-cell embryos and the contacting cell pair from eight-cell embryos, and examined whether other types of differentiation than the default epidermal type emerge. As shown in Table 2B and Table 3, we found the following. First, when the eight-cell embryos from which contacting cell pairs were prepared were fertilized 3 h earlier than two-cell embryos from which 2C cells were prepared, most of 2C cells (82.5%) in the triplets differentiated into cell types other than the epidermal type. Second, when the eight-cell embryos were fertilized at the same time as the two-cell embryos or when two-cell embryos were fertilized 3 h earlier than eight-cell embryos, the nonepidermal-type differentiation was obtained in 53% cases, and the epidermal-type differentiation was found in the remaining 47% cases. Furthermore, among clearly defined cases in which the 2C cells showed the electrical properties other than epidermal or nonexcitable cells, 13.5% of 2C cells became nonepidermal when two-cell embryos were fertilized 3 h earlier than eight-cell embryos, while it was 23.5% when two-cell embryos were fertilized at the same time as eight-cell embryos, and it was 53% when two-cell embryos were fertilized 3 h later than eight-cell embryos. In the Table 2B, the results from altogether six experimental series were collected and summarized. We drew the following conclusion from these results. As for the difference of developmental stages between the 2C cell and the cell pair, the batch
of two-cell embryos from which 2C cells are prepared must be fertilized at the same time or preferably 3 h later than the batch of eight-cell embryos from which cell pairs are prepared.

In addition, as for the cell contact timing, the contacting 2C cells in a triplet must be <8 h in development regardless of the developmental time difference to obtain nonepidermal-type induction, as described in the section of 2C cell preparation of Materials and Methods. When the contacting 2C cells were >8 h in development at the time of contact, the 2C cells always became the default epidermal type. Those cases were not included in the Table.

Spike potentials, voltage clamp experiments, and current-voltage relationship in 2C cells that were differentiated into the neural type. As shown Table 3, after 50 DHs, we examined 18 2C-aa triplets prepared under various timing conditions. When two-cell embryos were fertilized 3 h later or at the same time as eight-cell embryos, most 2C cells showed the nontunic and nonepidermal type. Traces in Fig. 1A, 2C-aa, illustrate spike potentials evoked in the 2C cell in a 2C-aa triplet at 80.5 DHs with constant current stimulation. The spikes showed characteristically small (<10 mV) or no overshoot and a sharp decay due to activation of A current-type K⁺ current, indicating the typical posterior neural type as previously described in an anterior quadrant cell, the neurally differentiated A3 cell in A3-aa triplets (51). The posterior neural-type spike potentials under constant current stimulation was also found in nonepidermally differentiated 2C cells within 2C-AA triplets. Non-epidermally differentiated 2C cells were morphologically distinct with no tunic coats as shown in Fig. 2B, 2C (post-N-type)-AA. Figure 3, A1 and B1 illustrate current traces recorded under voltage clamp in the neurally differentiated 2C cells induced in two 2C-AA triplets at early 50 DHs and at 79.5 DHs, respectively. The currents at 79.5 h showed large A current and apparently short-lasting small Na⁺ current characteristic of the posterior neural type, as described in our previous paper (51). At 50 h, the currents showed almost no inward current but exhibited a delayed outward current, revealing no significant A current, as described previously. However, if the current traces were corrected by subtracting the GJC transient at the beginning of the potential step as described in Fig. 3, the depressed inward peak of the Na⁺ current indicated the presence of A-type K⁺ current (vertical arrow in Fig. 3A2). In the current-voltage relationship (I-V) (Fig. 4, Aa and Ab), the half-maximum amplitude potential for Na⁺ inward current was −15 mV and the peak amplitude potential was +3 mV. The activation potential levels were apparently more positive than

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**Fig. 1. Na⁺ or Ca²⁺ spikes evoked by constant current stimulation (CC) in 2C cells of 4 types of 2C triplets. Traces were recorded in the 2C cell of 2C triplets at ~70 developmental hours (DHs).**

**A:** posterior neural-type (Post-N-type) Na⁺ spike recorded in a 2C cell within a 2C-aa triplet. The spike showed a relatively high threshold and sharp fall. **B:** anterior neural-type (Ant-N-type) Na⁺ spike recorded in a 2C cell within a 2C-aAaA triplet. **C:** Ca²⁺ spikes with a relatively long duration recorded in a muscularly differentiated 2C cell within a 2C-bb triplet. M-type, muscle cell type. **D:** Ca²⁺ spikes with a relatively long duration recorded in a muscularly differentiated 2C cell within a 2C-BB triplet. Upper and lower traces illustrate potential and current changes, respectively, in each record. The DHs of the 2C cell within a triplet are indicated in respective records. In C the bridge balance was adjusted at the beginning of the current pulse but at the end the balance was slightly off.
those of Na\(^+\) channel current in neurally induced a4.2 cells, because of the presence of large outward A current over inward Na\(^+\) inward current at the relatively negative potential of -40 mV. This point was reported previously in the neurally induced anterior neural type. C: a 2C-bb triplet at 47.5 DHs in which the 2C cell showed inductive differentiation into the muscle type. D: a 2C-BB triplet at 79.5 DHs in which the 2C cell showed inductive differentiation into the muscle type. The inductive pairs were prepared from 8-cell embryos fertilized 3 h in advance to 2-cell embryos from which the 2C cell was prepared, except in D in which 8-cell and 2-cell embryos were fertilized simultaneously. E, F, G, and H: 2C cells in triplets failed inductive differentiation and showed the default epidermal-type differentiation. The lower photos of E, F, G, and H show the same as illustrated in the corresponding upper photos after the process of edge detection. The photographs reveal clear presence of tunic coats on the surface of 2C cells, indicating epidermal-type differentiation. The DHs of the 2C cells in E, F, G, and H are 49.5, 76.5, 50, and 50, respectively. The 2-cell embryos from which the 2C cells were prepared and the 8-cell embryos from which inductive pairs were prepared had been fertilized simultaneously in all these cases of failed induction. The scale in the lower right (200 \(\mu\)m) applies for all photographs. Epi-type, epidermal cell type.

Fig. 2. Brightfield photographs of 2C triplets in which 2C cells showed successful or failed inductive differentiation into the 4 cell types. A: A 2C-aAaA triplet at 47.5 DHs in which the 2C cell showed inductive differentiation into the anterior neural type. B: A 2C-AA triplet at 50 DHs in which the 2C cell showed inductive differentiation into the posterior neural type. C: a 2C-bb triplet at 47.5 DHs in which the 2C cell showed inductive differentiation into the muscle type. D: a 2C-BB triplet at 79.5 DHs in which the 2C cell showed inductive differentiation into the muscle type. The inductive pairs were prepared from 8-cell embryos fertilized 3 h in advance to 2-cell embryos from which the 2C cell was prepared, except in D in which 8-cell and 2-cell embryos were fertilized simultaneously. E, F, G, and H: 2C cells in triplets failed inductive differentiation and showed the default epidermal-type differentiation. The lower photos of E, F, G, and H show the same as illustrated in the corresponding upper photos after the process of edge detection. The photographs reveal clear presence of tunic coats on the surface of 2C cells, indicating epidermal-type differentiation. The DHs of the 2C cells in E, F, G, and H are 49.5, 76.5, 50, and 50, respectively. The 2-cell embryos from which the 2C cells were prepared and the 8-cell embryos from which inductive pairs were prepared had been fertilized simultaneously in all these cases of failed induction. The scale in the lower right (200 \(\mu\)m) applies for all photographs. Epi-type, epidermal cell type.
Since the A current is characteristic to neural differentiation of neurally differentiated 2C cells within 2C-aa or 2C-AA triplets as in anterior quadrant (A3) cells described in the previous paper (51), development of the A current was used as a differentiation marker for the posterior neuronal type. According to the results of the previous paper, the A current expressed in the A3 cell showed an outwardly directed transient within 50 ms after the onset of a step pulse above -15 mV. Although at -15 mV the A current occurred simultaneously with the inwardly directed Na current, the ratio of the amplitude with its sign (negative values were assigned to inward-directed currents and positive values for outward-directed currents) at 50 ms after stimulation to the peak amplitude of the inward current at -15 mV (mostly within 10 ms after the step) was a good marker for the presence of the A current (Fig. 3A4). The ratio was larger than 0.25 for the cells that expressed only Na current and no A currents, such as for the a4.2 cell in the aA neural induction pair (51). The ratio was <0.25 if A currents were expressed and became significantly negative in the posterior neurally differentiated A3 cells. The same ratio was used as a simple indicator to illustrate whether the 2C cell expresses the A current and is posterior neurally differentiated in various triplets in the present experiment (see Fig. 9D). In Fig. 3, the 2C cells at early 50 DHs and at 79.5 DHs revealed A current index 0.2 and 0.9, respectively.
To induce the anterior neural type characterized by the absence of significant A current, we manipulated the neurally determined cell pair aA to contact with the 2C cell. In our previous studies (33, 51), we demonstrated that the aA pair in a cleavage-arrested eight-cell embryo is a simple neural induction model, and a4.2 cells always become the anterior neural type when they differentiate. In this study, as shown in Fig. 2A (2C-aAaA) and the schematic illustration in Fig. 12 (2C-aAaA), we manipulated the 2C cell to directly contact with two a4.2 cells and not with two A4.1 cells during the required induction time, i.e., 10 h from the beginning of contact. The 2C-aAaA traces in Fig. 1B illustrate the anterior N-type spike potentials with a large overshoot of >40 mV, which were evoked by constant current stimulation in the 2C cell in a 2C-aAaA triplet. Figure 5, A1 and B1 illustrates current traces under voltage clamp of an anterior neural-type 2C cell induced in a 2C-aAaA triplet at early 47.5 DHs and at 75.8 DHs. The currents at 75 h showed clear neuronal-type Na⁺ current and delayed rectifier K⁺ current without significant A current, showing characteristics of the anterior neural type, as described in our previous paper (51). The A current index was 0.4, >0.25 in this case. Around 50 h, the currents of the neuronal type showed relatively small inward current and a substantial amount of delayed outward current, as described previously. In the I-V relation illustrated in Fig. 4, Ba and Bb, the half-maximum potential for the Na⁺ inward current was −15 mV and the potential at which the Na⁺ inward current was maximum was −5 mV. These potential levels were the same as those of Na⁺ channel current in anterior neurally induced a4.2 cells (51). The initial pseudocapacitive transient at the beginning of voltage step in the traces at 47.5 h is due to GJC as described above. However, at 75 h the GJC component of the transient was reduced, assuring that the major currents under voltage clamp were derived from the penetrated 2C cell. In rare cases, it was noted the small inward-directed notches on the falling phase of Na⁺ current in the 2C cell, which were generated by electronic spread of spikes produced in the neighboring cells, suggesting neuronal differentiation of contacting a4.2 cells. In Fig. 5, A2 and B2, the initial portions of current traces were corrected by subtracting the respective neighboring-capacity-charging currents from the original traces shown in Fig. 5, A1 and B1, respectively. In Fig. 5A2, the initial outward transients superimposing on the earliest portion of small Na⁺ inward currents in 47.5 DHs traces were likely to be, at least in part, the reversed inward K⁺ rectifier current.

Differentiation of the 2C cell in contact with the aa cell pair. As shown Table 3, when two-cell embryos were fertilized 3 h later or at the same time as eight-cell embryos, most 2C cells showed the nonepidermal type without tunic coats, of which three were identified electrically as the posterior neuronal type and no 2C cells were identified as the anterior neural type. On the other hand, most 2C cells in the triplets in which the
two-cell embryos were fertilized 3 h ahead of the eight-cell embryos, and half of the 2C cells in the triplets in which the two-cell embryos were fertilized at the same time as the eight-cell embryos showed clear tunic coats and were identified as the epidermal type, except one 2C cell fertilized 3 h in advance showed posterior neural type. In all cases, including those in which the 2C cells became epidermal and nonepidermal, the a-a pairs in the triplets differentiated into the epidermal type as judged by the presence of clear tunic coats on the a-a pairs, as shown in Fig. 2E [2C(Epi)-aa].

In summary, the induction of 2C cells in the 2C-aa triplet into the posterior neural type was found to be clear, although it was not strictly DH difference dependent. In our previous experiments with the triplets, composed of a quadrant cell from four-cell embryos and a pair from eight-cell embryos, the anterior quadrant cell from four-cell embryos showed default commitment to the posterior neuronal type and the differentiation was facilitated by the contact with the aa cell pair. Similarly, we suspect that differentiation of the 2C cell into the posterior neuronal type in a 2C-aa triplet was not instructive, but rather permissive induction by the aa cell pair. However, we also noted that the posterior neural type was not the major type of autonomous differentiation of the 2C cell.

**Differentiation of the 2C cell in contact with the aa-aA cell pair.** Among 15 2C-aAaA triplets, we obtained four anterior neuronal types when 2C cell embryos were fertilized 3 h later than eight-cell embryos or at the same time as eight-cell embryos, as shown in Table 3 (2C-aAaA). As shown in Fig. 2A [2C(antN)-aAaA], 2C cells and aA pairs in neurally induced 2C-aAaA triplets were not covered with tunic coats, and a4.2 cells in the triplets became the neural type as described above. Manipulation of the 2C cell to be directly in contact with two a4.2 cells and not with two A4.1 cells during the required induction time was relatively difficult, and cases, in which the contact was morphologically confirmed, were rare. This was the reason why the successfully neurally induced 2C cells were a few in the 2C-aAaA triplets. It was noted that 2C cells, in which induction failed, differentiated epidermally or became nonexcitable, but did not differentiate into the posterior neural type [Table 3 (2C-aAaA)].

**Differentiation of the 2C cell in contact with the AA cell pair.** As shown Table 3, we examined 18 2C-AA triplets after 50 DHs of 2C cells with various timing conditions, most 2C cells in triplets in which two-cell embryos were fertilized 3 h later than eight-cell embryos, showed the nontunic and nonepidermal type, and in two cases, 2C cells were identified electrically as the anterior neural type. When two- and eight-cell embryos were fertilized at the same time, 2C cells from two triplets were identified as the posterior neural type, and those from two other triplets as the nonexcitable type. When two-cell embryos were fertilized 3 h ahead of...
eight-cell embryos, 2C cells in three out of five triplets showed
epidermal differentiation. In all cases, AA pairs in the triplets
did not become the epidermal type, but became nonexcitable.
Thus, the 2C cell in the triplet was induced to become the
neural type by the AA pair. The 2C cells differentiated into
either anterior or posterior neural type, but the latter was
dominant.

Spike potentials, Ca$^{2+}$ channel currents, and I-V relations in
the 2C cell differentiated into the muscular type. In Fig. 1C, the
current-clamp record from a nonepidermally differentiated 2C
cell that was induced in a 2C-bb triplet at 79.8 h revealed spike
potentials with an overshoot of 30 mV and a duration of 60 ms
at the 0 mV level, showing a relatively fast rise and slower
decay. The critical membrane potential was above −25 mV,
which was relatively more positive than those of neural spikes
described above. These spike potentials were neither posterior
neural nor anterior neural type judged from their threshold
potentials and spike shapes. Figure 6, A1 and B1 illustrates
current traces under voltage clamp recorded in the same non-
epidermally differentiated 2C cells induced in a 2C-bb triplet at
49.7 DHs and at 79.8 DHs, respectively. The traces at 79.8 h
showed clear inward currents with a slow rise and decay
compared with those of neuronal-type Na$^+$ currents at the
similar potential level and highly developed delayed rectifier
K$^+$ currents without significant A currents. At 49.7 h, the
currents were entirely inward at +3 mV, slow in rise, and long
lasting, which was characteristic of the L-type Ca$^{2+}$ current.
These currents have the same kinetic properties and voltage
dependency as those previously described in the differentiated
muscle-type B5.1 from a 16-cell embryo or B4.1 from an
eight-cell embryo, or B3, the posterior quadrant cell of a four-cell
embryo of the Halocynthia roretzi and aurantium, in
the previous papers when the external Ca$^{2+}$ concentration of
10 mM in the present experiments was taken into consideration
(32, 36, 37). In Fig. 6, A2 and B2 the current traces corrected
by subtracting the GJC transient current at the beginning of
voltage step are illustrated compared with original traces of A1
and B1, respectively. GJC transients in 2C-bb triplets were less
than the cases in 2C-AA or 2C-aAaA, because the contacting
inducer cell pair bb was differentiating into epidermal cells
with tunic coats and was different from the muscle-type 2C cell
in the same triplet, as shown in Fig. 2C (inductive differenti-
ation of 2C-bb triplet). The tunic coat was observed only on the
surface of bb pairs, avoiding the surface of the 2C cell, as
shown in Fig. 2C, inductive differentiation of a 2C-bb triplet.
The epidermally differentiated bb cell pairs did not show any
delayed rectifier K$^+$ currents. Thus, these findings confirm the
induction of the 2C cell in 2C-bb triplets into the muscle type.
The I-V relations for the 2C cell in a 2C-bb triplet are
illustrated in Fig. 7, Aa and Ab. The half-maximum potential

\[ I(V) = \frac{1}{2} I_{\text{max}} \cdot \left( \frac{V - V_{\text{th}}}{V_{\text{m}} - V_{\text{th}}} \right) ^{1/2} \]

Fig. 6. Current traces of the muscularly differenti-
ated 2C cells in contact with the b4.2-b4.2 pair under
voltage clamp. A1 and A2: traces from a muscularly
differentiated 2C cell in a 2C-bb triplet at 49.7 DHs.
The current traces at the beginning of the potential
steps in A1 were corrected by subtracting the pseudo-
capacitative current as described in the legend of Fig.
3. The corrected traces are illustrated in A2. B1 and
B2: traces from the same muscularrly differentiated
2C cell as shown in A1 and A2, at 79.8 DHs. In B2,
the corrected traces of B1 are shown. The 2C cells in
A1 and B1 developed 3 h later than the contacted bb
pairs and the 2C-bb triplet was the same between A1
and B1.
for the Ca$^{2+}$ inward current was −7 to −5 mV and the potential at which the Ca$^{2+}$ current was maximum was 7 to 12 mV. These potential levels were the same as those of L-type Ca$^{2+}$ channel currents in the differentiated muscle-type, cleavage-arrested cells of Halocynthiae when the external Ca$^{2+}$ concentration of 10 mM in the present experiment was taken into consideration.

Figure 1D illustrates another current-clamp record of a muscually differentiated 2C cell induced in a 2C-BB triplet at 56 h. The spike potentials showed an overshoot of 25 mV and a duration of 60 ms at the 0 mV level, revealing a relatively fast rise and slower decay similar to the muscually differentiated 2C cells in 2C-bb triplets. The critical membrane potential was also above −25 mV, which is relatively more positive than those of neural spikes described above. Figure 8, A1, B1, and C1 illustrate current traces of three muscually differentiated 2C cells induced in 2C-BB triplets at 31.5 DHs, at 56 DHs, and at 79.2 DHs, respectively. The currents at 56 h showed clear inward currents with a slow decay at the −25 mV level and a huge delayed rectifier K$^+$ current without a significant A current above the −30 mV level. At 31.5 h, the Ca$^{2+}$ current was clearly detected, although relatively small in amplitude. The currents were purely inward at +5 mV, and slow in rise and long lasting at any potential levels above the activation potential level, which was characteristic of the L-type Ca$^{2+}$ current, as described for the cases of the 2C-bb triplet at 49.7 DHs. The 2C cell was confirmed by the absence of tunic coats as nonepidermally and thus muscually differentiated at 50 DHs, although electrical recording was not successful. At 31.5 h, the egg-type Na$^+$ current with a more negative activation level than that of the neuronal-type Na$^+$ channel current in neurally differentiated cells was also detected. The egg Na$^+$...
The channel was previously described as characteristic to the ascidian egg and early embryonic cell membrane (49). Figure 8, A2, B2, and C2 shows the corrected traces by subtracting the pseudocapacitative transient through GJC at the beginning of voltage step corresponding to the original traces of Fig. 8, A1, B1, and C1, respectively. The corrected traces for 56 DHs and 79.2 DHs showed no significant difference from the original traces, suggesting that the transients due to GJC became less in magnitude after development. However, the corrected traces for early 31.5 DHs clearly revealed the L-type Ca\(^{2+}\)/H\(^+\) current and egg-type Na\(^+/H^+\) current, as described above for the case of 2C-bb triplets. In I-V relations illustrated in Fig. 7, Ba, Bb, and Bc, the half-maximum potential for the Ca\(^{2+}\) inward current was -5 mV and the potential at which the Ca\(^{2+}\) current became maximum was 7 mV in the case of 56 DHs. These potential levels were consistent with those of the L-type Ca\(^{2+}\) channel current in musculature differentiated cleavage-arrested cells of B3 of Halocynthiae four-cell embryos, B4.1 of eight-cell embryos and B5.1 of 16-cell embryos (32), when the external Ca\(^{2+}\) concentration of 10 mM in the present experiment was taken into consideration.

In the induced 2C-BB triplets, it is noted that no tunic coat was observed on the surface of the 2C cell, as shown in Fig. 2D, inductive differentiation of 2C-BB triplet. When the electrical excitability of the B4.1 cells in the triplets was examined, they were either nonexcitable or had an excitability with small Ca\(^{2+}\) and delayed K\(^+\)-type currents. The default differentiation of a single B4.1 cell separated from an eight-cell embryo showed either epidermal-like excitability with Ca\(^{2+}\) channels or the muscular-type Ca\(^{2+}\) spikes, as reported previously (32).

**Differentiation of 2C cells induced by bb cell pair contact.** As shown in Table 3, 2C-bb, we examined the 2C cells in 21 2C-bb triplets with various DH different conditions after 50 DHs. When two-cell embryos were fertilized 3 h later than eight-cell embryos, eight out of 10 2C cells showed the nonepidermal type without tunic coats, and among these eight cells, six cells were electrically identified as muscle cells.
However, there were two cases of epidermal-type differentiation out of those 10 2C cells. On the other hand, when two-cell embryos were fertilized 3 h in advance or at the same time as eight-cell embryos, most cells showed clear tunic coats and were identified as the epidermal type. In no cases, was neuronal-type differentiation found.

The differentiation of bb pairs in the triplets was always identified as the epidermal type judging from tunic coats, as shown in Fig. 2, C and G, inductive and failed inductive differentiation of 2C-bb. In summary, the induction of 2C cells into the muscle type in 2C-bb triplets was unambiguous because the muscle type was not default differentiation of the 2C cell, without exception.

Differentiation of 2C cells induced by B-B cell pair contact. As shown in Table 3, 2C-BB, we examined 2C cells in 13 2C-BB triplets with various DH difference conditions after 50 DHs. When two-cell embryos were fertilized 3 h later than eight-cell embryos, four out of five 2C cells differentiated into the nonepidermal type without tunic coats and were electrically identified unambiguously as the muscular type. When two-cell embryos were fertilized at the same time as eight-cell embryos, two out of seven were identified as the muscular type. When two-cell embryos were fertilized 3 h earlier than eight-cell embryos, a case was nonepidermal and nonexcitable. Neuronal-type differentiation was observed in no 2C cells examined in 2C-BB triplets as in 2C-bb triplets.

In all cases, the BB pairs in the triplets became nonexcitable or epidermal-like or muscular type, as reported previously in the isolated cleavage-arrested B4.1 cells (32). In summary, the muscular-type differentiation of 2C cells was unambiguously induced by the BB pair in the triplets.

Time course of ion channel expression in the 2C cell in triplets. In Fig. 9, A–C, the time courses of ion channel expression of neurally differentiated 2C cells in various types of triplets are illustrated. The expression of Na⁺ channels and delayed rectifier K⁺ channel started after 45 h in 2C cells differentiated into either anterior or posterior neural type, as described in the anterior quadrant A3 cells in A3-aa or A3-AA triplets reported previously (51). Development of the A current was relatively delayed compared with the Na⁺ channel current or delayed rectifier K⁺ current in the posterior-neurally differentiated 2C cells. This was also true for the posterior neural differentiation of anterior quadrant A3 cells described previously (51). Thus, we concluded that the expression, and possibly inductive process, of neuronal ion channels in 2C cells was similar to the anterior quadrant A3 cells in triplets previously described (51). In addition, Fig. 9D illustrates the changes in the A current index for 2C-aa, 2C-aA-aA, and 2C-AA triplets. The value 0.25 clearly separates between anterior-neural and posterior-neural types. However, in the 2C cell in 2C-AA triplets the A current index tended to be more negative as development progressed. Also, it was noted that at the early time around 50 DHs there was a clear indication of small A current as shown in Fig. 3A4, 2C cell in a 2C-AA triplet, 50 DHs.

In Fig. 10, A and B, the time courses of ion channel expression in 2C cells differentiated into the muscular type are illustrated in 2C-bb and 2C-BB triplets. The expression of Ca²⁺ channels in these cells, especially in the case of 2C-BB triplets, initiated as early as 30 h, while in 2C-bb triplets it seemed slightly delayed. Initial appearance of Ca²⁺ channels was earlier than that of neuronal Na⁺ channels. The L-type Ca²⁺ channels appeared in the epidermally differentiated 2C cell as well, as shown in Figs. 10C and 11. However, the epidermally-differentiated cells did not express the associated delayed rectifier K⁺ current. The only significant delayed

![Fig. 9. Developmental changes in the peak or steady amplitude of inward and outward currents in 2C cells in 2C-aa (A), 2C-aA-aA triplet (B), and 2C-AA triplet (C), and in the A current index (D). Peak amplitudes of inward currents at −15 mV representing the neuronal Na⁺ channel currents, the steady outward current at 20 mV measured during a 10-ms period from 190 ms to 200 ms after the voltage step representing the delayed rectifier K⁺ current at 20 mV and outward transient at 20 mV representing the A type K⁺ channel current were plotted against developmental time. Data represented by the circle symbols with the same color indicate the recordings obtained from the same 2C cell at a different DH. Some of the peak amplitudes of inward currents that were estimated by the maximum rate of rise of the spike potentials and the effective capacity of the penetrated 2C cells were also included in the plotting and those plots are indicated by asterisks on the upper right of symbols. In D the developmental changes in the A current index are shown for 2C cells in 2C-aa (A), 2C-aA-aA triplet (B), and 2C-AA triplet (C) with different symbols. The dotted line for 0.25 indicates the separation between anterior and posterior neural types.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00767.2006)
rectifier K⁺ current in Fig. 10C, 96 DHs, was derived from the rare posteriorly-neuralized 2C cell. Furthermore, the epidermal Ca²⁺ channel appeared much later, after 50 DHs and the maximally developed current amplitude was significantly less than that in the 2C cells differentiated into muscle cells. The Ca²⁺ channel expression in 2C cells was apparently similar to that of the posterior quadrant cell from four-cell embryos (B3), which differentiated in the muscular type in the isolated and cleavage-arrested condition although in rare cases, <20% of the population (32). However, 2C cells in isolation or in contact with another 2C cell never differentiated into muscle cells. Therefore, as shown above, the 2C cells in 2C-bb or 2C-BB triplets were definitely induced to differentiate into muscular-type cells, and thus the channel expression mechanism of induced 2C cells was different from that in the isolated posterior quadrant B3 cells. Delayed rectifier K⁺ channels in muscular-type 2C cells appeared after 50 h in both 2C-BB and 2C-bb triplets. There were the cases in which development of marked transient outward currents appeared in 2C-BB triplets with a delay of a few tens of hours after the appearance of the delayed rectifier K⁺ current. Further experiments are required to determine whether the transient outward current expression is characteristics of induction by B4.1 cell pairs. However, Nakajo and Okamura (25) recently reported the transient outward current due to Ca²⁺-induced K⁺ channels in ascidian larval muscle cells. Since we were recording the transient outward current in the presence of 10 mM Ca²⁺, the outward current that we recorded could be Ca²⁺-induced K⁺ currents characteristic of ascidian larval muscle cells. Although no neuronal-type Na⁺ channels appeared in differentiated muscle cells, expression of the egg-type Na⁺ channel was found at early stages of development from 10 to 50 h in any triplets or previously described as neuronally differentiated a4.2 cells or epidermally differentiated cleavage-arrested one-cell embryos (11, 40). The egg Na⁺ currents were different from those neuronal-type Na⁺ currents in the activation potential and the current decay time course (40), and its expression was considered to depend on maternally transferred mRNA, as described previously (11, 49).

**DISCUSSION**

In the present paper, we used the cleavage-arrested embryonic cell from *Halocynthia* two-cell embryos as a model for toti-potent embryonic stem cells for the controlled differentiation to induce four excitability types by forcing them to contact with the cell pairs from eight-cell embryos. The reason why we have chosen the cleavage-arrested 2C cell as a model is as follows. When an embryo is cleavage-arrested before its first cleavage, there is no way of knowing whether the embryo will take a normal differentiation pathway. However, from our experience of culturing intact *Halocynthia* embryos in mass, the embryo that showed symmetrical first-cleavage with the normal developmental time of 3 h at 9°C will hatch at the normal time and become a fully grown tadpole even in the case where artificially matured eggs derived from excised gonads were fertilized. Therefore, the use of normally cleaved 2C cells as a toti-potent stem cell model is the best way to eliminate abnormal blastomeres that might reveal abnormal differentiation.

Additionally, the reason why we have used the cell pairs as an inducer is as follows. In the past, we attempted to induce differentiation of a cleavage-arrested 2C cell into cell types different from the default epidermal type with the contact of a single cell from an eight-cell embryo and failed even under the most favorable DH difference between the 2C cell and inducer.
Thus, we gathered that the inductive power of a single cell from an eight-cell embryo was not quantitatively sufficient, and we used a cell pair from an eight-cell embryo as an inducer for a 2C cell throughout the present experiments.

The four types induced in the cleavage-arrested 2C cells were the epidermal, anterior neural, posterior neural, and muscular excitability types and were identified with the knowledge of previous results of membrane differentiation in *Halocynthia* cleavage-arrested early embryonic cells, as illustrated in Table 4. Inward rectifier K$^+$ and Ca$^{2+}$ currents accompanied with neither Na$^+$ nor delayed rectifier K$^+$ currents represented epidermally differentiated cells that mostly have transparent tunic coats covering the larval surface, which are easily observable under a microscope (12, 21). Thus, epidermally differentiated cells can be characterized by their electrical properties; however, in fully differentiated epidermal cells, the transparent tunic coats on the cell surface prevented successful electrical recording. Therefore, in most cases we identified the epidermal type by observing the tunic coats on the cell after 50 DHs (11). Na$^+$, delayed rectifier K$^+$, and/or A-type K$^+$ currents represent neuronally differentiated cells. It is well established in *Halocynthia* embryonic cells that neuronal differentiation can be identified when TuNaI-derived Na$^+$ channel currents, or Na$^+$ spikes are observed electrically, because TuNa I is the *Halocynthia* Na$^+$ channel gene that was demonstrated to be exclusively expressed in neurons of tadpole larvae with in situ hybridization (30, 39, 49). Electrical and zygotic Na$^+$ channel expression in the cleavage-arrested and neuralized cells is blocked by antisense cDNA of the TuNaI gene (13, 39, 42). Although the expression of synaptotagmin in pan neural cells (15) or gelsolin in peripheral neurons (29) is also useful for identification of neural characteristics in the ascidian.
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<td>Motoneuron</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Egg Na channel</td>
<td>Not yet identified</td>
<td>Early embryo (electrical)</td>
<td>Stages before 40 DH</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Delayed K channel</td>
<td>Kv2</td>
<td>Pan-neuronal</td>
<td>After 40 DH</td>
<td></td>
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<td></td>
<td></td>
<td>Significant A current before 70 DH</td>
<td>Kv1</td>
<td>Motoneuron</td>
<td>Later than K2 expression</td>
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<td></td>
<td></td>
<td>L-type Ca channel</td>
<td>TuCaI and/or other genes</td>
<td>Muscular, epidermal, neuronal</td>
<td>After 40 DH in neuronal cells</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Inward rectifier K channels</td>
<td>Possibly TuGIRKA(22)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Epidermal type</strong></td>
<td>Tail epidermal cells</td>
<td>No neural Na channels</td>
<td>Early embryo (electrical)</td>
<td>Stages before 40 DH</td>
<td></td>
<td>Tunic coat</td>
<td>11, 14, 36, 37</td>
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<td>Egg Na channel</td>
<td>Not yet identified</td>
<td>Early embryo (electrical)</td>
<td>Stages before 40 DH</td>
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<td></td>
<td></td>
<td>L-type Ca channel</td>
<td>TuCaI and/or other genes</td>
<td>Muscular, epidermal, neuronal</td>
<td>After 40 DH in epidermal cells</td>
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<td></td>
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<td>Ca-induced K channels</td>
<td>TuIRKAA(21)</td>
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<tr>
<td><strong>Muscular type</strong></td>
<td>Tail muscle cells</td>
<td>No neural Na channels</td>
<td>Early embryo (electrical)</td>
<td>Stages before 40 DH</td>
<td></td>
<td>ACh-esterase (Karnovsky), HrMA4, HrMHC</td>
<td>14, 32, 36, 37, 47</td>
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<td>Egg Na channel</td>
<td>Early embryo (electrical)</td>
<td>Stages before 40 DH</td>
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<td></td>
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<td>TuCaI</td>
<td>Muscular, epidermal, neuronal</td>
<td>After 25 DH in muscle cell</td>
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<td>Delayed K channels</td>
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<td>Early embryo (electrical)</td>
<td>Stages before 40 DH</td>
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<td></td>
<td>Ca-induced K channels</td>
<td>TuIRKAA or TuGIRKA</td>
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Kv2, voltage-dependent K+ channel; DH, developmental hours.
tadpole, electrical and zygotic Na\(^+\) channel expressions are sufficient to identify the neuronal cells in the Halocynthia embryonic system (Table 4).

The presence of the A-type K\(^+\) current with the Na\(^+\) channel current represents the posterior neural type, because the excitability of the cleavage-arrested A4.1 cell, which is the ancestor of larval motoneurons, was characterized by the A current (30), and motoneurons express the tunicate voltage-dependent K\(^+\) channel 1 (K\(_{A0}\)) that shows the A-type current in the Xenopus oocyte expression system (42) (Table 4). In addition, the cleavage-arrested b4.2 cells, which are ancestor cells of the peripheral sensory cells in the larval tail epidermis, revealed the electrical excitability with the A current by differentiation induced by the cell contact of A4.1 or by proteolytic enzymes (33, 34). However, from ascidian larvae, another A-current-type K\(^+\) channel gene, K\(_{A4}\), has been cloned and characterized recently (24) (Table 4). The expression of K\(_{A4}\) is pan-neuronal, which is apparently contradictory to the hypothesis that the A-type current characterizes the posterior neurons. However, so far the cleavage-arrested a4.2 cells, which are ancestor cells of neuronal cells locating anterior to the larval brain stem, revealed no A current before 80 DHs, although there was the possibility that the A current derived from K\(_{A4}\) might appear after a long process of maturation even in a4.2 cells (16, 24, 31). Thus, in the present experiment the posterior type was defined as the cells with the excitability characterized by the A current index < 0.25 at the ~15 mV level before 80 DHs. As shown in Fig. 9D, in the 2C cell in 2C-AA triplets, the A current index tended to be more negative as development progressed. Therefore, there is the possibility that posterior neuronal phenotype could be a more mature state, or some modified state of anterior neuronal phenotype, since A current appeared later than delayed rectifier K\(^+\) current. However, it was also noted that at the early time around 50 DHs, there was a clear indication of small A current as shown in Fig. 3A4, 2C cell in a 2C-AA triplet, 50 DHs, and that neutralized 2C cells in 2C-aAA triplets always stayed at the anterior neural-type state until 80 DHs, as far as A current index concerned, as shown Fig. 9D.

L-type Ca\(^{2+}\) currents and highly developed delayed rectifier K\(^+\) currents without Na\(^+\) currents represents fully differentiated muscle cells (Table 4). Molecular identity of the L-type Ca\(^{2+}\) current in Halocynthia larvae is known as TuCal and the in situ hybridization has demonstrated the expression of TuCal in muscular, epidermal, and neuronal cells (14, 36, 37) (Table 4). The musculary differentiated, cleavage-arrested early embryonic cells, such as B3, B4.1, and B5.1, express the L-type Ca\(^{2+}\) current at early stages before 40 DHs, and the delayed rectifier K\(^+\) current with slightly slower kinetics than that of the neuronal type (32, 47). This property is in contrast with the neuronal or epidermal type that expresses a relatively small L-type Ca\(^{2+}\) current of <3 nA in 10 mM Ca\(^{2+}\) seawater after 40 DHs (41). In addition, the musculary differentiated cells have no Na\(^+\) currents and no tunic coats, but are stained with Karnovsky for ACh-esterase (12, 32). The consistent cooccurrence of the staining of ACh esterase and electrophysiological identification of muscular differentiation has previously been established (32). Thus, the muscular-type differentiation is identified as the cells with no Na\(^+\) channels but with L-type Ca\(^{2+}\) channels and delayed rectifier K\(^+\) channels and without coverage of tunic coats (Table 4).

The identification of cellular differentiation by electrical excitability may not be popular these days. However, the electrical excitability quantitatively represents the combinatorial expression of various distinct kinds of ion channel molecules, and the channel molecules have been mostly identified as gene products at least in the ascidian tadpole larva. Furthermore, the ascidian larva has a relatively small number of genes for ion channels (38) and shows distinct excitabilities in the various types of early embryonic cells (49). Therefore, the electrophysiological measurements of the ion channel expression reveal unique terminal differentiation markers that can be measured in situ in the ascidian embryonic system.

In the present experiments, terminal differentiation markers were membrane excitability channels, and there are no markers for nonexcitable cells, such as cells of endodermal or notochordal lineage. Previous experiments have revealed that excitability measurements of cell membrane properties and immunohistochemical or histochemical staining of cytoplasmic properties always went in parallel during cellular differentiation of cleavage-arrested early Halocynthia embryonic cells (32). For example, the muscular excitability was always accompanied by Karnovsky staining and the epidermal excitability with 2C5 antibody staining. However, this exclusive-type differentiation does not guarantee the exclusion or inclusion of nonexcitable endodermal properties in the present experiment. In respect to the fundamental mechanism for cellular differentiation, it is required to ask more closely whether the classical hypothesis for the exclusivity for differentiation in a cell raised by Weiss (54) is applicable for the case of differentiation in cleavage-arrested blastomeres that artificially contain multiple cytoplasmic determinants (5).

In this study, inducers for the 2C cell in the 2C triplet were chosen from cleavage-arrested cell pairs in eight-cell embryos with which both previous classical embryological studies and our previous electrophysiological experiments have shown that each cell pair out of four cell types in the cleavage-arrested, eight-cell embryos exhibits uniquely defined developmental fates (49). Thus, the four types of cell pairs from ascidian eight-cell embryos represent the regionally segregated differentiation fates. In fact, it has been reported that isolated cells derived from cleavage-arrested, eight-cell embryos autonomously differentiate into regionally specific cell types that were verified electrophysiologically (32). On the other hand, specifically directed differentiation of mammalian embryonic stem cells was successfully achieved when embryonic stem cells were transplanted into a specific region of a normal whole embryo at right developmental timing (2, 56). Therefore, in the present experiments, we decided to determine whether this type of controlled differentiation of embryonic stem cells can be achieved during pychordate embryonic development. Indeed, the major differentiation types were induced in 2C cells derived from two-cell embryos by cell contact with early regionalized embryonic cell pairs from eight-cell embryos. Cleavage-arrested 2C cells from two-cell embryos either in isolation or in contact with each other differentiated into the epidermal type in nearly all cases (Fig. 11). Thus, nonepidermal-type differentiation of 2C cells in the triplets under various conditions observed in the present experiments is the evidence for instructive induction by contacting cell pairs derived from eight-cell embryos. Success of nonepidermal differentiation depended on the developmental time difference between 2C
cells from two-cell embryos and inducer cell pairs from eight-cell embryos. Batches of two-cell embryos used for 2C cell preparation must be fertilized at the same time or preferably 3 h later than that of eight-cell embryos. This timing condition was also critical in the case of neural induction of anterior quadrant A3 cell in A3-aa triplets as reported in our previous paper (51).

Mechanisms for cell type commitment during early ascidian embryonic development proposed in the past. Conklin (3, 4) demonstrated mosaic development of the ascidian embryo where the cytoplasmic factors within each blastomere decide the direction of its future development. This beautiful and clear view for the developmental mechanism is derived from careful observations and findings that distinctly colored cytoplasmic granules are differentially distributed among all blastomeres until the critical determination period of the ascidian embryo, such as the 114-cell stage (26). This view is unique and in contrast to the regulative development of higher vertebrates, such as mammals, where the blastomere at the initial developmental stages shows multipotentials, and its differentiation commitment is mainly guided by cell-cell interaction from neighboring cells (8). Thus, there is strict and clear distinction between mosaic and regulative development of animal embryos among various phyla.

On the other hand, according to the phylogenic view, the ascidian embryo is the prototype of vertebrate embryos, showing the tubular nervous system, notochord, and many other equivalent organs as those in vertebrates in the ascidian larval body plan (44). Furthermore, in respect to developmental mechanisms, the neural tissues in the ascidian tadpole larva do not differentiate autonomously but are induced by cell-cell interaction, as in the case of vertebrate neural induction, although the ascidian inducer tissue equivalent to Spemann’s organizer has not been clearly defined until recently (19, 20). According to Nishida (27), parts of both presumptive notochordal and presumptive neural tissues in A-line blastomeres are responsible for the inducer. Furthermore, notochordal and mesenchymal tissues in the ascidian larva also inductively differentiate as in the vertebrate mesodermal induction (27). However, ascidian notochordal and mesenchymal induction is not identical with vertebrate induction, because the other major mesodermal tissue, ascidian larval muscle cells, differentiates autonomously from the B-line blastomeres. Thus, the inducer for mesodermal descendants is not exclusively derived from ascidian presumptive endodermal regions (27, 28). In the present study, however, we have demonstrated that ascidian muscle differentiation is inductive from the beginning of development under the special cleavage-arrested condition.

Ascidian embryonic development further reveals many differences in details of molecular mechanisms from those of vertebrate embryos. Recently, the Ciona genome project uncovered that there are many ascidian genes homologous to those of higher vertebrates, while the genome size is minimum among chordates and protochordates (6). On the other hand, the ascidian genome is presumed to be the prototype of vertebrate genome (6, 43, 45). Therefore, even if developmental mechanisms showed functional homology, the entire molecular machinery has to be composed of fewer elemental genes, and addition of evolutionally unique mechanism after bifurcation from the protochordate and chordate common ancestor may have produced the difference between vertebrate and ascidian molecular development. For example, according to the recent progress on ascidian molecular and genomic studies, which elucidated much of those developmental mechanisms, FGF9/16/20 was finally determined as an endogenous inducer for neural, notochordal, and mesenchymal tissues (1, 27). The inducer was common for all three tissues unlike the cases in vertebrates, in which more than 20 members of FGFs are acting as inducers or growth factors during mammalian development of neural and other tissues (7). In ascidia, tissue specificity seems to be determined by maternally transferred mRNAs, the majority of which are transcription factor genes, such as Ci-Est for neural tissues and macho-1 for mesenchymal tissue (1, 27). Muscle differentiation is solely determined by the presence of maternal mRNA, macho-1, in the presumptive muscle region derived from the B-line lineage, although the presumptive muscle cells that are descendant of the b-line lineage seem to be induced by cell-cell interaction (27, 28).

Endoderm differentiation seems to be determined by the presence of β-catenin mRNA, the most important member in the Wnt signaling cascade (27). In vertebrate embryos, maternal β-catenin existing in unfertilized eggs is an important factor for mesodermal induction (17), while in ascidian embryos β-catenin mRNA is also of maternal origin and an essential endodermic cytoplasmic factor (27). Thus, in ascidian embryos, the modern version of cell-type specific determinants is consolidated by recently found various maternal mRNAs (27).

2C inductive triplets as a novel vertebrate embryonic stem cells model. In vertebrate embryonic cells, especially mammalian early embryonic cells, regulative development or multipotency was evident since embryonic stem cells, which are derived from early mammalian inner cell mass, are a homogeneous population, yet have the potential to differentiate into all kinds of somatic cells, which has made them a hopeful means for the use in regenerative medicine. However, the details of differentiation mechanisms in mammalian embryos are so complex that more time is required for elucidation of the whole story for controlled differentiation of embryonic stem cells (48, 56). One example of experimental success of controlled differentiation is reported in the case of transplantation of embryonic stem cells into the specialized region in a normal embryo at the right developmental time (2).

On the other hand, extensive whole genome studies especially for phylogenetic ancestors or prototype phyla could give us simple and elementary structures for the complex human developmental mechanisms, as exemplified in the case of the ascidian genomic studies that have elucidated many common molecular entities, such as FGF as an inducer for nervous tissue and for mesenchymal tissues, Wnt signaling, various transcription factors acting in the nervous system, such as BMPs, Ets, Pax, Pitx, and so on (27). However, due to apparent contradiction between mosaic and regulative development, direct comparison between ascidian and higher vertebrate embryonic development is not feasible. Thinking from a different perspective, it might be possible that the regulatory nature of mammalian embryonic stem cells is only apparent and controlled differentiation of embryonic stem cells would be impossible to achieve with exogenous factors supplied only from outside without changing distribution of the endogenous cytoplasmic factors. The solution in this direction may come from more physiological studies on mosaic eggs, such as those in ascidians.
In the present experiment, the cell-type-specific differentiation of embryonic 2C cells prepared from the ascidian mosaic egg was induced by contact with the cell pair derived from cleavage-arrested, eight-cell embryos. The epidermally committed aa pair in eight-cell embryos induced the posterior neural type; although infrequently, the AA pair induced the anterior neural type, and the bb and BB pairs induced the muscle type (see Fig. 12, a schematic of present experimental results). Except for epidermally deter-
mined aa or bb pairs, the induction types were apparently in accord with the types of default differentiation in isolation or developmental fates of inducer cells. As for aa and bb pairs, it is well known that a4.2 cells include anterior neuronal fate and b4.2 cells include the developmental fate for posterior tail muscle cells (27). And the cleavage-arrested and isolated B4.1 cells derived from eight-cell embryos differentiated into muscle cells autonomously, although infrequently, i.e., < 20% of cases (32). These experimental facts may be derived from the prototypic mechanism that governs ES cell differentiation when cells are transplanted in a specific region of the normal embryo. Although the ascidian egg is mosaic in nature, when all cytoplasmic determinants are fully mixed in the cleavage-arrested embryonic 2C cell, the cell acquires regulatory multifactorial potentials that the regulative egg has. Even in the normal ascidian embryo some distal tail muscle cells are inductively differentiated, and some members of mesenchymal cells are transformed to muscle cells when the effects of FGF are artificially blocked (27). Therefore, we conclude that the embryonic 2C cell derived from the mosaic egg can be a regulative embryonic stem cell model.

It is still possible that the mammalian embryonic stem cells have their toti-potential to differentiate into any types of somatic cells. However, even in mammals, it is now well known that there are many maternally transcribed messenger RNAs, acting as initial cytoplasmic polarizing agents in the fertilized egg (53). In this case, we speculate that apparently regulative properties of mammalian embryonic stem cells are derived from imperfect segregation of cytoplasmic factors. Furthermore, the homogeneity of embryonic stem cells is still not completely established yet in respect to the endogenous distribution of cytoplasmic factors. If embryonic stem cells are heterogeneous, the controllable differentiation may only be the matter of selection among embryonic stem cell subgroups with a different distribution of cytoplasmic factors. For this issue, extensive studies of expression profiles of embryonic stem cells may present a clear answer (53).

In the present study, we presented a simple multidirectionally controllable inductive system with cleavage-arrested embryonic cells derived from classical mosaic eggs of ascidians. The 2C cell from a two-cell embryo must have all presumptive fates for the whole tadpole larva, and their composition of all cytoplasmic factors must be uniform within the cell. Furthermore, the composition in a 2C cell must be common among the population of 2C cells derived from normally cleaved two-cell embryos. In other words, our 2C cell preparation must be biologically homogeneous in respect to differentiation capacity. The factors happened to be mixed in the cleavage-arrested condition. This condition allowed the 2C cells to differentiate into major cell types in the tadpole larva by inductive effects from contacting cell pairs. This potential is principally required for mammalian embryonic stem cells to be useful for future medical use. The early embryonic development of the ascidian embryo is therefore established as the prototype of vertebrates from classical comparative embryological studies and recent molecular and genomic studies. Thus, although the cleavage-arrested embryonic cells no longer have regenerative capacity, the 2C cell triplet is a model for the mammalian embryonic stem cell. The physiological analyses on these cell triplets would be useful for understanding of the elemental processes in embryonic stem cell differentiation.

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