Development 128, 2007-2017 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV2665

Binary specification of nerve cord and notochord cell fates in ascidian embryos

Takuya Minokawa[1,*], Kasumi Yagi[2], Kazuhiro W. Makabe[2] and Hiroki Nishida[1,‡]

[1]Department of Biological Sciences, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan
[2]Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
*Present address: Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125, USA
‡Author for correspondence (e-mail: hnishida@bio.titech.ac.jp)

Accepted 19 March 2001

SUMMARY

In the ascidian embryo, the nerve cord and notochord of the tail of tadpole larvae originate from the precursor blastomeres for both tissues in the 32-cell-stage embryo. Each fate is separated into two daughter blastomeres at the next cleavage. We have examined mechanisms that are responsible for nerve cord and notochord specification through experiments involving blastomere isolation, cell dissociation, and treatment with basic fibroblast growth factor (bFGF) and inhibitors for the mitogen-activated protein kinase (MAPK) cascade. It has been shown that inductive cell interaction at the 32-cell stage is required for notochord formation. Our results show that the nerve cord fate is determined autonomously without any cell interaction. Presumptive notochord blastomeres also assume a nerve cord fate when they are isolated before induction is completed. By contrast, not only presumptive notochord blastomeres but also presumptive nerve cord blastomeres forsake their default nerve cord fate and choose the notochord fate when they are treated with bFGF. When the FGF-Ras-MAPK signaling cascade is inhibited, both blastomeres choose the default nerve cord pathway, supporting the results of blastomere isolation. Thus, binary choice of alternative fates and asymmetric division are involved in this nerve cord/notochord fate determination system, mediated by FGF signaling.

Key words: Ascidian embryogenesis, Nerve cord, Notochord, Developmental fate, Autonomous differentiation, Induction, Default fate

INTRODUCTION

Ascidians (subphylum Urochordata) are regarded as an extant group that branched from a common ancestor with the other chordates, including cephalochordates and vertebrates (Wada and Satoh, 1994). The organization of ascidian larvae shows basic features of the chordate body plan. In particular, the notochord and dorsal neural tube are considered to be essential characteristics of chordata. The phylogenic position and simple structure of the tadpole larva make ascidian embryogenesis a fascinating study subject from both ontogenic and phylogenic viewpoints.

The development of ascidian embryos has been well studied (reviewed by Satoh, 1994; Nishida, 1997). Previous investigations have elucidated the cellular and subcellular mechanisms of fate determination of most major larval tissues, including epidermis, endoderm, muscle, mesenchyme, notochord and brain. The tissue-determining factors localized in the egg cytoplasm play essential roles in the fate determination of muscle, endoderm and epidermis (Nishida, 1992b; Nishida, 1993; Nishida, 1994a). On the other hand, cell interactions are responsible for the fate determination of brain, notochord and mesenchyme cells (Rose, 1939; Nishida, 1991; Nakatani and Nishida, 1994; Kim and Nishida, 1999). Particular interest has concentrated on the mechanisms that underlie the formation of the notochord. Inductive interaction is involved in notochord formation. Recent advances have enabled us to understand the molecular basis of fate determination and differentiation of the notochord (Nakatani et al., 1996; Corbo et al., 1997; Fujiwara et al., 1998; Yasuo and Satoh, 1993; Yasuo and Satoh, 1998; Takahashi et al., 1999).

However, the fate determination mechanism of the nerve cord has not been elucidated so far. The central nervous system (CNS) of the ascidian larva consists of a brain vesicle in the cranial region and a nerve cord in the trunk (formerly brain stem) and in the tail (formerly spinal cord) regions (Fig. 1A; Crowther and Whittaker, 1992; Nicol and Meinertzhagen, 1991). The brain vesicle is derived from a4.2 (anterior-animal) blastomeres of the 8-cell-stage embryo (Fig. 1B). The nerve cord in the tail consists of four rows of ependymal glial cells: dorsal, left, right and ventral. The lateral and ventral rows are derived from A4.1 (anterior-vegetal) blastomeres; the dorsal row is derived from b4.2 (posterior-animal) blastomeres (Fig. 1B; Nishida, 1987). Fig. 1F summarizes the notochord and nerve cord cell lineages derived from A4.1, which are relevant to this study. A6.2 and A6.4 blastomeres at the 32-cell stage give rise to both notochord and nerve cord (Fig. 1C,F). At the next cleavage, they divide into notochord precursors (A7.3 and

A7.7) and nerve cord precursors (A7.4 and A7.8; Fig. 1D). Their developmental fates are restricted to giving rise to notochord or nerve cord. The only exception is the A7.8 cell, which also has a muscle fate. After the next division, the A8.16 cell of the 110-cell embryo still has nerve cord and muscle fates, but the A8.15 cell is restricted to form nerve cord (Fig. 1E,F). These nerve cord precursors participate in neural plate and neural tube formation in the posterior half of neurulae (Satoh, 1978).

Previous studies have shown how the notochord is specified in ascidian embryos. Inductive interaction is responsible for notochord specification. Induction is initiated at the 32-cell stage, with presumptive endoderm blastomeres and neighboring notochord precursors as inducers. Basic fibroblast growth factor (bFGF) is a potent inducing molecule. Ras is involved in the signal transudation. The signaling eventually promotes *Brachyury* expression in the fate-restricted notochord blastomeres at the 64-cell stage (Yasuo and Satoh, 1993; Nakatani and Nishida, 1994; Nakatani and Nishida, 1997; Nakatani et. al., 1996).

In contrast, little is known of how the nerve cord fate is specified. Okada et al. used expression of a voltage-gated sodium channel gene, *TuNaI*, as a neuronal differentiation marker, and demonstrated that the neuronal differentiation in partial embryos derived from A4.1 blastomeres occurs without induction from another blastomeres of 8-cell-stage embryo (Okada et al., 1997). However, the role of cell interactions responsible for nerve cord fate specification after the 8-cell stage and the mechanism of the separation of nerve cord and notochord fates are still unclear. In addition, the lack of adequate molecular markers of nerve cord differentiation that are expressed homogeneously in most cells of the posterior nerve cord has prevented further analysis of nerve cord fate specification. Recently, genes that are widely expressed in the larval CNS, including A-line caudal nerve cord, have been isolated. *HrETR-1* and *HrTBB2* are expressed in most of the cells in the CNS and its precursors (Miya and Satoh, 1997; Yagi and Makabe, 2001). Therefore, we investigated the fate determination mechanism of the nerve cord using mainly *HrETR-1* and, supportively, *HrTBB2* as molecular markers.

Our results showed that (1) cell-cell interaction is not involved in fate determination of the A-line nerve cord; (2) the default fate of the presumptive nerve cord/notochord blastomeres of the 32-cell embryo is nerve cord; and (3) FGF signaling suppresses the nerve cord fate and promotes the notochord fate, and is involved in a binary choice of the alternative fates and asymmetric division.

MATERIALS AND METHODS

Embryos

Adult *Halocynthia roretzi* were collected near the Asamushi Marine Biological Station and the Otsuchi Marine Research Center. Naturally spawned eggs were artificially fertilized and reared in Millipore-filtered seawater containing 50 μg/ml streptomycin sulfate and 50 μg/ml kanamycin sulfate at 11°C.

Isolation of blastomeres

Embryos were manually devitellinated with tungsten needles and reared in 0.9% agar-coated plastic dishes filled with seawater. Blastomeres were identified and isolated from embryos with a fine glass needle under a stereomicroscope (Olympus SZH-10). Isolated blastomeres were cultured separately as partial embryos in agar-coated plastic dishes.

Continuous dissociation of embryonic cells

Cells were continuously dissociated after the first cleavage, as described previously (Nishida, 1992a) with some modifications. Fertilized eggs were transferred into Ca^{2+}-free sea water, such that daughter cells were continuously separated. The Ca^{2+}-free seawater consisted of 435 mM NaCl, 9.3 mM KCl, 24.5 mM $MgCl_2$•$6H_2O$, 25.5 mM $MgSO_4$•$7H_2O$, 2.15 mM $NaHCO_3$ and 0.2 mM ethylene-glycol-*bis*(oxyethylenenitrilo)-tetra-acetic acid (EGTA). Dissociation was monitored at frequent intervals and facilitated by agitation or gentle pipetting. Dissociated cells were then fixed at the neural plate stage and processed for whole-mount in situ hybridization.

Treatment with bFGF, MEK inhibitor and FGFR inhibitor

From the 24- to early 32-cell stages, presumptive nerve cord/notochord blastomeres (A6.2 and A6.4) were isolated and then cultured in seawater containing 0.1% bovine serum albumin (BSA) and 0.2-2 ng/ml recombinant human bFGF (Amersham, Buckinghamshire, UK). At the 44-cell stage, bFGF-treated blastomeres divided once to become partial embryos consisting of two blastomeres. At the 64-cell stage, the two-celled partial embryos were washed several times with seawater and then immediately treated with 2.5 μg/ml cytochalasin B (Aldrich Chemical, Milwaukee, WI) to arrest further cell division. The concentration of bFGF sufficient to fully induce notochord varied from 0.2 to 2 ng/ml, depending on batches of eggs and season (more tended to be required towards the end of the spawning season). We just used the batches in which applied bFGF promoted a full inductive response that was evaluated by the expression of the notochord-specific antigen.

To inhibit the FGF-Ras-mitogen-activated protein kinase (MAPK) signaling pathway, which is essential for the induction of notochord, the MAPK kinase (MEK) inhibitor (U0126, Promega, Madison, WI, USA) and FGF-receptor (FGFR) inhibitor (SU5402, Calbiochem-Novabiochem, San Diego, CA) were used. U0126 and SU5402 were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at –80°C. The stock solutions were diluted with seawater to the final concentration just before use. Whole embryos at the 24-cell stage (just before the initiation of notochord induction) were transferred to seawater containing 2 μM MEK inhibitor or 2 μM FGFR inhibitor. This concentration is enough to inhibit notochord formation by the A6.2 and A6.4 blastomeres (G. J. Kim and H. N., unpublished). At the early 64-cell stage (after the completion of notochord induction), embryos were washed several times with seawater, then blastomere pairs (A7.3+A7.4 and A7.7+A7.8) were co-isolated. They were subsequently treated with cytochalasin B.

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was carried out according to Wada et al. (Wada et al., 1995) with following modifications. To avoid accidental loss of tiny partial embryos, hybridization and washing were carried out in 96-well plates (Cat. No. 256073, Silent Screen Plate, Nalge Nunc, Naperville, IL), the bottom of which is sealed with nylon membrane with 3.0 μm pores so that liquid can be sucked out from the bottom. The specimens were treated with 1-5 μg/ml proteinase K in phosphate-buffered saline containing 0.1% Tween 20 for 15 minutes at 37°C before hybridization. Clearing with benzyl alcohol and benzyl benzoate was omitted because the partial embryos were small and clear enough.

HrETR-1 (Yagi and Makabe, 2001), which is an ascidian homolog of vertebrate *ETR-1* and encodes an RNA-binding protein of the Elav family, was used as a molecular marker for nerve cord fate determination. *HrTBB2* (Miya and Satoh, 1997), which is a nervous tissue-specific β-tubulin gene, was also adopted as another nerve cord

marker. The expression of *HrETR-1* was monitored at the neural-plate stage. The expression at this stage was stronger than at other stages, including the tailbud stage. At this stage, all A-line progenitors of lateral and ventral rows of nerve cord strongly expressed *HrETR-1*. The expression of *HrTBB2* was monitored at the middle-tailbud stage when the expression was strongest.

The monoclonal antibody 5F1D5 recognizes a notochord-specific antigen, Not-1 (Nishikata and Satoh, 1990). Indirect immunohistochemistry was carried out with Alexa-Fluor-488-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) by the standard method.

RESULTS

Nerve cord fate is specified autonomously

We used expression of *HrETR-1*, a gene that is preferentially expressed in the CNS, as a molecular marker for nerve cord differentiation (Fig. 1G-I). The neural plate formed in the dorsal region of the embryos. *HrETR-1* was intensively expressed in the neural plate, including the regions of brain and palps that are derived from a4.2 blastomeres, and the region of the posterior nerve cord that is derived from A4.1 blastomeres (Fig. 1G). The descendants of B4.1 and b4.2 did not express *HrETR-1*. All specimens for examination of *HrETR-1* expression were fixed at this stage. The nerve cord of the tailbud consisted mainly of four rows of ependymal glial cells running along the anterior-posterior axis. At the tailbud stage, the expression of *HrETR-1* became weaker but continued in the lateral rows in the nerve cord (Fig. 1H,I).

To examine whether the determination of nerve cord cell fate is an autonomous process or requires cell-cell interactions with other blastomeres, we manually isolated presumptive nerve cord blastomeres derived from A4.1 at various stages between the 8- and 76-cell stages (Fig. 1F). The blastomeres were isolated within 20 minutes of completion of the previous cell division at 11°C. The isolated blastomeres were cultured separately as partial embryos until the neural-plate stage. The presence of nerve cord cells in the partial embryos was monitored from the expression of *HrETR-1*.

Table 1 and Fig. 2 summarize the results of isolation at various stages. When the A4.1 (anterior-vegetal) blastomeres were isolated at the 8-cell stage, several cells in the A4.1 partial embryos expressed *HrETR-1* (Figs 1B,F, 2A). However, when the a4.2 (anterior-animal) blastomeres were isolated, the partial embryos did not express *HrETR-1* (Fig. 2B), although the descendants of a4.2 in normal embryos did (Fig. 1G). These results suggest that cell-cell interaction is necessary for the expression of *HrETR-1* in the brain and palp precursors, but not in the nerve cord precursors. When the b4.2 (posterior-animal) blastomeres and the B4.1 (posterior-vegetal) blastomeres were isolated, the derived partial embryos did not express *HrETR-1* (Fig. 2C,D).

Next, the A-line presumptive nerve cord blastomeres were isolated at the 16-cell stage. Several cells in the partial embryos derived from A5.1 and A5.2 expressed *HrETR-1* (71% and 69% of cases, respectively; Fig. 2E). When the A6.2 and A6.4 blastomeres were isolated at the 24-cell stage, the resulting partial embryos expressed *HrETR-1* in 93% and 100% of cases, respectively (Fig. 2F). Isolation at this stage resulted in partial embryos in which most of constituent cells expressed *HrETR-1* (lower left-hand partial embryo in Fig. 2F), although in approximately one third of the cases, the expression of *HrETR-1* was relatively weak or negative in some cells of the partial embryos (upper right-hand partial embryo in Fig. 2F). When A7.4 and A7.8 blastomeres were isolated at the 44-cell stage, the resulting partial embryos expressed *HrETR-1* in 90% and 100% of cases, respectively (Fig. 2G). When A8.7, A8.8, A8.15 and A8.16 blastomeres were isolated at the 76-cell stage, the resulting partial embryos expressed *HrETR-1* in 96%, 81%, 100% and 100% of cases, respectively (Fig. 2H). Thus, all partial embryos derived from A-line nerve cord precursor blastomeres expressed *HrETR-1* in isolation.

Table 1. Expression of *HrETR-1* in partial embryos derived from isolated blastomeres

Stage of isolation	Blastomere*	Partial embryos expressing *HrETR-1*/embryos examined
8 cell	**A4.1**	62/64 (97%)
	a4.2	0/67 (0%)
	b4.2	1/36 (3%)
	B4.1	0/59 (0%)
16 cell	**A5.1**	124/175 (71%)
	A5.2	56/81 (69%)
24 cell	**A6.2**	126/136 (93%)
	A6.4	64/64 (100%)
44 cell	**A7.4**	37/41 (90%)
	A7.8	31/31 (100%)
76 cell	**A8.7**	23/24 (96%)
	A8.8	17/21 (81%)
	A8.15	28/28 (100%)
	A8.16	6/6 (100%)

Blastomeres were identified and isolated manually with a fine glass needle, then allowed to develop until neural-plate stage.

*Blastomeres of nerve cord lineage are indicated in bold. (See Fig. 1 and Satoh (1979) for nomenclature and positions of blastomeres in the *Halocynthia* embryo.)

The results of the blastomere isolation experiments did not exclude the possibility that cell-cell interactions before the 8-cell stage and after the 76-cell stage are important for the determination of nerve cord cell fate. To inhibit cellular interactions completely, continuous cell dissociation was performed. Thirty devitellinated fertilized eggs were transferred to Ca^{2+}-free seawater at the one-cell stage, cultured until the neural-plate stage and fixed. During culture, frequent agitation or gentle pipetting was used to keep the embryonic cells dissociated. Out of 2428 cells examined, 82 (3.4%) expressed *HrETR-1*. This indicates that *HrETR-1* is expressed in certain cells even without cell-cell interaction.

The results of the blastomere isolation experiments suggested that cell interaction is not necessary for the expression of *HrETR-1* in the descendants of A4.1, but that it is necessary in the descendants of a4.2. To confirm whether the dissociated cells expressing *HrETR-1* were exclusively A-line cells, we separately examined *HrETR-1* expression in dissociated cells in descendants of the animal blastomeres (a4.2 and b4.2) and vegetal blastomeres (A4.1 and B4.1). The third cleavage divides embryos into animal and vegetal hemispheres. The divisions are unequal, slightly shifting to the animal pole (Satoh, 1979). Consequently, the smaller blastomeres are a4.2 and b4.2 (animal blastomeres), and the larger ones are A4.1 and B4.1 (vegetal blastomeres). In cell dissociation experiments, just after the third cleavage (when

the two daughter blastomeres are still attached to each other), the blastomeres were sucked in and out one by one by mouth pipette and were dissociated into two. Each blastomere was classified as animal or vegetal by its size, then they were separately cultured again in the dissociated state. Dissociated cells derived from the animal blastomeres rarely expressed *HrETR-1* (5/1031: 0.5%, Fig. 3A). In contrast, 100 out of the 1230 (8.1%) dissociated cells derived from the vegetal blastomeres expressed *HrETR-1* (Fig. 3B). The difference was statistically significant ($P<0.001$, χ^2 test). These results confirm that no cell interactions were involved in *HrETR-1* expression in the A-line presumptive nerve cord cells.

Presumptive notochord blastomeres assumed nerve cord fate without completion of notochord induction

Presumptive notochord blastomeres (A7.3 and A7.7) isolated at the 64-cell stage differentiate into notochord, but isolates (A6.2 and A6.4) at the 32-cell stage do not (Nakatani and Nishida, 1994; Nakatani et al., 1996). However, the precise time when isolated blastomeres acquire the ability to develop autonomously into notochord has not been determined. We isolated the notochord precursors at the 44-cell stage, just after the sixth cleavage in relevant blastomeres. The division generates the presumptive notochord and nerve cord blastomeres as the two daughters (Fig. 1F).

The A7.3 and A7.7 blastomeres, whose fate is already restricted to give rise to only notochord, were isolated at both the 44- and 64-cell stages (Table 2; Fig. 4). When the presumptive notochord blastomeres were isolated at the 44-cell stage, over 80% of the partial embryos did not express notochord features, as judged by both their morphology and the expression of the Not-1 antigen (Fig. 4A,C). An aliquot of partial embryos expressed *HrETR-1* in 73% of cases (Fig. 4E, arrows). In contrast, when they were isolated at the 64-cell stage, cells in most partial embryos showed notochord features, as judged by their morphology, such as elongated cell shape and the presence of a vacuole (Fig. 4B, arrowheads), and the expression of the Not-1 antigen (Fig. 4D). This result is consistent with previous work (Nakatani and Nishida, 1994). These partial embryos did not express *HrETR-1* (Fig. 4F). These results suggest that notochord induction is not completed just after the sixth cleavage, although the induction is initiated during the 32-cell stage, and that notochord precursors assume a default nerve cord fate without the accomplishment of the inductive processes.

It has long been known that ascidian embryos develop various kinds of differentiation markers, even if cell division is arrested with cytochalasin B after cleavage stages (Whittaker, 1973). The notochord-specific antigen Not-1 is also expressed when cleavage arrest is started after the 32-cell

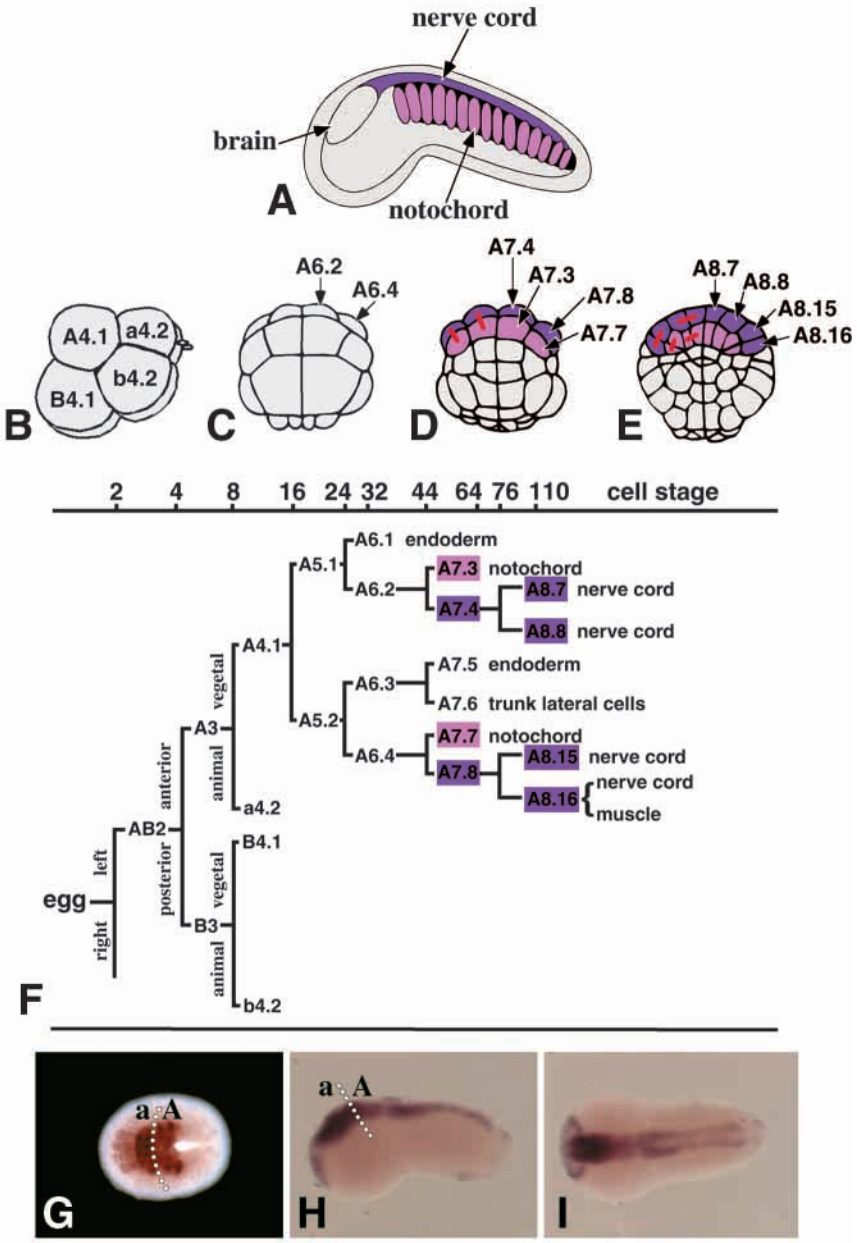

Fig. 1. Cell lineages of posterior nerve cord and notochord, and expression pattern of *HrETR-1*. (A-E) Schematic diagrams illustrating ascidian embryos. (A) Tailbud embryo showing nerve cord and notochord. (B) Eight-cell embryo (lateral view; anterior is upwards and vegetal pole is towards the left). (C-E) 32-, 64- and 110-cell embryos, respectively (vegetal views; anterior is upwards). Presumptive nerve cord blastomeres are colored purple. Presumptive notochord blastomeres are colored pink. Short red bars between blastomeres indicate daughter cells generated by the previous cleavages. (F) Lineage tree showing nerve cord and notochord lineages. Only lineage of bilateral half is shown. (G-I) Spatial expression of *HrETR-1*, as revealed by whole-mount in situ hybridization. (G) Neural plate stage embryo (dorsal view; anterior is towards the left). Expression is seen in most cells of the neural plate, including both the presumptive nerve cord region derived from A4.1 blastomeres and the presumptive brain/palp regions derived from a4.2 blastomeres. White dots indicate the boundary between a4.2 derivatives and A4.1 derivatives. (H,I) Early tailbud embryo: (H) lateral view; (I) dorsal view. Expression is evident in cells of the larval nervous system, including brain, posterior nerve cord and palps.

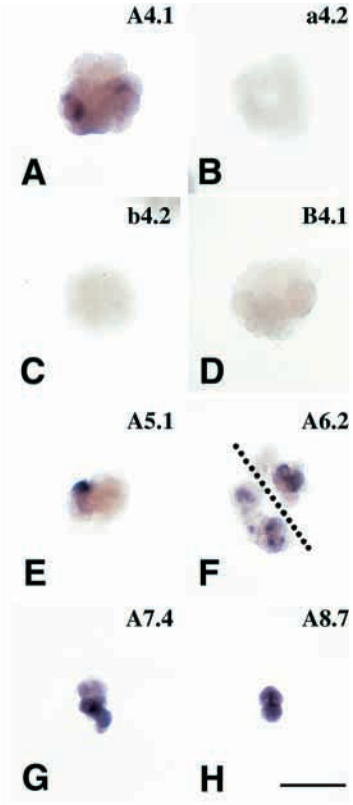

Fig. 2. (A-D) Expression of *HrETR-1* in partial embryos derived from isolated blastomeres at the eight-cell stage. Isolated blastomere is shown in each panel. (A) Partial embryo derived from A4.1 blastomere, which has nerve cord fate. Several cells in the partial embryo express *HrETR-1*. (B) Partial embryo derived from a4.2 blastomere, which has brain and palp fates. No expression of *HrETR-1* is observed. (C) Partial embryo derived from b4.2 blastomere. (D) Partial embryo derived from B4.1 blastomere. (E-H) Partial embryos derived from presumptive nerve cord blastomeres at the 16- (E), 24- (F), 44- (G) and 76-cell (H) stages. All express *HrETR-1*. In F, two partial embryos are shown. Scale bar: 100 μm.

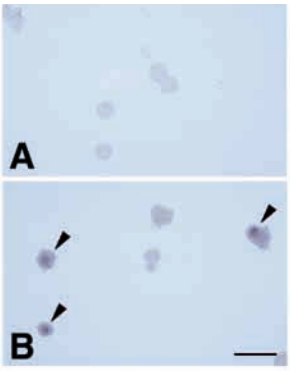

Fig. 3. Expression of *HrETR-1* in fully dissociated cells originating from blastomeres in animal hemisphere (A) and in vegetal hemisphere (B). Cells expressing *HrETR-1* (arrowheads) are present only in B. Scale bar: 100 μm.

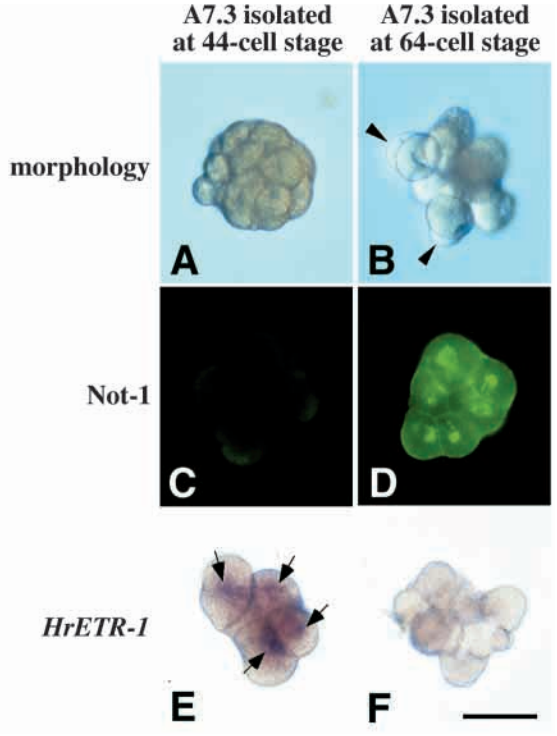

Fig. 4. Expression of notochord and nerve cord markers in partial embryos derived from A7.3 presumptive notochord blastomeres isolated at 44-cell stage (A,C,E) and at 64-cell stage (B,D,F). (A,B) Morphological observation. Arrowheads indicate cells with typical morphology of notochord cells in partial embryos. (C,D) Expression of Not-1 antigen. (E,F) Expression of *HrETR-1*. Arrows indicate expression of *HrETR-1*. Scale bar: 50 μm.

stage (Nishikata and Satoh, 1990). We may also expect *HrETR-1* expression in cleavage-arrested embryos. Therefore, we took the opportunity to confirm whether the default fate in nerve cord/notochord precursor blastomeres is to nerve cord. The presumptive nerve cord/notochord blastomeres (A6.2 and A6.4) are unequally divided at the sixth cleavage (Yasuo and Satoh, 1998). Owing to the differences in blastomere size between sister blastomeres, we were able to distinguish the smaller presumptive nerve cord blastomeres (A7.4 and A7.8) from the larger presumptive notochord blastomeres (A7.3 and A7.7), even after isolation from embryos.

First, the presumptive nerve cord/notochord blastomeres (A6.2 and A6.4) were isolated between the 24- and 32-cell stages before notochord induction, allowed to divide once and treated with cytochalasin B at the 64-cell stage (Fig. 5A). The Not-1 antigen was not expressed in either blastomere (Fig. 5B;

Table 2. Expression of notochord- and nerve cord-specific features in partial embryos derived from presumptive notochord blastomeres

Stage of isolation	Blastomere*	Partial embryos with notochord features/embryos examined		Partial embryos with *HrETR-1* expression/embryos examined¶
		Notochord morphology‡	Not-1§	
44 cell	A7.3	9/46 (20%)	1/25 (4%)	28/44 (64%)
	A7.7	5/44 (11%)	2/28 (7%)	44/54 (81%)
64 cell	A7.3	61/62 (98%)	42/43 (98%)	0/53 (0%)
	A7.7	38/41 (93%)	37/39 (95%)	4/46 (9%)

*Both A7.3 and A7.7 are presumptive notochord blastomeres.
‡Isolates were cultured until hatching stage and examined by light microscope.
§Isolates were cultured until early-tailbud stage and examined by immunohistochemistry.
¶Isolates were cultured until neural-plate stage and examined by in situ hybridization.

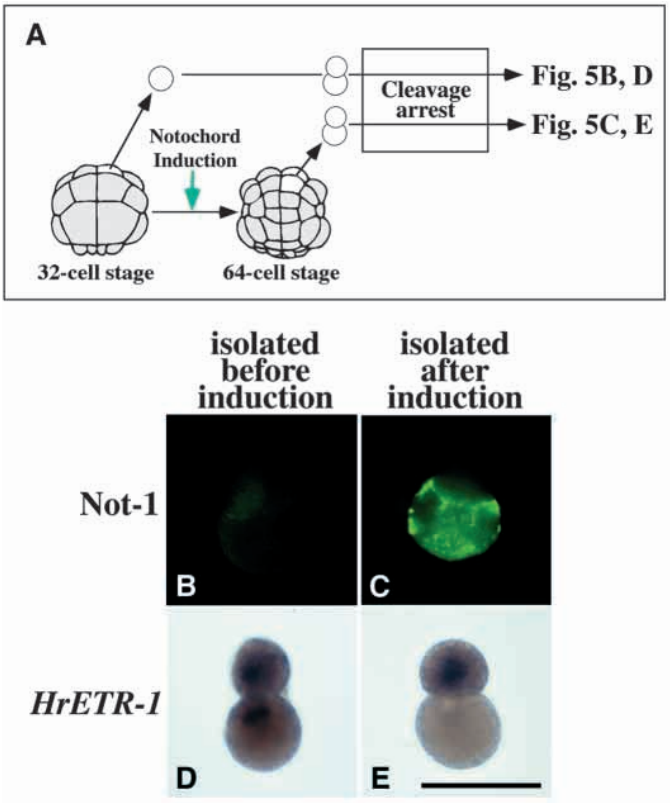

Fig. 5. Expression of notochord and nerve cord markers in two-celled partial embryos. (A) Diagram of the experimental procedure to make two-celled partial embryos. See text for details. (B,D) Two-celled partial embryos isolated before notochord induction. (B) Expression of Not-1 antigen. No expression is observed in either blastomere. (D) Expression of *HrETR-1*. Both blastomeres show *HrETR-1* expression. (C,E) Two-celled partial embryos isolated after notochord induction. (C) Expression of Not-1 antigen. The larger blastomere expresses Not-1. The smaller shows no trace of Not-1 expression. (E) Expression of *HrETR-1*. The smaller blastomere expresses *HrETR-1*. The larger one shows no *HrETR-1* expression. Scale bar: 100 μm.

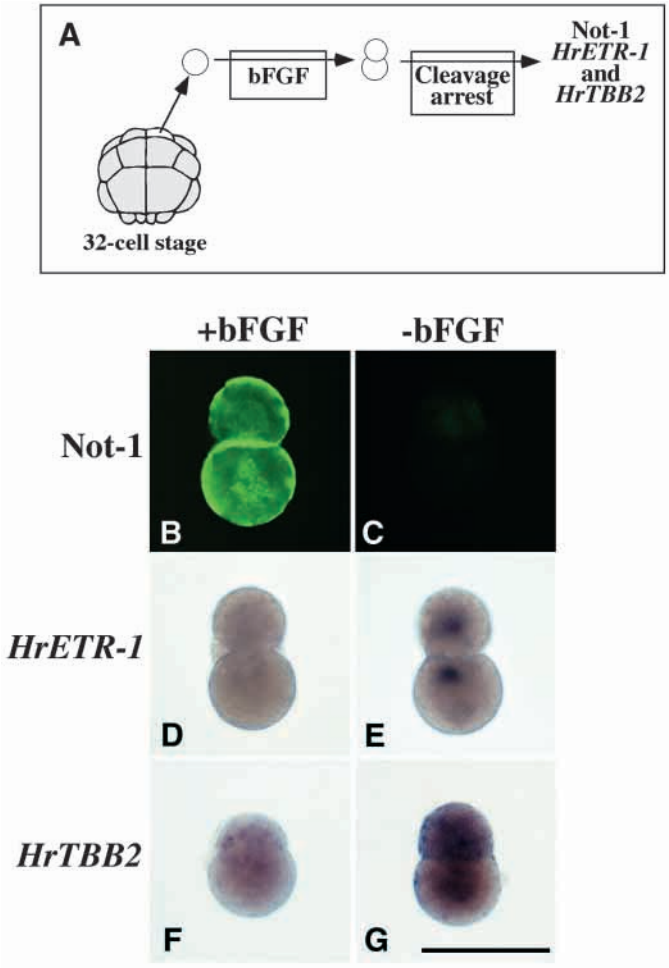

Fig. 6. Expression of notochord and nerve cord markers in two-celled partial embryos treated with bFGF. (A) Diagram to show the experimental procedure to make two-celled partial embryos treated with bFGF. (B,D,F) Expression of tissue-specific markers in two-celled partial embryos treated with bFGF. (B) Expression of Not-1 antigen. Both presumptive notochord blastomere (larger blastomere) and presumptive nerve cord blastomere (smaller one) show strong Not-1 expression. (D,F) Expression of *HrETR-1* (D) and *HrTBB2* (F) is not seen in either blastomere. (C,E,G) Expression of tissue-specific markers in two-celled partial embryos without FGF treatment. (C) Not-1 antigen is not expressed in either blastomere. (E,G) Both blastomeres show *HrETR-1* (E) and *HrTBB2* (G) expression. Scale bar: 100 μm.

Table 3A). By contrast, *HrETR-1* was clearly expressed in both blastomeres in all cases (Fig. 5D). Thus, both daughter blastomeres of the nerve cord/notochord blastomere chose the nerve cord fate without inductive influence.

Second, the A7.3+A7.4 or A7.7+A7.8 pairs were co-isolated at the 64-cell stage and treated with cytochalasin B to arrest successive cleavages (Fig. 5A). The larger (notochord) blastomeres in the two-celled partial embryo eventually expressed Not-1 (Fig. 5C; Table 3A), and only the smaller (nerve cord) cells expressed *HrETR-1* (Fig. 5E). Thus, after the completion of induction, each blastomere followed its fate in normal embryogenesis, even in the isolated or cleavage-arrested state.

Both promotion of notochord fate and suppression of nerve cord fate were induced by bFGF treatment

Treatment with bFGF of presumptive nerve cord/notochord blastomeres that had been isolated before induction promotes the formation of the notochord (Nakatani et al., 1996). When the blastomeres are treated with 0.2–2 ng/ml of bFGF at the 32-cell stage, all the descendants of presumptive nerve cord/notochord blastomeres differentiate into notochord. Therefore, it is likely that the presumptive nerve cord blastomeres (A7.4 and A7.8) are converted to notochord when the inductive signal is administered from all over the induced blastomere surface. To examine whether the expression of nerve cord marker genes in the presumptive nerve cord blastomere was suppressed by treatment with bFGF, we isolated the presumptive nerve cord/notochord blastomeres (A6.2 and A6.4), treated them with bFGF and arrested cell divisions after the 64-cell stage (Fig. 6A, Table 3B).

When the presumptive nerve cord/notochord blastomeres (A6.2 and A6.4) were treated with bFGF (0.2-2 ng/ml),

both blastomeres in the cleavage-arrested two-celled partial embryos expressed Not-1 in most cases (Fig. 6B; Table 3B). The expression of *HrETR-1* as well as *HrTBB2* (nervous tissue-specific tubulin gene) in the two-celled partial embryos were suppressed in both blastomeres in most cases (Fig. 6D,F).

In control experiments, when the presumptive nerve cord/notochord blastomeres (A6.2 and A6.4) were treated with seawater containing only BSA, neither blastomere in the cleavage-arrested two-celled partial embryos expressed Not-1 (Fig. 6C; Table 3B), and the nerve cord marker genes were expressed in both blastomeres (Fig. 6E,G). These results indicate that the nerve cord fate in the presumptive nerve cord blastomere was suppressed by treatment with bFGF. The results reveal a binary choice of developmental pathways in the nerve cord/notochord system.

Suppression of notochord fate and promotion of nerve cord fate were induced by treatment with inhibitors of MEK and FGFR

A previous study has indicated that Ras protein is involved in signal transduction in notochord induction (Nakatani and Nishida, 1997). Notochord formation is inhibited when the Ras signaling pathway is disrupted by the injection of dominant negative Ras protein (Nakatani and Nishida, 1997) or by treatment with MEK and FGFR inhibitors (G. J. Kim and H. N., unpublished). To further confirm whether the presumptive notochord blastomeres choose the default nerve cord fate when the components of the FGF-Ras-MAPK signaling pathway is disrupted, we examined cleavage-arrested two-celled partial embryos treated with MEK or FGFR inhibitors.

Whole embryos were treated with MEK inhibitor from the 24- (or 32-) to 64-cell stage for about 80 minutes to inhibit notochord induction. Then the daughter blastomeres of A6.2 and A6.4 (A7.3+A7.4 and A7.7+A7.8, respectively) were co-isolated at the 64-cell stage from the embryos, and successive cleavages were arrested by treatment with cytochalasin B (Fig. 7A; Table 3C). Neither blastomere of the resulting two-celled partial embryos expressed Not-1 (Fig. 7B; Table 3C), but the nerve cord marker genes were expressed in both blastomeres (Fig. 7E,H). Similar results were also obtained from the same kind of experiments using FGFR inhibitor (Fig. 7C,F,I; Table 3C).

The blastomeres were co-isolated from 64-cell embryos treated with seawater containing DMSO, which was used to dissolve the inhibitors. In this control experiment, the larger blastomere in the resulting two-celled partial embryos expressed Not-1 (Fig. 7D; Table 3C), and the nerve cord marker genes were expressed in the smaller blastomere of the embryo (Fig. 7G,J).

To examine the periods of sensitivity to the inhibitors, we performed the following experiment. The notochord and nerve cord precursors were co-isolated at the 64-cell stage from the untreated embryos and treated with each inhibitor and cytochalasin B. After 80 minutes, the two-celled partial embryos were washed several times with seawater and cultured in seawater containing only cytochalasin B. In these experiments, inhibitors of MEK and FGFR had no effect, neither did the DMSO treatment (Table 3C), demonstrating that these inhibitors have a specific temporal sensitive period before the 64-cell stage. These results coincide well with previous observations that notochord induction is completed by the 64-cell stage. Thus, notochord precursors assumed the nerve cord fate when FGF-Ras-MAPK signaling did not occur.

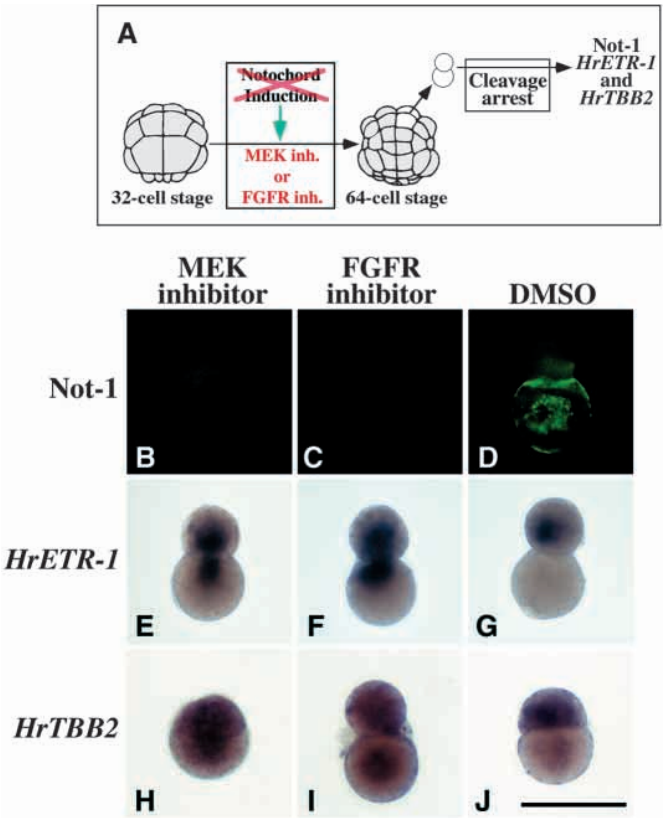

Fig. 7. Expression of notochord and nerve cord markers in two-celled partial embryos treated with MEK inhibitor and FGFR inhibitor. (A) Diagram of the experimental procedure to make two-celled partial embryos treated with inhibitors (inh.) of FGF signaling. (B,E,H) Treatment with MEK inhibitor. (B) Not-1 is not expressed in either blastomere. (E,H) Both blastomeres in two-celled partial embryo show *HrETR-1* (E) and *HrTBB2* (H) expression. (C,F,I) Treatment with FGFR inhibitor. (C) Not-1 is not expressed in either blastomere. (F,I) Both blastomeres show *HrETR-1* (F) and *HrTBB2* (I) expression. (D,G,J) Treatment with DMSO. (D) The larger blastomere (notochord blastomere) expresses Not-1. The smaller one (nerve cord blastomere) does not. (G) Only smaller blastomere expresses *HrETR-1*. (J) Only smaller blastomere expresses *HrTBB2*. Scale bar: 100 μm.

DISCUSSION

We have investigated the mechanisms of fate determination of nerve cord cells derived from A-line blastomeres using experiments involving blastomere isolation, cell dissociation, bFGF treatment and treatment with signaling inhibitors. Nerve cord fate was determined autonomously in A-line. The default fate of isolated presumptive notochord blastomeres was to nerve cord. When FGF signaling was inhibited, the presumptive notochord blastomeres also chose the default nerve cord fate. Presumptive blastomeres of notochord and nerve cord assumed the notochord pathway when they were treated with bFGF. The blastomeres always chose either the induced notochord or default nerve cord fate.

Autonomous specification of nerve cord cell fate

Based on an analogy to vertebrate neural induction, it is reasonable to speculate that the nerve cord in the tail of

Table 3. Expression of tissue-specific markers in two-celled partial embryos treated with bFGF and inhibitors

			Expression of Not-1 antigen (%)				Expression of *HrETR-1* (%)				Expression of *HrTBB2* (%)			
	Stage of isolation	Treatment	*n*	Both blastomeres	One blastomere	Neither blastomere	*n*	Both blastomeres	One blastomere	Neither blastomere	*n*	Both blastomeres	One blastomere	Neither blastomere
(A) None	32 cell	–	51	0	0	**100**	36	**100**	0	0	0	N.D.	N.D	N.D.
	64 cell	–	56	0	**100**	0	64	3	**97**	0	0	N.D.	N.D	N.D.
(B) bFGF	32 cell	bFGF	38	**89**	11	0	28	0	7	**93**	12	0	0	**100**
	32 cell	BSA	34	0	6	**94**	41	**100**	0	0	16	**100**	0	0
(C) Inhibitors	64 cell	FGFR inhibitor from 24-cell stage	59	0	2	**98**	52	**98**	2	0	12	**100**	0	0
	64 cell	FGFR inhibitor from 64-cell stage	16	0	**100**	0	18	0	**100**	0	0	N.D.	N.D.	N.D.
	64 cell	MEK inhibitor from 24-cell stage	60	0	0	**100**	58	**100**	0	0	8	**100**	0	0
	64 cell	MEK inhibitor from 64-cell stage	27	0	**100**	0	29	0	**100**	0	0	N.D.	N.D.	N.D.
	64 cell	DMSO	18	0	**100**	0	14	0	**100**	0	19	0	**100**	0

n, number of partial embryos examined.
Both blastomeres: both blastomeres in a two-celled partial embryo expressed the tissue-specific marker.
One blastomere: one blastomere expressed the tissue-specific marker. In this, larger blastomeres always expressed Not-1 antigen, while smaller blastomeres always expressed *HrETR-1* and *HrTBB2*.
Neither blastomere: neither blastomere expressed a tissue-specific marker.
N.D., not determined.

ascidian larvae is also induced. But this was not the case, because blastomere isolation and cell dissociation experiments indicated that the specification of nerve cord fate is governed by cell autonomous mechanism. The nerve cord of ascidian larvae is formed by neural tube closure, as in vertebrates. Our study, using *HrETR-1* and *HrTBB2* expression as molecular markers, does not indicate whether the morphogenic process of neural tube formation in ascidian larvae requires cell interactions or not. Our results indicate that the initial step of A-line nerve cord specification during cleavage stage is autonomous.

There was a difference in responses between the a-line (presumptive brain vesicle) and A-line (presumptive nerve cord) blastomeres in the blastomere isolation and dissociation experiments. The expression of *HrETR-1* was abolished in the a-line cells, but it was still present in the A-line cells. These results indicate that the mechanism responsible for the expression of *HrETR-1* is different between the lineages. Previous studies have also showed differences in neuron formation between the a-line and A-line. Neural differentiation in a4.2 required induction by A-line cells when neuronal differentiation was evaluated molecularly by the expression of *TuNaI*, a neuron-specific sodium channel gene, and physiologically by the expression of neuron-specific active membrane potential (Okado and Takahashi, 1990; Okamura et al., 1993; Okamura et al., 1994). By contrast, isolated A4.1 blastomeres autonomously give rise to neurons (Okada et al., 1997). Therefore, not only *HrETR-1* (an early pan-neural marker) expression but also neuronal differentiation occurs autonomously in A-line cells. Thus, CNS specification in ascidian larvae operates differently in a- and A-descendants: induction is involved only in brain formation. The dorsal row of nerve cord is derived from b-line cells. The fate determination mechanism of b-line nerve cord was not elucidated in the present study because *HrETR-1* and *HrTBB2* are not expressed in the b-line nerve cord cells (Fig. 1G).

In *Xenopus*, the formation of neural tissue is inhibited by the epidermal inducer bone morphogenetic protein (BMP4), which is expressed throughout the ectoderm. BMP4 activity is antagonized by molecules such as chordin, noggin and follistatin in the process of neural induction. These molecules act directly by binding to the BMP4 protein, preventing BMP from activating its receptor (Sasai and De Robertis, 1997, and references therein). When animal cap cells are dissociated, neural tissue forms, because BMP is lost, presumably by dilution with culture medium. Addition of BMP to culture medium can inhibit neuralization of dissociated cells, promoting the formation of epidermis (Wilson and Hemmati-Brivanlou, 1995). In our experiments, dissociated vegetal cells expressed *HrETR-1*. A similar loss of an inhibitory molecule may have happened in the dissociation procedure. We cannot completely rule out this possibility. However, it is probably not the case in ascidian embryos for the following reasons. First, BMP/chordin antagonism is not involved in ascidian CNS formation. Overexpression of BMP and chordin does not affect CNS formation (S. Darras and H. N., unpublished). Second, cell isolation and recombination experiments clearly reveal that neural formation from a-line cells requires inductive interaction (Okado and Takahashi, 1990; Okamura et al., 1993; Okamura et al., 1994). Nevertheless, these cells failed to express *HrETR-1* in the dissociated state, whereas A-line cells showed autonomous expression of the gene. Third, it is hard to imagine that nerve cord fate is specified by release from a neural inhibitory molecule because, as shown in the last half of this study, notochord fate is induced by FGF signaling as early as the 32-cell stage, and nerve cord fate can be regarded as the default fate in this system. Thus, we conclude that the specification of A-line nerve cord fate is an autonomous process in ascidian embryos.

Primary muscle, epidermis and endoderm of ascidians are specified autonomously. The presence of localized determinants of these tissues in fertilized egg cytoplasm has been shown by cytoplasmic transplantation and deletion experiments (Nishida, 1992b; Nishida, 1993; Nishida, 1994a). However, transplantation and deletion of anterior-vegetal ooplasm that would be inherited by nerve cord precursor blastomeres did not show any effect on embryonic

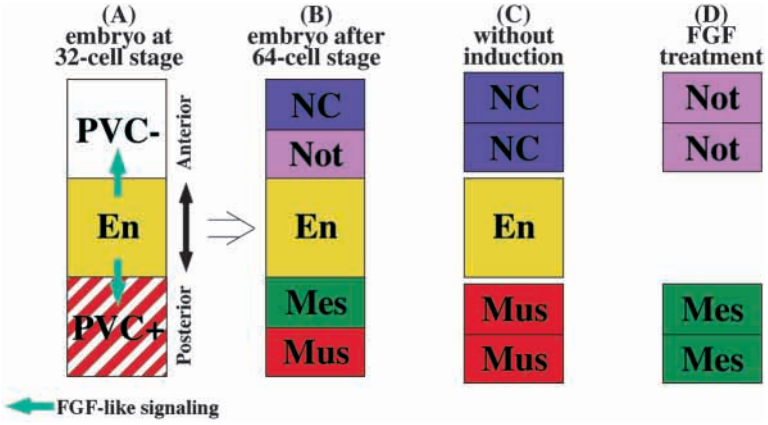

Fig. 8. A directed signal and asymmetric division model of tissue specification mechanism in the vegetal hemisphere of the ascidian embryo. The model is applicable to both the anterior and posterior margins of the vegetal hemisphere. (A) Schematic drawing representing embryo at 32-cell stage. Endoderm precursors (En) emanate inductive FGF-like signal (green arrows) to neighboring anterior and posterior blastomeres and polarize them. Posterior-vegetal cytoplasm (PVC; red oblique lines) causes different responsiveness in posterior marginal cells. (B) Asymmetric divisions occur at the 64-cell stage. In the anterior region, one daughter cell that faces the inducer and does not have the PVC assumes notochord fate (Not). In the posterior region, one daughter cell that faces the inducer and contains the PVC adopts mesenchyme fate (Mes). (C) Without inductive signal, both daughter blastomeres in the anterior region assume default nerve cord fate (NC), and those in the posterior region assume default muscle fate (Mus). (D) When isolated blastomeres receive FGF signal all over the surface, both daughter cells develop into notochord or mesenchyme, depending on absence or presence of PVC.

development (Nishida, 1994b). This result suggests that there is no specific ooplasmic determinant localized to the anterior-vegetal region for nerve cord formation. By contrast, it is intriguing that when posterior-vegetal ooplasm is removed, mirror image duplication of the anterior half occurs in the posterior half (Nishida, 1994b). Cells in the vegetal marginal zone in ascidian embryos seem to be destined for nerve cord if they do not contain posterior-vegetal cytoplasm factors and do not receive endoderm signal.

Default fate of nerve cord/notochord blastomeres

Nakatani and Nishida have confirmed that the isolated nerve cord/notochord blastomeres (A6.2 and A6.4) of 32-cell embryos do not differentiate into notochord, muscle, epidermis or endoderm (Nakatani and Nishida, 1994). They point out that the partial embryos may differentiate into nerve cord simply because of the close relationship of notochord and nerve cord in the cell lineage tree (Fig. 1F). However, it was hard to examine the possibility without a molecular marker for nerve cord formation at that time. In this study, we have examined the possibility using *HrETR-1* as the marker. The presumptive notochord blastomere (A7.3 and A7.7) isolated at the 44-cell stage did not express notochord features and expressed *HrETR-1*. When the presumptive nerve cord/notochord blastomeres were isolated at the 32-cell stage, both daughter blastomeres eventually expressed *HrETR-1*. Inhibition of FGF signaling also caused both daughter blastomeres to express nerve cord marker genes. These results clearly showed that the default fate of nerve cord/notochord blastomeres is nerve cord. By contrast, when treated with bFGF, both blastomeres assumed the notochord fate. The blastomeres always adopted either nerve cord fate or notochord fate. This result suggests that the intracellular signaling cascade from the FGF receptor to MAPK is located upstream of molecules responsible for promotion of notochord fate and suppression of default nerve cord fate.

The induction of notochord has to be initiated during the 32-cell stage, and the cells acquire the ability of autonomous notochord differentiation at the 64-cell stage (Nakatani and Nishida, 1994; Nakatani and Nishida, 1999; Nakatani et al., 1996). In this study, we examined the timing of completion of the inductive process in detail. When the presumptive notochord was isolated at the 44-cell stage, the descendants could not differentiate into notochord. This indicates that notochord induction is not complete until several minutes after the sixth cleavage in normal embryos.

A possible model for fate determination mechanism in the vegetal marginal zone in ascidian embryos

On an ascidian blastula fate map (Nishida, 1987; Kim et al., 2000), five tissue-forming regions are aligned along the anterior-posterior axis. From the anterior, they are nerve cord, notochord, endoderm, mesenchyme and muscle precursors. This and previous studies have clarified how these tissues are specified at the 32- and 64-cell stages. We propose a simple model in which directed signals and asymmetric division play a crucial role in fate specification in both anterior and posterior vegetal marginal zones (Fig. 8).

In the posterior region, the precursor of both mesenchyme and muscle in the 32-cell embryo divides into mesenchyme and muscle precursors at the sixth cleavage (Fig. 8A,B). Without the inductive influence of the endoderm, both daughter cells adopt the muscle fate (Fig. 8C). When the mother cell is isolated from the embryo at the 32-cell stage and is treated with bFGF, both daughter cells adopt the mesenchyme fate (Fig. 8D; Kim and Nishida, 1999; Kim et al., 2000). Therefore, one can see a striking parallel between what happens in the anterior and posterior regions. In the posterior region, the default fate is muscle and the induced fate is mesenchyme. In the anterior region, the default fate is nerve cord and the induced fate is notochord. In both regions, separation of default and induced fates occurs at the 64-cell stage, endoderm blastomeres are the inducers and FGF-Ras-MAPK signaling mediates the induction.

In the normal embryo, only one of two daughter cells that face the endoderm follows the induced fates. The other assumes the default fates. When the mother cell is treated with bFGF, such that the cell receives the signaling molecule all over its surface, both daughters assume the induced fates. Therefore, directed induction from the endoderm is required for the fate separation in the two daughter cells. Previous studies have indicated that the inductive processes are initiated during the 32-cell stage in both anterior and posterior regions (Nakatani and Nishida, 1994; Nakatani and Nishida, 1999; Nakatani et al., 1996; Kim and Nishida, 1999; Kim et al., 2000), although the induction is completed several minutes after the sixth division. Therefore, an FGF-like signal emanating from the

endoderm blastomeres polarize the mother cells at the first step and cause asymmetric division, so that a daughter cell neighboring endoderm cells assumes the induced fates and the other adopts the default fates.

It has previously been reported that the presumptive nerve cord/notochord blastomeres of the 32-cell stage embryos can induce notochord differentiation each other (Nakatani and Nishida, 1994). To reconfirm this observation, a pair of presumptive nerve cord/notochord blastomeres (A6.2 and A6.4) was co-isolated at the 24- and early 32-cell stages, allowed to divide once, and cell divisions were arrested after the 64-cell stage. Expression of both markers of notochord and nerve cord was observed in the partial four-celled embryos (data not shown). It is difficult to explain this result by our model. Therefore, we cannot completely rule out the possibility that there is an endogenous polarity in the presumptive nerve cord/notochord blastomeres, and the subsequent asymmetric division is biased by the polarity when the inductive signal is supplied.

There is no difference in the FGF-like inducing signals from the endoderm between the anterior and posterior regions. But cells in both regions respond differently to the endoderm signal. Transplantation of egg cytoplasm revealed that the difference in responsiveness is caused by the cytoplasm of the responding blastomeres, which is inherited from the egg (Kim et al., 2000). The posterior-vegetal cytoplasm (PVC) of eggs confers the muscle fate on the posterior blastomeres. When a blastomere receives an FGF-like signal, it becomes mesenchyme. Without the PVC, the blastomere assumes the nerve cord fate. When the blastomeres receives the signal, it becomes notochord. Thus, the directed signal and asymmetric division operate in both anterior and posterior regions and are responsible for the arrangement of nerve cord, notochord, endoderm, mesenchyme and muscle precursors (in this order from anterior to posterior).

Recently, it has been reported that a Notch-Su(H) pathway might responsible for notochord differentiation in another ascidian *Ciona intestinalis* (Corbo et al., 1998). It is possible that the mechanism responsible for nerve cord/notochord fate determination is not common in all ascidian species.

In the present study, the specification of nerve cord fate has been analyzed. This now enables us to understand total cellular and subcellular mechanisms involved in fate specification of all of the major tissues (epidermis, brain vesicle, nerve cord, muscle, notochord, mesenchyme and endoderm) in *Halocynthia* embryos.

We thank the staff of the Asamushi Marine Biological Station and the Otsuchi Marine Research Center for their help in collecting live ascidians, and the staff of the Misaki Marine Biological Station for their help in maintaining them. We are also grateful to our colleagues, especially Dr T. Nishikata for providing monoclonal antibody 5F1D5, Dr T. Miya for providing plasmid containing *HrTBB2* cDNA, instruction on in situ hybridization and valuable discussion, and Mr S. Darras and Dr P. Lemaire for valuable discussion and suggestion. This work was supported by the Research for the Future program from the Japanese Society for the Promotion of Science (96L00404) and a grant from the HFSP organization (RG-0299/98).

REFERENCES

Corbo, J. C., Levine, M. and Zeller, R. W. (1997). Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*. *Development* **124**, 589-602.

Corbo, J. C., Fujiwara, S., Levine, M. and Gregorio, A. D. (1998). Suppressor of Hairless activates Brachyury expression in the *Ciona* embryo. *Dev. Biol.* **203**, 358-368.

Crowther, R. J. and Whittaker, J. R. (1992). Structure of the caudal neural tube in an ascidian larva: vestiges of its possible evolutionary origin from a ciliated band. *J. Neurobiol.* **23**, 280-292.

Fujiwara, S., Corbo, J. C. and Levine, M. (1998). The Snail repressor establishes a muscle/notochord boundary in the *Ciona* embryo. *Development* **125**, 2511-2520.

Kim, G. J. and Nishida, H. (1999). Suppression of muscle fate by cellular interaction is required for mesenchyme formation during ascidian embryogenesis. *Dev. Biol.* **214**, 9-22.

Kim, G. J., Yamada, A. and Nishida, H. (2000). An FGF signal from endoderm and localized factors in the posterior-vegetal egg cytoplasm pattern the mesodermal tissues in the ascidian embryo. *Development* **127**, 2853-2862.

Miya, T. and Satoh, N. (1997). Isolation and characterization of cDNA clones for β-tubulin genes as a molecular marker for neural cell differentiation in the ascidian embryo. *Int. J. Dev. Biol.*, **41**, 551-557.

Nakatani, Y. and Nishida, H. (1994). Induction of notochord during ascidian embryogenesis. *Dev. Biol.* **166**, 289-299.

Nakatani, Y. and Nishida, H. (1997). Ras is an essential component for notochord formation during ascidian embryogenesis. *Mech. Dev.* **68**, 81-89.

Nakatani, Y. and Nishida, H. (1999). Duration of competence and inducing capacity of blastomeres in notochord induction during ascidian embryogenesis. *Dev. Growth Differ.* **41**, 449-453.

Nakatani, Y., Yasuo, H., Satoh, N. and Nishida, H. (1996). Basic fibroblast growth factor induces notochord formation and the expression of *As-T*, a *Brachyury* homolog, during ascidian embryogenesis. *Development* **122**, 2023-2031.

Nicol, D. and Meinertzhagen, I. A. (1991). Cell counts and maps in the larval central nervous system of the ascidian *Ciona intestinalis* (L.). *J. Comp. Neurol.* **309**, 415-429.

Nishida, H. (1987). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. *Dev. Biol.* **121**, 526-541.

Nishida, H. (1991). Induction of brain and sensory pigment cells in the ascidian embryo analyzed by experiments with isolated blastomeres. *Development* **112**, 389-395.

Nishida, H. (1992a). Developmental potential for tissue differentiation of fully dissociated cells of the ascidian embryo. *Roux's Arch. Dev. Biol.* **201**, 81-87.

Nishida, H. (1992b). Regionality of egg cytoplasm that promotes muscle differentiation in embryo of the ascidian, *Halocynthia roretzi*. *Development* **116**, 521-529.

Nishida, H. (1993). Localized regions of egg cytoplasm that promote experssion of endoderm-specific alkaline phosphatase in embryos of the ascidian *Halocynthia roretzi*. *Development* **118**, 1-7.

Nishida, H. (1994a). Localization of egg cytoplasm that promotes differentiation to epidermis in embryos of the ascidian *Halocynthia roretzi*. *Development* **120**, 235-243.

Nishida, H. (1994b). Localization of determinants for formation of the anterior-posterior axis in eggs of the ascidian *Halocynthia roretzi*. *Development* **120**, 3093-3104.

Nishida, H. (1997). Cell fate specification by localized cytoplasmic determinants and cell interactions in ascidian embryos. *Int. Rev. Cytol.* **176**, 245-306.

Nishikata, T and Satoh, N. (1990) Specification of notochord cells in the ascidian embryo analyzed with a specific monoclonal antibody. *Cell Differ. Dev.* **30**, 43-53.

Okada, T., Hirano, H., Takahashi, K. and Okamura, Y. (1997). Distinct neuronal lineages of the ascidian embryo revealed by expression of a sodium channel gene. *Dev. Biol.* **190**, 257-272.

Okado, H. and Takahashi, K. (1990). Induced neural-type differentiation in the cleavage-arrested blastomere isolated from early ascidian embryos. *J. Physiol.* **427**, 603-623.

Okamura, Y., Okado, H. and Takahashi, K. (1993). The ascidian embryo as a prototype of vertebrate neurogenesis. *BioEssays* **15**, 723-730.

Okamura, Y., Ono, F., Okagaki, R., Chong, J. A. and Mandel, G. (1994). Neural expression of a sodium channel gene requires cell-specific interactions. *Neuron* **13**, 937-948.

Rose, S. M. (1939). Embryonic induction in the ascidia. *Biol. Bull.* **77**, 216-232.

Sasai, Y. and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182**, 5-20.

Satoh, N. (1978). Cellular morphology and architecture during early morphogenesis of the ascidian egg: An SEM study. *Biol. Bull.* **155**, 608-614.

Satoh, N. (1979). Visualization with scanning electron microscopy of cleavage pattern of the ascidian eggs. *Bull. Mar. Biol. St. Asamushi Tohoku University* **16**, 169-178.

Satoh, N. (1994). *Developmental Biology of Ascidians*. Cambridge, UK: Cambridge University Press.

Takahashi, H., Hotta, K., Erives, A., Di Gregorio, A., Zeller, R. W., Levine, M. and Satoh, N. (1999). *Brachyury* downstream notochord differentiation in the ascidian embryo. *Genes Dev*. **13**, 1519-1523.

Wada, H. and Satoh, N. (1994). Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequences of 18S rDNA. *Proc. Natl. Acad. Sci. USA* **91**, 1801-1804.

Wada, S., Katsuyama, Y., Yasugi, S. and Saiga, H. (1995). Spatially and temporally regulated expression of the LIM class homeobox gene *Hrlim* suggests multiple distinct functions in development of the ascidian, *Halocynthia roretzi*. *Mech. Dev.* **51**, 115-126.

Whittaker, J. R. (1973). Segregation during ascidian embryogenesis of egg cytoplasmic information for tissue-specific enzyme development. *Proc. Nat. Acad. Sci. USA* **70**, 2096-2100.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331-333.

Yagi, K. and Makabe, K. W. (2001). Isolation of an early neural maker gene abundantly expressed in the nervous system of the ascidian, *Halocynthia roretzi*. *Dev. Genes Evol.* **211**, 49-53.

Yasuo, H. and Satoh, N. (1993). Function of vertebrate *T* gene. *Nature* **364**, 582-583.

Yasuo, H. and Satoh, N. (1998). Conservation of the developmental role of *Brachyury* in notochord formation in a urochordate, the ascidian *Halocynthia roretzi*. *Dev. Biol.* **200**, 158-170.