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#### ESSAY

## Determination at the Last Cell Cycle before Fate Restriction

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### INTRODUCTION

During embryogenesis, the developmental fates of embryonic cells are gradually restricted. Eventually, the developmental fate of a cell is restricted such that it can give rise to only a single type of cells. In other words, descendants of such a cell, referred to as a primordial cell, contribute exclusively to formation of a single type of tissue. However, restriction of developmental fate does not provide any information about the time when determination occurs in the cell in question.

The determinative state of cells can be defined operationally only by isolation and grafting experiments. A determined cell continues to develop autonomously according to its developmental fate after isolation from the embryo or after grafting to any other region of an embryo. A series of such isolation and grafting experiments, carried out at different stages, usually reveals the time at which the fate of particular embryonic cells are unequivocally determined (Slack, 1991).

Numerous embryological experiments have demonstrated that, in many cases, cell fate is determined by intercellular interactions. In particular, embryonic induction involves the interaction between "inducing" cells and "responding" cells, as a result of which the fate of the responding cells is determined. In this process, the fate of the responding cells is controlled by the signal(s) emitted from the inducing cells (Gurdon, 1987; Slack, 1993). In classical embryology, most studies of embryonic induction were performed in vertebrates, for example, studies of mesoderm induction (Nieuwkoop, 1969; 1973) and neural induction (Spemann and Mangold, 1924; Spemann, 1938) in amphibian embryos. Embryos of vertebrates consist of a large number of cells compared with those of invertebrates. The inductive interactions are generally assumed to determine the fate of large groups of cells, with all descendants of the induced cells following a single common developmental pathway.

Inductive interactions and the timing of determination have recently been investigated in detail in invertebrate embryos, namely, those of ascidians and a nematode. In these animals embryogenesis involves only small numbers of cells. The cell lineages during embryogenesis are invariant and have been described in detail (Sulston *et al.*, 1983; Nishida, 1987). The determination and induction of certain cell types have been analyzed at the single-cell level, and it has been shown that the inductive interactions occur during the cell cycle immediately prior to the cell division during which the developmental fate is restricted to a single type of cell. In two cases, it has been clearly demonstrated that induction affects the fate of only one of the two daughter cells of the cell that has been exposed to the inductive influence. These studies serve to emphasize the importance of the events that happen in the last cell cycle prior to fate restriction. In this review, several examples of such cases will be described and the nature of the determination that occurs prior to fate restriction will be discussed.

## THREE EXAMPLES OF THE TEMPORAL RELATIONSHIP BETWEEN DETERMINATION AND FATE RESTRICTION IN ASCIDIAN DEVELOPMENT

Embryogenesis of ascidians can be regarded as a typical example of mosaic development. Most of the blastomeres exhibit developmental autonomy upon isolation from the embryo. However, some blastomeres cannot develop autonomously in isolation, an observation that suggests a requirement for intercellular interactions. Systematic series of isolation of such blastomeres at various stages allowed identification of the times at which the blastomeres acquire the capability for autonomous development, namely, the timing of determination (Nishida and Satoh, 1989; Nishida, 1990; Nakatani and Nishida, 1994). In the examples discussed below, the temporal relationship between determination and fate restriction is unusual.

#### Sensory pigment cells

The brain of the ascidian larva comprises two pigment cells, known as the ocellus and otolith melanocytes. Analysis of cell lineage has shown that two bilateral pigment-lineage cells in the animal hemisphere give rise to these melanocytes (Nishida, 1987). Formation of these melanocytes requires the inductive influence of the adjacent vegetal blastomeres (Rose,

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1939; Reverberi et al., 1960; Nishida and Satoh, 1989).

There are nine cell divisions in the pigment cell lineage from the zygote to the terminally differentiated melanocyte, as shown in Fig. 1A. In order to define the stage at which the determination occurs, embryos were dissociated into single cells at various stages, and then the dissociated cells were cultured until control larvae hatched (Nishida and Satoh, 1989). Embryos that had been dissociated before 9 hr of development (the beginning of gastrulation) failed to differentiate melanocytes. By contrast, if the dissociation was performed after 10 hr (middle gastrula), some of the dissociated cells showed evidence of differentiation to melanocyte. A cell cluster derived from a single cell of a 10 hr embryo contained two melanocytes and a mass of non-melanocytic cells. By contrast, a cell cluster derived from a single cell dissociated at 11 hr consisted of only two melanocytes (Fig. 1A). Therefore, it appeared that the determination occurred before the developmental fate was restricted to formation of melanocytes. This conclusion is supported by the lineage tree as well as by the fact that, after dissociation at 10 hr, cell clusters consisted of both of melanocytes and non-melanocytes, a result that indicated that the determined cell divided once to form a melanocyte precursor cell and a non-melanocyte precursor cell. By the way, we note also that, in normal embryogenesis, one of the daughter cells of the a9.49 cell is prevented from differentiating into melanocyte by another specific cellular interaction that occurs later in development (Nishida and Satoh, 1989).

#### Secondary muscle

There are 21 muscle cells on each side of the tail of the tadpole larva of the ascidian Halocynthia roretzi. The 14 cells of the anterior and middle parts of the tail are designated primary muscle cells and the 7 cells in the posterior part are called the secondary muscle cells. Blastomeres of the primary muscle lineage show developmental autonomy even at early cleavage stages, while those of the secondary muscle lineage do not. In order to define the stage at which the cells acquire the capacity for autonomous development, secondary muscle lineage blastomeres were identified and isolated from the embryos at various stages (Nishida, 1990). In Figure 1B, the lineage of the secondary muscle that is derived from the A7.8 blastomere of the 64-cell embryo is shown. There are nine cell divisions in this lineage from the zygote to the terminally differentiated muscle cells. Two muscle cells are produced in this lineage (Nishida, 1987).

When the A7.8 cells were isolated, they never developed into muscle. When A8.16 cells were isolated immediately after the seventh division, no muscle formed. By contrast, isolation of the A8.16 cells at 9 hr resulted in partial embryos that consisted of two differentiated muscle cells, as well as nonmuscle cells (Fig. 1B). The lineage tree and the fact that the partial embryo consisted of both muscle and non-muscle cells indicated that determination of muscle fate had occurred part of the way through the cell cycle immediately before the eighth cell division. Then the eighth cell division yielded a muscle precursor cell and a non-muscle (spinal cord) precursor cells.

#### Secondary notochord

The tadpole larva of ascidians has 40 notochord cells in the tail. Of these cells, 32 in the anterior and middle parts of the tail are derived from the primary lineage, while 8 in the posterior part originate from the secondary lineage (Nishida, 1987). Blastomeres of both notochord lineages fail to develop into notochord in isolation (Nakatani and Nishida, 1994). The inductive interactions that occur in the primary notochord precursor cells have been analyzed in detail and they are described in the next section. In studies of the secondary notochord lineage, similar experiments involving the isolation of blastomeres at various stages were carried out. The results are summarized in Figure 1C. There are nine cell divisions in this notochord lineage and, eventually, four notochord cells are produced in this lineage.

When B6.2 cells were isolated at the 32-cell stage, no differentiation of notochord was observed. When B7.3 cells were isolated at the 64-cell stage, the resultant partial embryos consisted of four differentiated notochord cells, as well as non-notochord cells. When B8.6 cells were isolated at the 110-cell stage, the partial embryos consisted of four notochord cells exclusively (Nakatani and Nishida, 1994). Resembling the other above-mentioned examples, these results show that determination occurs during the last cell cycle prior to the restriction of the developmental fate to notochord.

## INDUCTION OF PRIMARY NOTOCHORD IN ASCIDIAN EMBRYOS

As mentioned in the previous section, there are 32 primary notochord cells in the ascidian larva. These cells are derived from the four primordial notochord cells of the 64-cell embryos after three subsequent divisions (Fig. 2). When A6.2 cells were isolated at the 32-cell stage, notochord did not form. However, when A7.3 cells were isolated at the 64-cell stage, the resultant partial embryos consisted of eight differentiated notochord cells (Nakatani and Nishida, 1994). In the cited study, the involvement and timing of inductive interaction were investigated in detail by recombining isolated blastomere at various stages, as described below.

First, inducer cells of notochord were identified by recombining the isolated presumptive notochord cells (A6.2) with various neighboring cells. Then a responding cell and an inducing cell were isolated at the early 32-cell stage, and they were recombined after various time intervals (Fig. 2, bottom). When cells were recombined immediately after isolation, notochord differentiation was observed. However, when they were recombined at the late 32-cell stage or at the 64-cell stage, it was too late for induction to occur. Therefore, inductive interactions must have been initiated at the early 32-cell stage. Recently, it was found that notochord can be induced by the treatment of cells with basic fibroblast growth factor (bFGF) instead of by recombination with inducer cells. The sensitivity to bFGF increased during the 32-cell stage and then decreased



Fig. 1. Three examples of the timing of determination and fate restriction during ascidian embryogenesis. (A) Events during development of the sensory pigment cell lineage. (B) Events during development of the secondary muscle lineage. (C) Events during development of the secondary notochord lineage. Numbers in circles indicate the rounds of cell division in the relevant lineage up to the final cell division. In the middle part in each figure, a lineage tree is shown. Letters with numbers, such as a7.13, are the names of blastomere. The relevant lineage is indicated by thick lines. Non-relevant lineage is indicated by broken lines and cell divisions are omitted. In the lower part in each figure, the results of the dissociation and isolation of embryonic cells are shown. A downward arrow shows when the dissociation and isolation were carried out. In the partial embryos, differentiated melanocytes, muscle cells, and notochord cells are shown in black in the respective figures.



Fig. 2. Timing of induction and fate determination in the primary notochord lineage during ascidian embryogenesis. For explanation, see legend to Figure 1. At the bottom of this figure, the stages of recombination in which the A6.2 cell isolated at the early 32-cell stage was recombined with an inducer cell, as well as the resultant partial embryos, are shown.

rapidly when the sixth cell division started (Nakatani, Yasuo, Satoh, and Nishida, unpublished data). All of these results indicate that inductive interactions occur during the 32-cell stage. At the 32-cell stage, the developmental fate of the notochord precursor (A6.2) cells has not yet been restricted exclusively to notochord (Fig. 2). The cells still contain spinal cord fate. During the sixth cleavage, the fates to give rise to notochord and spinal cord are inherited separately by the two daughter cells, so that the fate of one daughter cell (A7.3) is restricted to notochord. Thus, in the primary notochord lineage, the involvement and timing of induction can be demonstrated directly by the recombination experiments. It is clear that the inductive interactions take place during the last cell cycle before fate restriction.

#### INDUCTION OF GUT IN C. elegans EMBRYOS

The gut of *C. elegans* consists of a tube of 20 cells. The entire gut originates from the so-called E blastomere of the 8-cell embryo (Fig. 3). During the early cleavage stage, the EMS blastomere of the 4-cell embryo divides longitudinally to yield two cells, the E and MS cells. All of the progeny of the E blastomere contribute to gut. Therefore, the fate of the E blastomere is restricted to gut. By contrast, the progeny of the MS cell contribute to the posterior half of the pharynx, some anterior body-wall muscle, and some neurons.

Induction is required for establishment of gut fate. The induction of gut in *C. elegans* is one of the most intensively analyzed cellular interactions in invertebrates that have been examined to date by micromanipulative approach. Isolation



Fig. 3. Induction of gut during embyrogenesis in *C. elegans.* (A) 4cell stage. (B) 8-cell stage. Lateral views. Anterior is to the left, and dorsal is up. During the 4-cell stage, the posterior P2 blastomere induces the ventral EMS blastomere to form gut (arrow). Consequently, at the next division to the 8-cell stage, the E daughter blastomere is produced posteriorly with respect to the MS daughter blastomere. The fate of the E blastomere is restricted to formation of gut, namely, all of the descendants of the E blastomere are gut cells. of EMS cell at the early 4-cell stage prevents gut differentiation. An EMS cell that is isolated at the late 4-cell stage differentiates gut autonomously. Recombination of an early-isolated EMS cell with the posterior P2 cell rescues the gut formation (Goldstein, 1992). For differentiation to gut, the recombination with the inducing P2 cell must be carried out at least 3 min before the EMS cell divides (the length of the cell cycle of the EMS cell is approximately 15 min). The EMS cell can only respond to gut induction during a single cell cycle. (Goldstein, 1995a). Thus, gut fate is determined by the inductive interaction between EMS and P2 cells during the 4-cell stage.

Experiments involving the blastomere recombination have shown that the P2 cell induces the formation of an E (primordial gut) cell on the side of the EMS cell with which the P2 cell is in contact (Goldstein, 1993). In the normal embryo, the P2 cell makes contact with the posterior side of the EMS cell, from which the E cell is formed. When a P2 cell is placed on the opposite side of the EMS cell, gut forms from the descendants of what would normally have been the MS cell. Thus, cell contact between the inducing cell and the responding cell polarizes the mother cell, and one of the two daughter cells assumes the induced fate. Moreover, both daughters of the EMS cell assume the fate of an MS cell in the absence of induction by the P2 cell, indicating that the fate of the MS cell is a "default" fate (Goldstein, 1995a). The direction of the division axis of the EMS cell is also oriented by the contact with the P2 cell (Goldstein, 1995b). Contact-dependent orientation of the mitotic spindle occurs such that the cleavage plane emerges perpendicular to the direction of induction. Consequently, only one daughter of the EMS cell faces the inducing cell, with the other daughter cell facing away from the site of contact with the inducing cell. This arrangement ensures that only one daughter cell is fated to gut. Thus, determination of gut fate is accomplished by a contactdependent inductive interaction prior to fate restriction, and only one daughter cell of the induced mother cell inherits the capacity to form gut.

#### **CONCLUDING REMARKS**

Several examples of fate determination have been discussed here in which cellular interaction appears to be involved. In each case, determination seems to occur during the last cell cycle immediately before fate restriction. Moreover, in two examples, it is clear that inductive interactions take place prior to fate restriction. These studies were carried out with invertebrate embryos that contain a small number of cells. Cellular interactions and determination occur within small number of cells and they could, therefore, be analyzed at the single-cell level. The results reveal novel aspects of induction different from those observed in vertebrates. In vertebrates, it has been postulated that inductive interactions generally determine the fate of large groups of cells, with all descendants of the induced cells assuming a single common developmental pathway (Fig. 4A). The number of cases studied so far in invertebrates is too small to allow us to postulate the general significance of determination prior to fate restriction. It is now important to study the timing of induction, in particular in cases where induction occurs within small number of cells and in a contact-dependent manner.

Non-equivalent or asymmetric cell divisions that produce two distinct cells play a fundamental role in generating different types of cell. In the cases discussed herein, only one daughter cell of a mother cell that was subject to an inductive influence became fated to give rise to a certain cell type (Fig. 4B). This phenomenon has several important implications for the understanding of the way in which developmental fate is determined. Presumably, the inductive influence causes some localized change in the mother cell. An obvious hypothesis is that particular cytoplasmic factors are captured at the site of contact with the inducer cell, as suggested by Goldstein (1995a) in the induction of gut in C. elegans. Alternatively, particular factors might be activated near the contact site, causing one daughter to differentiate differently than the other. Recently, the Numb and Prospero proteins that are involved in Drosophila neurogenesis (Rhyu et al., 1994; Knoblich et al., 1995; Hirata et al, 1995) and the Notch protein that is involved in vertebrate neurogenesis (Chenn and McConnell, 1995) were shown to be inherited by one daughter cell in nonequivalent cell divisions. Asymmetrically segregated Numb and Prospero proteins are required for asymmetric fates. Although it is unknown whether cellular interactions are involved in these processes, the translocation of the Numb and Prospero proteins in a particular direction occurs during the cell cycle just before cell division. Another attractive possibility is that only one of the daughter chromosome or DNA that will be inherited by one of the daughter cell might be modified during the cell cycle of the mother cell, although there is no evidence to support this conjecture. In prospective views, it is clearly important to examine the events that occur during the last cell cycle in mother cells.

Finally, in view of the striking similarity between notochord induction in ascidians and gut induction in the nematode, it is noteworthy that the inducing cell and the responding cell are produced from a common mother cell by a single previous



Fig. 4. (A) Inductive influences (arrows) determine the fate of groups of cells usually depending on the position of the responding cells rather than on their lineage. All the progeny of the induced cells assume a common developmental fate, which is represented by A. (B) The mother cell is subject to an inductive influence (arrow). Consequently, only one daughter cell assumes a fate B that is different from the fate of the other daughter cell.

cleavage. Interactions between the inducing and the responding daughter cells result in polarization of the responding daughter cell. Then one granddaughter cell with a unique identity is produced. These processes serve as a mechanism to generate cell diversity among the progeny of a single cell.

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