Commitment of Cell Fate in the Early Zebrafish Embryo

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When do single cells in the early zebrafish embryo become irreversibly committed to a specific fate? Work with lineage tracing and fate mapping has shown that the marginal cells of the blastoderm give rise to hypoblast-derived fates (mesoderm and endoderm). However, experiments described here show that these marginal blastoderm cells remain pluripotent and uncommitted throughout the late blastula and early gastrula stages. Embryonic cells become committed to a hypoblast-derived fate at mid-gastrulation. Time-lapse photographic analysis reveals that committed cells, when transplanted heterotopically and heterochronically, can migrate along atypical pathways to reposition themselves within a more correct environment.

In many higher animals, the earliest cells of the embryo are initially pluripotent, that is, developmentally unrestricted and capable of expressing a number of possible phenotypes. However, different regions of the embryo eventually become "committed," or restricted in potential so that their continued differentiation is confined to certain developmental pathways.

One of the major goals of developmental biology is to characterize the cellular changes that accompany and produce a developmentally committed state (*1*). The expression of regulatory genes in *Drosophila* correlates with and may underlie cell commitment (*2*). Transplantation experiments in the fly revealed that a single cell can autonomously maintain a committed state in a foreign cellular environment (*3*). Vertebrate genes, such as *MyoD* (*4*), that may help to specify future fates have been characterized, and transplantation experiments similar to those performed in *Drosophila* are now possible in vertebrate embryos (*5*).

Lineage tracing experiments in the zebrafish (*Brachydenio rerio*) have revealed that the future, tissue-specific identities of embryonic cells can be predicted first at the onset of gastrulation (Fig. 1). Cells at or near the margin of the blastoderm involute during gastrulation to become the hypoblast layer, which later generates derivatives that have long been characterized as mesodermal and endodermal. Conversely, cells in the more animal pole regions of the blastoderm do not involute and always remain superficial to the hypoblast. These cells form the epiblast layer, which gives rise to all ectodermal structures of the embryo (*6*).

We have transplanted single cells at various stages of development between different regions in the gastrula of zebrafish and analyzed the development of their clonal progeny (*7*). The purpose of these manipulations was to ascertain whether a transplanted cell would express a fate appropriate for its new position, showing that it was pluripotent, or whether the cell was already committed to the fate of its old position at the time of transplantation. Through control experiments (Table 1), we established a base line of response to the transplantation methods and showed that cells taken from different donor sites (epiblast versus hypoblast) can display differing responses to the same cellular environments. We also studied in a vertebrate embryo, where cells are highly motile (*8*) and development depends on cell-cell interactions (*9*), how a single cell responds when completely surrounded by new cellular neighbors.

At the onset of gastrulation (*5h*), single cells from the marginal region of labeled donor embryos were transplanted into the animal pole region of unlabeled host embryos. Most of the transplanted cells, which normally would have expressed hypoblast-layer fates, gave rise to progeny typical of the epiblast, such as neurons or retinal cells (Fig. 2). None of the transplanted cells expressed a fate exclusive to the hypoblast, although some transplanted cells formed mesenchyme, a cell type that can stem from either epiblast-derived neural crest cells or hypoblast-derived mesoderm (*10*) (Table 1 and Fig. 2H).

Therefore, marginal cells, transplanted from *5h* embryos, developed in accordance with their new position and formed progeny cells that were morphologically indistinct from their surrounding epiblast-derived neighbors (Table 1). Morphogenesis was also appropriate for the new position. For example, if a transplanted cell developed as eye lens, the progeny cells were located only on one side of the host embryo, as are cells that normally form eye structures. However, a transplanted cell that populated the brain region formed a bilaterally distributed clone, a normal characteristic of neural tissues (*11*) (Fig. 2B). Therefore, under these experimental conditions, zebrafish marginal blastoderm cells at the onset of gastrulation were uncommitted and able to express a number of possible fates according to their positions within the embryo.

To ascertain when marginal zone cells become committed, we transplanted single cells from the hypoblast layer, which arises from the involution of marginal cells, of successively older donor embryos (*6.5h* to *8h*) into the animal poles of *5h* host embryos (*12*) (Table 1). When cells that had just involuted into the hypoblast (*6.5h*) were transplanted, about one-third of the cells continued to express their original hypoblast fate and about one-third expressed an epiblast-derived fate. Although it has been reported that cells often enter mitosis upon involution (*6*), no correlation between commitment and cell divisions was drawn in this study. However, almost all of the transplanted cells gave rise to multiple progeny cells (Fig. 2).

Most of the cells transplanted from mid-gastrula (*8h*) hypoblasts into the animal poles of *5h* host embryos retained the hypoblast fate, forming derivatives such as muscle and endothelial cells (Table 1 and Fig. 2). This result is in marked contrast to results of experiments with embryos at the onset of gastrulation (*5h*), in which none of the transplanted cells expressed a hypoblast fate. With respect to the effects of transplanta-

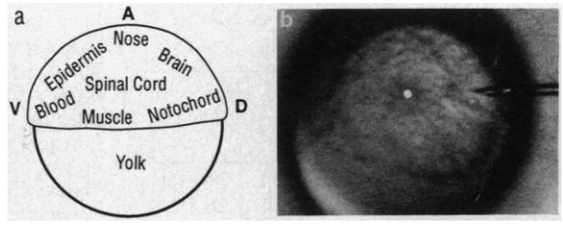

Fig. 1. (**a**) Zebrafish fate map before the onset of gastrulation [5.2*h*, where *h* is hours after fertilization at 28.5°C; redrawn from (*6*)]. At this stage, the blastoderm, in the form of an inverted bowl, sits atop the large yolk cell, which is ~600 μm in diameter. Ectodermal fates are derived from the animal-pole regions of the blastoderm, which become the epiblast. Mesodermal and endodermal precursors are located in the more vegetal, marginal regions of the blastoderm, which involute during gastrulation to form the hypoblast (*6*). A, animal pole; V, future ventral region; D, future dorsal region. (**b**) Animal pole view of an unlabeled host embryo at the onset of gastrulation. A single cell from a labeled donor embryo has been transplanted into the animal pole region with the transplantation capillary shown to the right.

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tion, we conclude that cells from mid-gastrulas had become committed to a hypoblast layer fate. In all of these experiments, the transplanted cell gave rise to progeny of only one type, that is, of either a hypoblast- or an epiblast-derived fate. Clones of mixed hypoblast and epiblast fates were never observed, which suggests that the committed state is inherited autonomously by all the progeny of the original, transplanted precursor.

Transplanted mid-gastrula (8*h*) cells not only retained their hypoblast fate but also became more correctly located within the hypoblast-derived structures of the host embryo. Although transplanted into the epiblast layer, an involuted hypoblast cell never formed an inappropriately located cell type, such as a muscle cell within the brain. These results suggest that at some time during embryogenesis, either the transplanted cell or its progeny migrated out of the epiblast-derived region and into the hypoblast-derived region of the host embryo. Video time-lapse recording of transplanted, labeled cells (*13*) showed that a pre-gastrulation (5*h*) pluripotent margin cell, when transplanted into the animal pole region, remained within the epiblast layer eventually to form ectodermal progeny. However, a mid-gastrulation (8*h*) committed hypoblast cell, when transplanted into the animal pole region, migrated during gastrulation across the epiblast-hypoblast boundary and entered the hypoblast layer of the host, where it later gave rise to progeny expressing a typical hypoblast fate (Fig. 3). Younger, pluripotent cells did not exhibit this behavior when transplanted; only older, committed cells reentered their tissue layer of origin.

Table 1. Results of single cell transplants. Abbreviations: AP, animal-pole region of the blastoderm; MZ, marginal-zone of the blastoderm; and HY, involuted hypoblast region of the blastoderm. Donor and host ages, as well as the source and transplant sites, are indicated in the first column. Host embryos were scored between 24*h* and 48*h* when the identities of the labeled progeny cells were ascertained (*10*). The progeny from any single transplanted cell exclusively expressed only one type of fate; mixed clones were never seen. The percentage of transplanted cells that gave rise to a given fate is indicated, with the actual number of cases in parentheses.

Donor/host	Epiblast-derived fate	Mesenchyme	Hypoblast-derived fate
5*h* AP/5*h* AP	100 (23)	0 (0)	0 (0)
5*h* MZ/5*h* MZ	0 (0)	17 (3)	83 (15)
8*h* HY/8*h* HY	0 (0)	15 (2)	85 (11)
8*h* AP/8*h* HY	100 (15)	0 (0)	0 (0)
5*h* MZ/5*h* AP	89 (16)	11 (2)	0 (0)
6.5*h* HY/5*h* AP	35 (6)	30 (5)	35 (6)
8*h* HY/5*h* AP	5 (1)	10 (2)	85 (17)

We suggest that, as a part of the transition to the committed state, a cell may acquire new, layer-specific adhesive molecules on its surface. A similar example of cellular segregation has been seen in mixed cultures of embryonic amphibian cells (*14*).

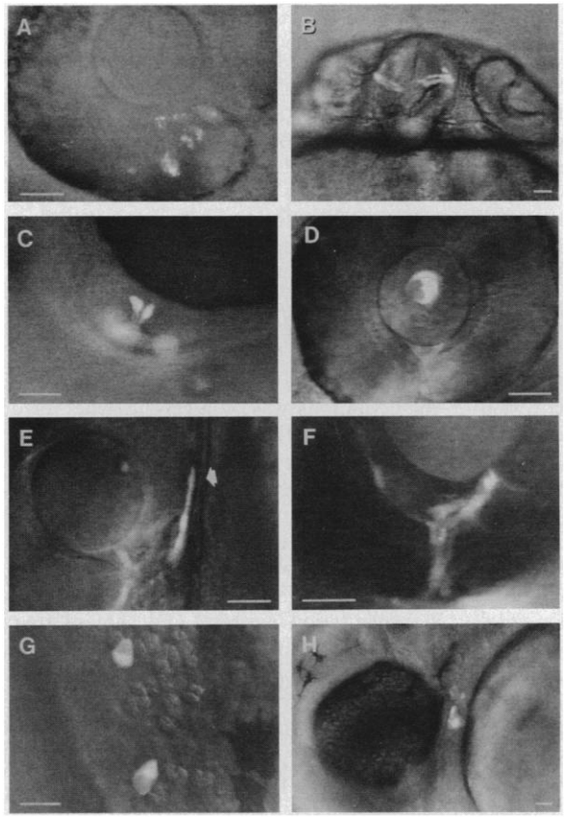

Fig. 2. Progeny derived from single transplanted cells. (A to D) Ectodermal fates and (E to G) mesodermal fates. (**A**) Labeled retinal cells in the eye of the zebrafish. (**B**) Face-on view of an embryo showing the laterally placed eyes and fluorescently labeled forebrain structures. (**C**) Labeled neurons in the olfactory bulb located near the darkly pigmented eye. (**D**) Labeled lens cells of the eye. (**E**) White arrow indicates one labeled muscle cell, in this case in the future inferior-oblique muscle of the eye. (**F**) Labeled endothelial cells forming blood vessels in the eye. (**G**) Labeled hatching gland cells that migrate over the anterior part of the yolk cell and release enzymes during hatching. (**H**) Cells characterized as mesenchymal in Table 1. These labeled cells remain undifferentiated by 48*h* and are typically located in the region of loose cells between the pigmented eye cell and the yolk cell. (A to G) Recorded in 24*h* to 30*h* embryos; (H) 48*h* embryo. Scale bars, 25 μm.

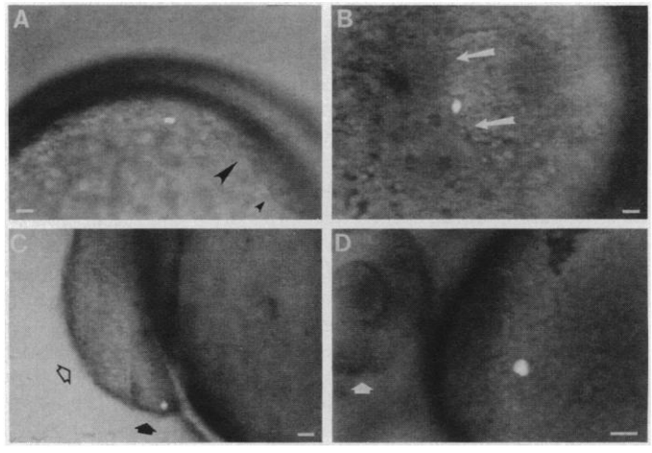

Fig. 3. Time-lapse data showing that committed hypoblast cells migrate out of the epiblast region after transplantation (n = 3), whereas uncommitted cells remain within the epiblast region (n = 4). (**A**) A hypoblast cell from an 8-hour donor embryo was previously transplanted, as in Fig. 1, into the animal pole of a 5-hour host embryo. This host is now at 7 hours of development. The transplanted and labeled cell is in the epiblast near the animal pole region at the top of the figure; dorsal is to the right, vegetal pole is toward the bottom of the figure. The small arrowhead points to the outer edges of the extending hypoblast, and the large arrowhead points to the most anterior limit of the hypoblast, which has not yet reached the animal pole. (**B**) View looking straight down on the animal pole region; dorsal is to the right. The leading edge of the involuted and extending hypoblast region, which is migrating in the direction of the white arrows, has reached the animal pole, where the region comes into contact with the transplanted cell. The transplanted cell has now moved through the epiblast layer and crossed the hypoblast-epiblast boundary to enter the hypoblast layer of cells. (**C**) Same embryo at 11 hours of development with the body axis in profile. The future head region (open arrow) is to the left and the large yolk cell is to the right. The transplanted cell can now be seen clearly in the hypoblast layer within a triangularly shaped embryonic structure known as the "polster," or head pillow region (black arrow). (**D**) Same embryo at 24 hours, orientation as in (C). The white arrow points to the eye. The labeled cell, although transplanted into an epiblast region, has formed a hypoblast-layer fate, namely a hatching gland cell, similar to that shown in Fig. 2G. Scale bar, 25 μm.

When large numbers of dissociated amphibian cells from two different tissue layers, such as definitive mesoderm and ectoderm, were mixed and allowed to reaggregate, they gradually sorted out into monotypic groups. In that experiment, the mesodermal cells regrouped to form an inner bolus completely surrounded by the darker, pigmented ectodermal cells. The acquired ability of cells to separate themselves out may represent a system of differential cell-adhesive properties among various tissues (*15*) or, alternatively, a system of differential migratory behaviors.

We do not yet understand what intrinsic cellular changes underlie the abilities of transplanted hypoblast cells to segregate from their new epiblast neighbors. However, we hypothesize that in the zebrafish, the commitment of these cells may entail some developmental change in their ability to distinguish between various cell-cell interactions. The nature of the molecular and biochemical changes that are initiated by these cellular interactions remains uncertain.

REFERENCES AND NOTES

1. Commitment events can never be inferred through observations alone, their identification requires the experimental manipulation of the cell or region in question. Therefore, a test of commitment is a test of the response of a cell or region to an experimentally produced condition. However, a particular cell may react quite differently to various experimental procedures. In addition, an experimental test may elicit different responses from the same cell at different stages of development. Therefore, in this report we have adopted the definition of Weisblat and Stent for cell commitment only "with respect to a particular experimental perturbation" [D. A. Weisblat, in *Message to Mind*, S. S. Easter, K. F. Barald, B. M. Carlson, Eds. (Sinauer, Sunderland, MA, 1988); G. S. Stent, *Philos. Trans. R. Soc. London Ser. B* **312**, 3 (1985)].
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7. Embryos were obtained from natural crosses of a closed but outbred laboratory stock and manually dechorionated with the use of watchmaker's forceps. Most embryos were raised at 28.5°C, and some were kept at 25°C for various durations to produce embryos at specific stages of development. Microinjection electrodes were back-filled with the fluorescent dye Rhodamine-Dextran (approximate molecular weight 10,000, 5% in 0.2 M KCl; Molecular Probes), which was microinjected into the yolk cells of donor embryos before the eight-cell stage. The injected dye spread through intercellular cytoplasmic connections to all cells of the blastoderm. Transplantation capillaries were pulled from 10-μl microcapillary tubes (VWR), and the tips of the capillaries were either machine-beveled or broken off to an inner diameter of 10 to 25 μm. The tips of capillaries were polished and tooled with a microforge to the shape shown in Fig. 1. Donor and host embryos were positioned on a depression slide in 3% methyl cellulose dissolved in 10% modified Hank's balanced salt solution. Individual cells were drawn directly from labeled donor embryos into the capillary through back pressure. The transplantation capillary was then inserted into an unlabeled host embryo, and the labeled donor cells were gently expelled. Host embryos were raised at 28.5°C in 10% Hank's embryo medium plus antibiotics (5000 units of penicillin and 5 mg of streptomycin per liter; Sigma).
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10. The identities of labeled cells were ascertained on the basis of position and morphology at 24*h* and 48*h*; because the zebrafish embryo is optically clear, it is relatively easy to characterize fluorescently labeled cells in whole, live embryos [C. B. Kimmel and R. M. Warga, *Dev. Biol.* **124**, 269 (1987)]. Cells characterized as mesenchymal were undifferentiated at 48*h* and were located in the loose, head mesenchyme regions of the embryo.
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12. To keep the test conditions constant in the experiments shown on rows 5 through 7 in Table 1, we always placed transplanted cells into the animal pole of a 5*h* host embryo.
13. The results from the time-lapse data were not included in Table 1. For time-lapse video recording, embryos were mounted between cover slips and viewed with both Nomarski and epifluorescence optics. Images were stored on an optical-disk recorder (Panasonic TQ-3031F), and separate fluorescent and white-light images were combined with the software program Axovideo (Axon Instruments). Images were photographed with the use of a GCC Colorfast slide maker.
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