Development 127, 2853-2862 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV3184

An FGF signal from endoderm and localized factors in the posterior-vegetal egg cytoplasm pattern the mesodermal tissues in the ascidian embryo

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Accepted 17 April; published on WWW 13 June 2000

SUMMARY

The major mesodermal tissues of ascidian larvae are muscle, notochord and mesenchyme. They are derived from the marginal zone surrounding the endoderm area in the vegetal hemisphere. Muscle fate is specified by localized ooplasmic determinants, whereas specification of notochord and mesenchyme requires inducing signals from endoderm at the 32-cell stage. In the present study, we demonstrated that all endoderm precursors were able to induce formation of notochord and mesenchyme cells in presumptive notochord and mesenchyme blastomeres, respectively, indicating that the type of tissue induced depends on differences in the responsiveness of the signal-receiving blastomeres. Basic fibroblast growth factor (bFGF), but not activin A, induced formation of mesenchyme cells as well as notochord cells. Treatment of mesenchyme-muscle precursors isolated from early 32-cell embryos with bFGF promoted mesenchyme fate and suppressed muscle fate, which is a default fate assigned by the posterior-vegetal cytoplasm (PVC) of the eggs. The sensitivity of the mesenchyme precursors to bFGF reached a maximum at the 32-cell stage, and the time required for effective induction of mesenchyme cells was only 10 minutes, features similar to those of notochord induction. These results support the idea that the distinct tissue types, notochord and mesenchyme, are induced by the same signaling molecule originating from endoderm precursors. We also demonstrated that the PVC causes the difference in the responsiveness of notochord and mesenchyme precursor blastomeres. Removal of the PVC resulted in loss of mesenchyme and in ectopic notochord formation. In contrast, transplantation of the PVC led to ectopic formation of mesenchyme cells and loss of notochord. Thus, in normal development, notochord is induced by an FGF-like signal in the anterior margin of the vegetal hemisphere, where PVC is absent, and mesenchyme is induced by an FGF-like signal in the posterior margin, where PVC is present. The whole picture of mesodermal patterning in ascidian embryos is now known. We also discuss the importance of FGF induced asymmetric divisions, of notochord and mesenchyme precursor blastomeres at the 64-cell stage.

Key words: Ascidian embryogenesis, Mesodermal patterning, Tissue induction, bFGF, Ooplasmic determinants, Notochord, Mesenchyme

INTRODUCTION

Localized factors in egg cytoplasm and cell interactions, especially inductive events, play crucial roles in the specification of cell fate during early embryogenesis of various kinds of animals. In vertebrates, the mesodermal region is induced by growth factors originating from vegetal cells (Asashima, 1994; Conlon et al., 1994; Feldman et al., 1998). The mesodermal region is patterned by influences from the dorsal organizer, whose formation involves localized dorsal determinants (Lemaire and Gurdon, 1994; Heasman, 1997; Mizuno et al., 1999). In ascidians, a primitive chordate, localized ooplasmic factors and inductive interactions are also involved in the specification of cell fate in early embryos (Venuti and Jeffery, 1989; Meedel, 1992; Satoh, 1994; Nishida, 1997). The larval structure of ascidians is relatively simple, and the major mesodermal tissues of the tadpole larva are notochord, muscle and mesenchyme. As shown in Fig. 1C, larval muscle cells lie laterally on both sides of the notochord, which is aligned in the center of the tail. On each side of the trunk region, there is a cluster of mesenchyme cells. As in frog embryos, these mesodermal tissues are derived from the marginal zone of the blastula, and the endoderm originates from the central zone of the vegetal hemisphere (Fig. 1A,B). Muscle formation is a cell-autonomous process (Reverberi and Minganti, 1946; Nishida, 1992a), and localized ooplasmic factors specify muscle fate in the posterior region of the marginal zone in the vegetal hemisphere (Nishida, 1992b; Marikawa et al., 1994). In contrast, recent studies have revealed that cellular interactions play important roles in the specification of notochord and mesenchyme fates (Nakatani and Nishida, 1994; Kim and Nishida, 1999).

Most notochord cells (primary notochord cells) originate from the anterior region of the marginal zone. Presumptive notochord blastomeres isolated from early 32-cell embryos do not develop into notochord cells (Nakatani and Nishida, 1994).

Formation of notochord cells is induced by cellular interactions with adjacent endoderm precursors and with neighboring notochord precursors themselves at the 32- and early 64-cell stages. Treatment with bFGF, but not activin, of isolated presumptive notochord blastomeres induces the expression of the *Brachyury* homolog, and eventually results in notochord differentiation (Nakatani et al., 1996). The Ras signaling cascade is involved in transduction of this signal (Nakatani and Nishida, 1997).

In the posterolateral region, cellular interaction with adjacent endoderm precursors during the 32- and early 64-cell stages is essential for formation of mesenchyme cells. Presumptive mesenchyme blastomeres isolated from early 32-cell embryos fail to differentiate into mesenchyme cells, and all descendants develop into muscle cells. A signal that originates from endoderm precursors induces mesenchyme fate and suppresses muscle fate. Thus, without this endoderm signal, the blastomeres assume the muscle pathway (Kim and Nishida, 1999).

We have been interested in how mesodermal patterning is achieved during ascidian embryogenesis. In this report, we show that the anterior and posterior marginal blastomeres respond in distinct ways to the same endoderm signal and develop into notochord and mesenchyme cells, respectively. bFGF also induces mesenchyme formation and suppresses muscle fate in mesenchyme precursor blastomeres. Segregation of the posterior-vegetal egg cytoplasm is essential for mesenchyme formation and brings about the difference in responsiveness to an FGF-like endoderm signal.

MATERIALS AND METHODS

Animals and embryos

Naturally spawned eggs of the ascidian *Halocynthia roretzi* were artificially fertilized with a suspension of nonself sperm and raised in Millipore-filtered (pore size 0.45 μm) seawater containing 50 μg/ml streptomycin sulfate and 50 μg/ml kanamycin sulfate at 13°C. At 13°C, tadpole larvae hatch approximately 35 hours after fertilization.

Isolation and recombination of blastomeres

Fertilized eggs were manually devitellinated with sharpened tungsten needles and reared in 0.9% agar-coated plastic dishes filled with seawater. Identified blastomeres were isolated from embryos with a fine glass needle under a stereomicroscope. Isolated blastomeres were cultured separately to develop into partial embryos.

At the early 32-cell stage (between 10 and 40 minutes after formation of the B6.2 blastomeres), the desired blastomeres were isolated from embryos. In blastomere recombination experiments, isolated blastomeres were made to adhere firmly to each other by treatment with 30% (w/v) polyethylene glycol (PEG) dissolved in water. After treatment for 30 seconds, the adhered blastomeres were washed and transferred to seawater and cultured as partial embryos. Recombined cells adhered firmly to each other, but fusion of the cells never occurred.

Treatment with growth factors

At the early 32-cell stage, presumptive mesenchyme blastomeres were isolated and cultured in seawater that contained 0.1% bovine serum albumin (BSA) and bFGF or activin A. Recombinant human bFGF was purchased from Amersham (UK). Recombinant human activin A was kindly provided by Dr M. Asashima (University of Tokyo, Japan).

Deletion and transplantation of egg cytoplasm

Deletion and transplantation of egg cytoplasm was carried out as described previously (Nishida, 1994). Fertilized eggs, after the second phase of ooplasmic segregation, were oriented using the positions of the polar bodies and the transparent myoplasm. Egg fragments containing the PVC, which was 8-15% of the total egg volume, were removed from the eggs by severing with a fine glass needle. The eggs were cultured as PVC-deficient embryos. For transplantation of the egg cytoplasm, egg fragments containing PVC that had been severed from the eggs were transplanted to the anterior-vegetal region of other intact eggs using PEG- and electric field-mediated fusion. The fused eggs were cultured as PVC-transplanted embryos. In the PVC-deficient and PVC-transplanted embryos, the cells divided with a normal cleavage schedule.

Immunofluorescence staining

Formation of mesenchyme cells in the partial embryos was monitored by staining with the Mch-3 monoclonal antibody. The Mch-3 antibody specifically recognizes small particles in mesenchyme cells of *Halocynthia* larvae (Kim and Nishida, 1998). The monoclonal antibody Mu-2 was used for monitoring muscle formation. The Mu-2 antibody recognizes the myosin heavy chain in tail muscle cells of *Halocynthia* larvae (Nishikata et al., 1987). The specimens were fixed after the hatching stage (10 minutes in methanol at –20°C). The monoclonal antibody Not-1 recognizes a component of the notochordal sheath that is secreted by notochord cells (Nishikata and Satoh, 1990). At the tailbud stage (approximately 24 hours after fertilization), this antibody is strictly specific for notochord cells. Therefore, specimens for notochord formation were fixed and immunostained at the tailbud stage. Indirect immunofluorescence detection was carried out by standard methods using fluorescein isothiocyanate-conjugated secondary antibodies (Cappel, Ohio, USA). The Mu-2 and Not-1 antibodies were kindly provided by Dr T. Nishikata (Konan University, Japan).

In situ hybridization

In situ hybridization was done with a digoxigenin-labeled *HrMA4* antisense probe as described previously (Kim and Nishida, 1999). *HrMA4* encodes *Halocynthia* larval muscle actin and was kindly provided by Dr N. Satoh (Kyoto University, Japan).

RESULTS

In ascidian embryos, it has been shown that notochord and mesenchyme fates are determined by inductive cell interactions (Nakatani and Nishida, 1994; Kim and Nishida, 1999). Induction of notochord and mesenchyme shares several common features. (1) Inductive interactions occur during the 32- and early 64-cell stages. (2) Recombination with a neighboring endoderm blastomere is sufficient for notochord and mesenchyme induction. For example, recombination of the A6.1 (endoderm) blastomeres with the A6.2 (notochord) blastomeres, and of the B6.1 (endoderm) blastomeres with the B6.2 (mesenchyme) blastomeres, results in notochord and mesenchyme formation in the latter blastomeres, respectively (Fig. 1). (3) Induced cells of 32-cell embryos respond to the signal(s) by asymmetric divisions that produce daughter cells with distinct fates. The daughter cell that faces the inducing endoderm blastomere assumes a notochord or mesenchyme fate. For example, the induced A6.2 blastomere divides into the A7.3 (notochord) and A7.4 (nerve cord) cells (Fig. 1D), and the induced B6.2 blastomere divides into the B7.3 (mesenchyme) and B7.4 (muscle) cells (Fig. 1E).

No difference in inducing activity between endoderm blastomeres

Endoderm induces notochord in the anterior region of embryos (upper side in Fig. 1B), whereas it induces mesenchyme in the posterior region (lower side in Fig. 1B). How are these two kinds of tissue induced in different regions by endoderm signal(s)? One possibility is that anterior and posterior endoderm precursors have different inducing characteristics. The A6.1 and A6.3 anterior endoderm precursors may produce a notochord-inducing signal, while the B6.1 posterior endoderm precursors emanate a mesenchyme-inducing signal. An alternative possibility is that the notochord and mesenchyme precursor blastomeres respond differently to the same endoderm signal. To investigate this issue, the mesenchyme (B6.2) and notochord (A6.2) precursor blastomeres were isolated at the early 32-cell stage, and then recombined with anterior (A6.1) and posterior (B6.1) endoderm precursors, respectively (Fig. 2). The results are summarized in Table 1.

When the B6.2 mesenchyme precursors were recombined with the A6.1 anterior endoderm blastomeres, the resulting partial embryos expressed the mesenchyme-specific Mch-3 antigen in 38% of cases (Fig. 2A). The percentage of the partial embryos that differentiated into mesenchyme cells was similar to that in control recombination of B6.2 blastomeres and B6.1 posterior endoderm blastomeres (40%). As a negative control, recombinants of B6.2 blastomeres with b6.6 epidermis precursors showed no expression of mesenchyme-specific features. These results indicate that both anterior and posterior endoderm precursors have the ability to induce mesenchyme cells.

Similarly, 31% of the resulting partial embryos expressed the notochord-specific Not-1 antigen when A6.2 notochord precursors were recombined with B6.1 posterior endoderm blastomeres (Fig. 2B). The proportion was similar to that in control recombination of A6.2 blastomeres and A6.1 anterior endoderm blastomeres (47%). When A6.2 blastomeres were recombined with a6.5 epidermis precursors, the partial embryos never developed notochord-specific features. Thus, both anterior and posterior endoderm blastomeres have notochord-inducing ability. These results suggest that there is no qualitative difference between anterior and posterior endoderm blastomeres in their ability to induce mesenchyme and notochord cells.

To ascertain that the mesenchyme and notochord precursor blastomeres show different responsiveness, notochord differentiation in mesenchyme recombinants (B6.2 + A6.1) and mesenchyme differentiation in notochord recombinants (A6.2 + B6.1) were examined (Table 1). In both cases, expression of the tissue-specific antigens was never observed. Therefore, the type of tissue induced completely depends on the responding blastomeres, and not on the inducing endoderm blastomeres.

bFGF induces mesenchyme as well as notochord formation

The mesenchyme and notochord precursor blastomeres respond differently to endoderm signal(s). Do they respond to the same signaling molecule or to distinct molecules that

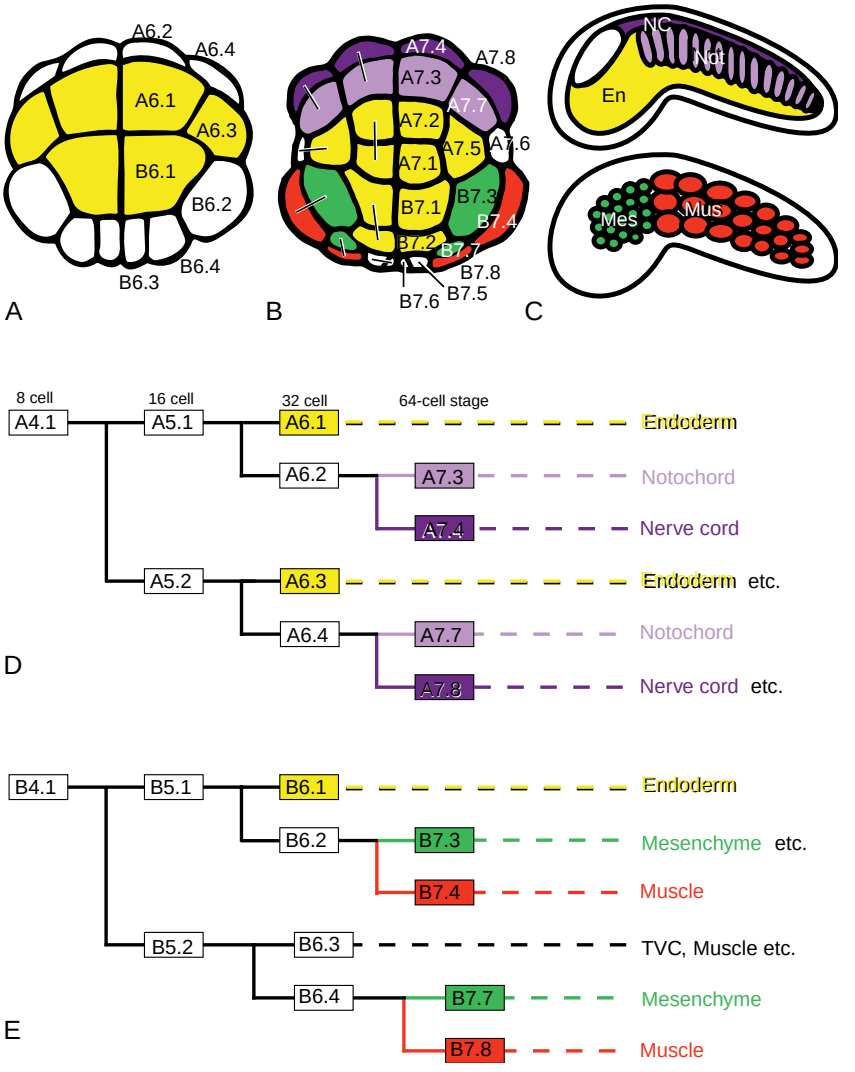

Fig. 1. Diagrams showing the fates of cells in the vegetal hemisphere in ascidian embryos (A-C). The name of each blastomere is indicated. Endoderm (En)-lineage cells are colored yellow. Mesenchyme (Mes)-lineage cells are shown in green and muscle (Mus)-lineage cells in red. Notochord (Not)- and nerve cord (NC)-lineage cells are colored pink and purple, respectively. (A) The 32-cell embryo. Vegetal view. Anterior is up. (B) The 64-cell embryo. Blastomeres connected with a bar are sister blastomeres. (C) The tailbud embryo. Lateral view. Anterior is to the left. Upper and lower diagrams illustrate midsagittal and parasagittal sections, respectively. (D,E) Lineage trees in the vegetal hemisphere. As development is bilaterally symmetrical, one side of the embryo is shown. (D) Lineage tree that is relevant to the primary notochord lineage and that starts from the anterior-vegetal (A4.1) blastomere of the 8-cell embryo. (E) Lineage tree starting from the posterior-vegetal (B4.1) blastomere, from which mesenchyme and primary muscle cells originate. TVC, trunk ventral cells.

Table 1. Expression of tissue-specific antigens in recombined blastomeres

Recombined blastomeres*	Expression of tissue-specific antigens	
	Mch-3	Not-1
Mesenchyme precursor		
B6.2+A6.1 (anterior endoderm)	38% (*n*=39)	0% (*n*=15)
B6.2+B6.1 (posterior endoderm)	40% (*n*=35)	
B6.2+b6.6 (epidermis)	0% (*n*=18)	
Notochord precursor		
A6.2+B6.1 (posterior endoderm)	0% (*n*=16)	31% (*n*=42)
A6.2+A6.1 (anterior endoderm)		47% (*n*=34)
A6.2+a6.5 (epidermis)		0% (*n*=14)

All blastomeres were separately isolated at the early 32-cell stage. They were immediately recombined and cultured, and then expression of the tissue-specific antigens was examined.

*The mesenchyme and notochord precursors are indicated to the left of the +, while recombined blastomeres are shown to the right of the + with their fates in parentheses.

induce notochord and mesenchyme cells? It has been shown that bFGF induces notochord formation in ascidians (Nakatani et al., 1996). Therefore, we examined whether bFGF was also effective in inducing mesenchyme formation.

Mesenchyme precursor blastomeres were isolated manually at the early 32-cell stage and then cultured in seawater that contained bFGF at various concentrations. When the B6.2 isolates were treated with bFGF, a significant proportion of partial embryos showed mesenchyme differentiation, even when the concentration of bFGF was lowered to 0.02 ng/ml (Fig. 3A,B; Table 2). Similar results were obtained using another mesenchyme precursor, B6.4 (data not shown).

When the results were compared with those for notochord induction in A6.2 blastomeres (right column in Table 2), the B6.2 mesenchyme precursors had almost the same dose dependency profile for bFGF treatment. We also treated the isolated mesenchyme precursors with activin A (10 ng/ml), but no mesenchyme formation was observed. This is similar to the observation that activin A is not able to induce notochord formation in presumptive notochord blastomeres (Nakatani et al., 1996).

When A6.2 notochord precursor blastomeres were treated with bFGF at 2.0 ng/ml, mesenchyme differentiation was never observed (*n*=21). Similarly, B6.2 mesenchyme precursors never developed into notochord cells after treatment with bFGF (*n*=25). Therefore, the mesenchyme and notochord precursor blastomeres respond differently to the same signaling molecule, bFGF, by formation of mesenchyme and notochord, respectively. When the isolated A4.1, a4.2 and b4.2 blastomeres from 8-cell embryos were treated with bFGF, the partial embryos never expressed the mesenchyme-specific antigen. Thus, blastomeres without the mesenchyme lineage did not have the capacity to form mesenchyme cells upon treatment with bFGF.

The period of sensitivity to bFGF

To determine the period during which mesenchyme precursors are sensitive to bFGF, B6.2 blastomeres were isolated between 10 and 40 minutes after formation of the blastomeres, and treatment with bFGF at 2.0 ng/ml was initiated at various times between the early 32-cell stage and the late 64-cell stage (Fig. 4). Sensitivity was gradually lost during the 64-cell stage. In most cases, the partial embryos differentiated into mesenchyme cells, when treatment with bFGF was started at the 32-cell stage. When treatment with bFGF was started at the late 64-cell stage, mesenchyme formation was observed in only 20% of cases, and the positive partial embryos contained only a few mesenchyme cells.

In the next experiment, pulse treatments were carried out at the late 32-cell stage. The durations of the pulse treatment were 10 minutes or 30 minutes (Fig. 4, bottom). The treated specimens were immediately washed with seawater. Differentiation of mesenchyme cells was detected in a significant proportion of specimens with both 10-minute and 30-minute treatments. These results indicate that 10 minutes is sufficient for effective induction of mesenchyme cells. The temporal profile of the sensitive period for mesenchyme induction by bFGF is similar to that of notochord induction by bFGF (Nakatani et al., 1996), although the sensitive period for

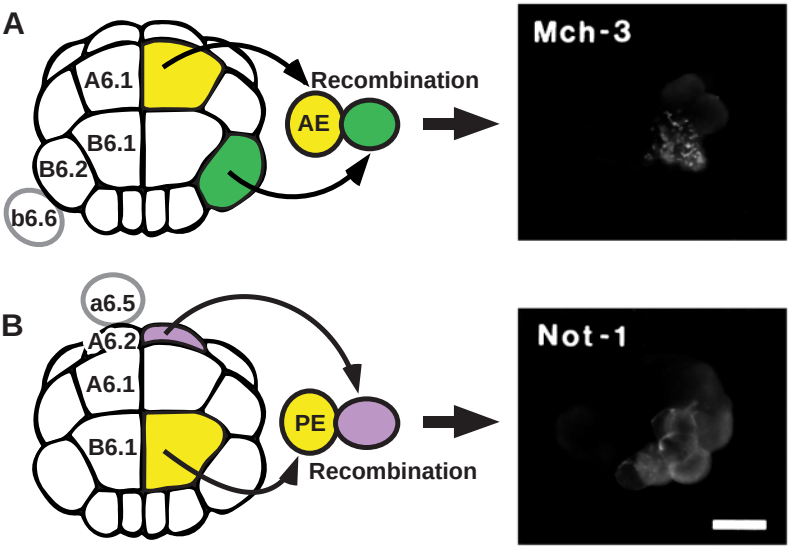

Fig. 2. Diagrams showing the relative position of the blastomeres relevant to recombination experiments at the early 32-cell stage. Resultant partial embryos are shown on the left. Presumptive endoderm blastomeres are colored yellow. The mesenchyme precursor blastomere is shown in green and the notochord precursor blastomere in pink. The b6.6 and a6.5 presumptive epidermis blastomeres indicated outside each diagram of the 32-cell embryo are cells in the animal hemisphere. Each blastomere was isolated from early 32-cell embryos and recombined. (A) The B6.2 mesenchyme precursor blastomere was recombined with the A6.1 anterior endoderm precursor (AE). The partial embryo expressed the mesenchyme-specific Mch-3 antigen. (B) The A6.2 notochord precursor blastomere was recombined with the B6.1 posterior endoderm precursor (PE). The partial embryo showed expression of the notochord-specific Not-1 antigen. Scale bar, 50 μm.

Table 2. Expression of tissue-specific antigens in isolated blastomeres after treatment with bFGF

	B6.2 (mesenchyme precursor)		A6.2 (notochord precursor)
Concentration of bFGF (ng/ml)	Mch-3/*n*	Mu-2/*n*	Not-1/*n*
2.0	100% (95%)/21	0% (0%)/12	100% (100%)/16
1.0	100% (83%)/12	10% (0%)/10	100% (100%)/19
0.5	100% (60%)/15	46% (0%)/13	100% (100%)/20
0.2	98% (26%)/50	100% (0%)/12	100% (67%)/36
0.02	91% (0%)/22	N.D.	96% (12%)/26
0.002	0% (0%)/16	N.D.	13% (0%)/15
0.0	3% (0%)/35	100% (100%)/15	3% (0%)/32

The B6.2 and A6.2 blastomeres were isolated from early 32-cell embryos and cultured in seawater containing 0.1% BSA and various concentrations of bFGF. The B6.2 partial embryos were examined with the Mch-3 and Mu-2 antibodies for the differentiation of mesenchyme and muscle cells, respectively. The A6.2 partial embryos were examined with the Not-1 antibody for notochord formation. The percentages indicate the proportion of the partial embryos that expressed the tissue-specific antigens in at least a part of the partial embryos. The percentages in parentheses indicate the proportion of the partial embryos in which all constituent cells expressed the tissue-specific antigens. N.D., not determined.

mesenchyme induction is slightly extended to the later stage compared with notochord induction.

Suppression of muscle fate by bFGF

The developmental fate of the B6.2 and B6.4 blastomeres is not yet restricted exclusively to mesenchyme at the 32-cell stage, and the blastomeres still retain a muscle fate (Fig. 1E). Without induction by endoderm precursor blastomeres, all descendants of the B6.2 and B6.4 blastomeres develop into muscle cells. An inductive signal from endoderm suppresses muscle fate and promotes mesenchyme formation (Kim and Nishida, 1999).

We examined muscle formation in bFGF-treated B6.2 partial embryos with the Mu-2 anti-myosin antibody. Without treatment, every cell in the partial embryos expressed the Mu-2 antigen in all cases (Fig. 3F; Table 2, percentages in parentheses). Treatment with bFGF suppressed expression of the Mu-2 antigen in a dose-dependent manner. At 2.0 ng/ml, every cell in the partial embryos expressed the Mch-3 antigen (Fig. 3A), but not the Mu-2 antigen (Fig. 3D). At a medium concentration, 0.2 ng/ml, most partial embryos expressed both the Mch-3 (Fig. 3B) and the Mu-2 (Fig. 3E) antigens. Similar results were obtained with another mesenchyme precursor, B6.4 (data not shown). In these experiments, the cells expressing Mch-3 were always smaller and the cells expressing Mu-2 were always larger, as is the case for the mesenchyme and muscle cells of normal tadpole larvae.

Suppression of muscle fate by bFGF was also substantiated by observation of expression of the muscle actin gene, *HrMA4*. In normal embryos, *HrMA4* transcripts are first detected in the B6.2 blastomeres at the 32-cell stage (Fig. 5A). After division, *HrMA4* expression is down-regulated in the B7.3 mesenchyme precursors, but continues in the B7.4 muscle precursors, at the 64-cell stage (Fig. 5B; Satou et al., 1995). However, without a signal from endoderm, both daughter cells continue to express *HrMA4* transcripts (Kim and Nishida, 1999). The B6.2 isolates were treated with 2.0 ng/ml bFGF, and then they were fixed at the late 64-cell stage and the expression of *HrMA4* was examined by in situ hybridization. In the 2-celled partial embryos, neither of the daughter blastomeres expressed *HrMA4* transcripts (Fig. 5C) in 90% of cases (n=21). Faint signals were detected in only 2 cases. Thus, expression of the muscle-specific actin gene was immediately down-regulated by treatment with bFGF. We confirmed that *HrMA4* transcripts were observed in both daughter blastomeres without bFGF treatment (Fig. 5D).

PVC is essential for mesenchyme specification

Presumptive mesenchyme and notochord blastomeres differ in their responsiveness to endoderm signal and bFGF. What causes this difference in responsiveness? Notochord is derived mainly from the anterior-vegetal blastomeres (A-line cells) of embryos, whereas mesenchyme originates from the posterior-vegetal blastomeres (B-line cells). Nishida (1994) reported that the PVC of eggs after the second phase of ooplasmic

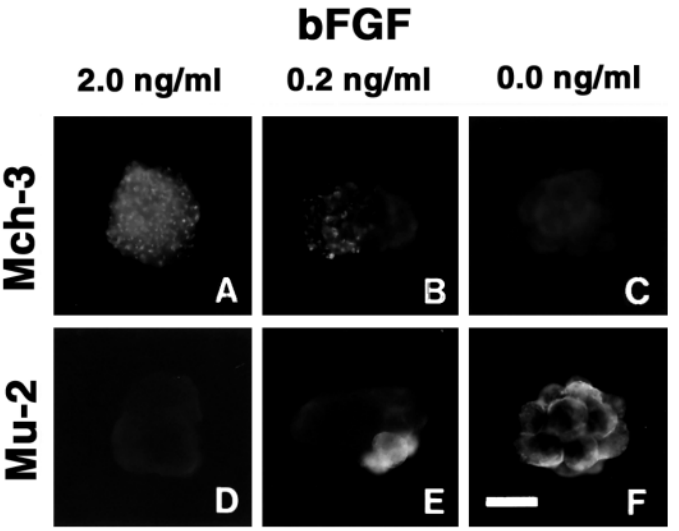

Fig. 3. Expression of the Mch-3 (A-C) and Mu-2 (D-F) antigens visualized by immunofluorescence in partial embryos that were derived from manually isolated presumptive mesenchyme-muscle blastomeres at the early 32-cell stage. The blastomeres were cultured in seawater containing 2.0 ng/ml bFGF (A,D), 0.2 ng/ml bFGF (B,E), and without bFGF (C,F). When the B6.2 isolates were treated with 2.0 ng/ml bFGF, all constituent cells of the resulting partial embryos expressed mesenchyme-specific features (A), but not muscle-specific features (D). When the isolates were treated at medium concentration (0.2 ng/ml), expression of both the Mch-3 (B) and Mu-2 (E) antigens was observed in each partial embryo. Without bFGF, the partial embryos never expressed the Mch-3 antigen (C), and every cell of the partial embryos was stained with the Mu-2 antibody (F). Scale bar, 50 μm.

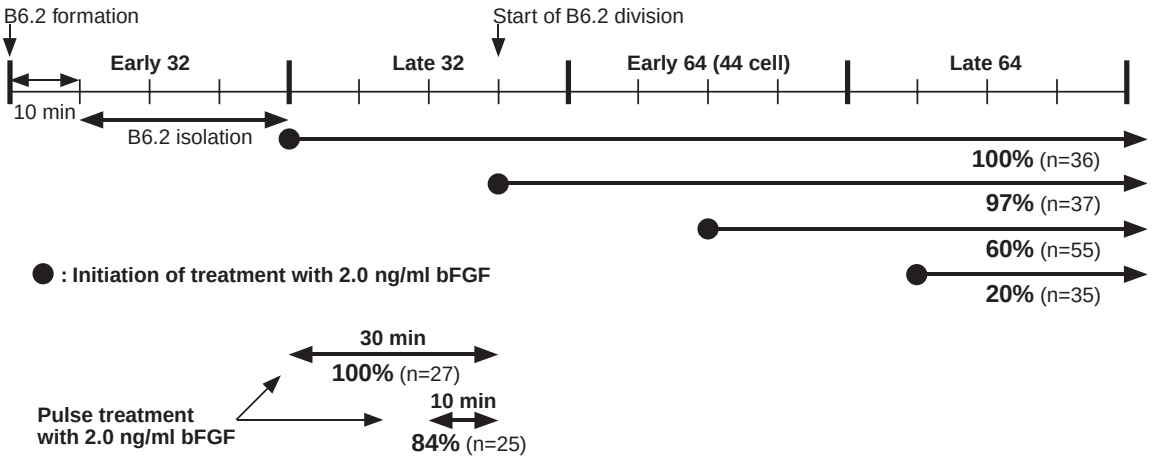

Fig. 4. Timetable and results of experiments designed to examine the period of sensitivity to bFGF. The B6.2 blastomeres were isolated at the early 32-cell stage, and then treatment with 2.0 ng/ml bFGF was initiated at various times between the early 32-cell stage and late 64-cell stage. The isolated B6.2 blastomeres were also pulse-treated with bFGF at the late 32-cell stage for 10 minutes or 30 minutes. The blastomeres treated with bFGF were allowed to develop into partial embryos. Mesenchyme formation was monitored by expression of the Mch-3 antigen.

segregation plays crucial roles in autonomous specification of muscle fate and suppression of notochord induction in the posterior-vegetal blastomeres of embryos. Here, we examined mesenchyme formation in PVC-deficient and PVC-transplanted embryos.

Fig. 6 illustrates experiments involving removal of egg cytoplasm. In these experiments, the blastomeres of the animal hemisphere were removed from the embryos at the 8-cell stage, so that the developed embryos lacked epidermis cells. This is because the larval tunic secreted by epidermis cells prevents the Mch-3 antibody from penetrating into the whole-mount preparation. Without removal of egg cytoplasm, isolated vegetal hemispheres developed the Mch-3 antigen in 81% of cases. When the PVC was removed just after the second phase of ooplasmic segregation, the Mch-3 antigen was expressed in only 4% of cases (Fig. 8A). In control experiments, the anterior-vegetal cytoplasm (AVC) was removed. The Mch-3 antigen was observed in 96% of cases. These results indicated that the PVC is required for mesenchyme formation.

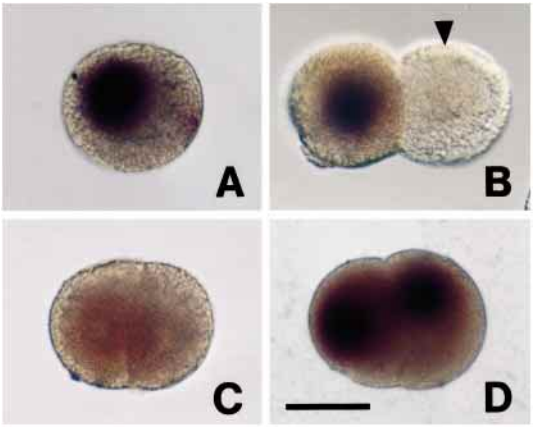

Fig. 5. Expression of the *HrMA4* muscle actin transcripts, as visualized by in situ hybridization. (A) *HrMA4* is clearly expressed in the isolated B6.2 blastomere from a 32-cell embryo. (B) The B7.3 and B7.4 blastomeres (two daughters of the B6.2 blastomere) were isolated together at the late 64-cell stage. A blastomere (arrowhead) that is likely to be the B7.3 blastomere does not express detectable transcripts of *HrMA4*. (C,D) B6.2 blastomeres were isolated at the early 32-cell stage and cultured in seawater with 2.0 ng/ml bFGF (C), and without bFGF (D), until control embryos reached the late 64-cell stage, and were then fixed. (C) *HrMA4* signals were not detected in either daughter blastomere. (D) *HrMA4* signals were detected in both daughter blastomeres. Scale bars, 50 μm.

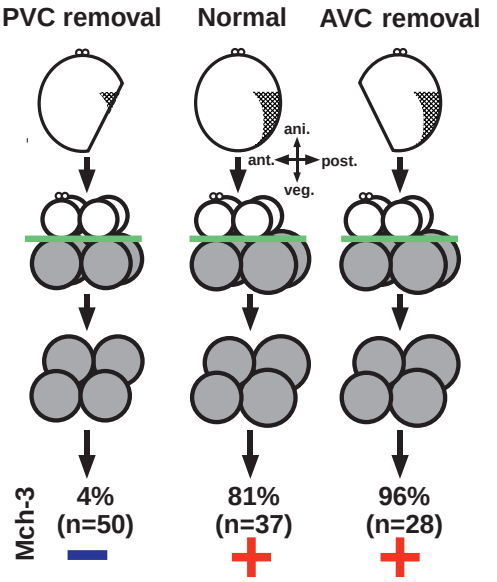

Fig. 6. Experiments involving removal of egg cytoplasm. (Left) The PVC of the fertilized eggs was removed after the second phase of ooplasmic segregation. The PVC-deficient 8-cell embryos were bisected into animal and vegetal halves, and then the vegetal halves were reared as partial embryos. The resulting partial embryos scarcely expressed the Mch-3 antigen. (Center) Partial embryos derived from the vegetal halves of normal 8-cell embryos expressed the Mch-3 antigen. (Right) Control experiment. The vegetal halves of AVC-deficient embryos developed the Mch-3 antigen.

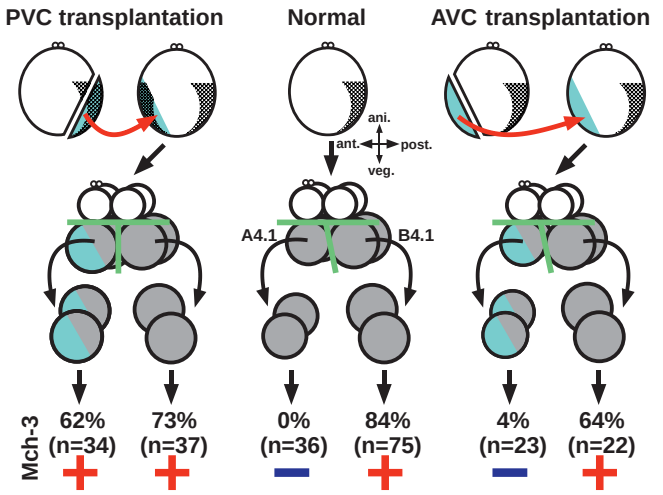

Fig. 7. Experiments involving transplantation of egg cytoplasm. (Left) PVC fragments stained with Nile Blue B were transplanted to the anterior-vegetal region of another intact egg. Anterior- and posterior-vegetal blastomeres were separately isolated from the transplanted 8-cell embryos. Partial embryos that originated from the anterior-vegetal blastomeres of the PVC-transplanted embryos showed ectopic formation of mesenchyme cells. (Center) Partial embryos that were derived from the anterior-vegetal blastomeres of normal embryos never formed mesenchyme cells. (Right) Control experiment. Transplantation of AVC had no effect on mesenchyme formation.

This hypothesis was further confirmed by transplantation of the PVC (Fig. 7). In these experiments, the anterior-vegetal (A4.1 pair) blastomeres and the posterior-vegetal (B4.1 pair) blastomeres were separately isolated from 8-cell embryos. Without transplantation of cytoplasm, mesenchyme formation was observed only in the posterior-vegetal partial embryos (Fig. 7, central column; Kim and Nishida, 1998), as expected from cell lineage and fate map. In transplantation experiments, PVC fragments severed from eggs were stained with Nile Blue B and then fused to the anterior-vegetal region of another intact egg. The transplanted eggs had the PVC in both the anterior and posterior sides. In this case, ectopic mesenchyme formation was observed in the anterior-vegetal partial embryos in 62% of cases (Fig. 8B). Transplantation of the AVC has no effect on mesenchyme formation.

In these experiments, cytoplasmic transplantation was carried out before the start of cleavage. In another type of experiment, the PVC fragments of eggs were directly transplanted to the isolated anterior-vegetal (A4.1) blastomeres from 8-cell embryos. The PVC-transplanted A4.1 blastomeres eventually expressed the Mch-3 antigen in 63% of cases (n=30; Fig. 8C). Transplantation of the AVC had no effect. These results suggest that the transplanted PVC made the anterior-vegetal blastomeres competent to be induced to form mesenchyme cells.

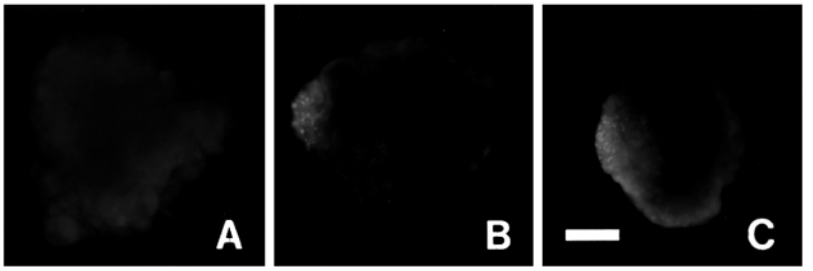

Fig. 8. Expression of mesenchyme-specific antigen in the partial embryos that originated from PVC-deficient and PVC-transplanted embryos. (A) Vegetal partial embryo of the PVC-deficient embryo. No expression of the Mch-3 antigen is observed. (B) Anterior-vegetal partial embryo of the PVC-transplanted embryo. Ectopic expression of the Mch-3 antigen is observed. (C) PVC were directly introduced to A4.1 blastomeres that were isolated from 8-cell embryos. The resulting partial embryo showed ectopic expression of the Mch-3 antigen. Scale bar, 50 μm.

DISCUSSION

Difference in the responsiveness of induced blastomeres

In the present study, we have demonstrated that there is no difference between the inducing abilities of the anterior and posterior endoderm blastomeres in mesenchyme and notochord induction during ascidian embryogenesis. All of the endoderm precursor blastomeres were able to emanate the signal that induces mesenchyme and notochord cells in the marginal zone of the vegetal hemisphere. The kind of tissue induced, namely mesenchyme or notochord, depended on the signal-receiving blastomeres. Our previous study (Nishida, 1994) showed that, when the PVC was removed, the embryos developed ectopic notochord cells in the posterior marginal zone. Anteriorly transplanted PVC suppresses notochord formation in the anterior part. In the present study, we examined mesenchyme formation in PVC-deficient and PVC-transplanted embryos. Removal of the PVC inhibited mesenchyme formation, and transplantation of the PVC to the anterior region of other eggs resulted in ectopic mesenchyme cells in the anterior region. Thus, the difference in responsiveness of the posterior mesenchyme and anterior notochord precursor blastomeres is caused by the presence or absence of the PVC, respectively (Fig. 9A). Blastomeres in the posterior marginal zone, which contain the PVC, develop into mesenchyme cells when they receive the signal from endoderm. Without this interaction, they differentiate into muscle cells (Kim and Nishida, 1999). Blastomeres in the anterior marginal zone, which do not have the PVC, develop into notochord cells when they receive the signal. It is still unknown what kind of tissue they differentiate into without the signal. If the situation is similar in the anterior and posterior regions, one plausible possibility is that they differentiate into nerve cord cells when they do not receive the signal.

The molecular functions of the PVC in the formation of mesenchyme cells and in inhibition of notochord formation have not yet been elucidated. It is possible that particular molecules localized in the PVC modify signal transduction in mesoderm induction and cause distinct tissue formation in the responding blastomeres. A part of these processes may be mediated by ascidian *snail*, a repressor of *Brachyury* expression. Expression of ascidian *snail* preferentially starts in the mesenchyme-muscle

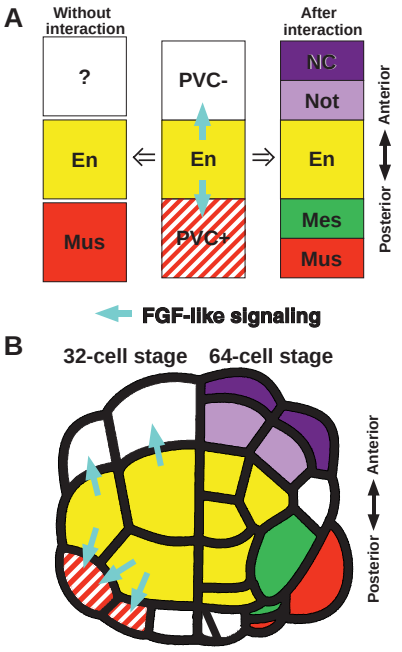

Fig. 9. Mesodermal tissues in ascidian embryos are patterned by the PVC and an FGF-like signal that originates from endoderm. (A) PVC (red oblique lines) causes the different responsiveness in marginal cells. (Right) Cells that do not have the PVC will develop into notochord cells (Not) when they receive an FGF-like signal originating from endoderm (En). Cells that contain the PVC will differentiate into mesenchyme cells (Mes) when they receive an FGF-like signal. (Left) Without signaling, every descendant of blastomeres with the PVC developed into muscle cells (Mus). It is not known what kind of tissue is formed from blastomeres without the PVC when they do not receive the signal. One possibility is nerve cord cells (NC). (B) Asymmetric divisions in the vegetal hemisphere. (Left half) The 32-cell stage. (Right half) The 64-cell stage. In the anterior marginal zone, an FGF-like signal originating from endoderm at the 32-cell stage causes asymmetric divisions into notochord and nerve cord precursors of the 64-cell embryo. In the posterolateral marginal zone, an FGF-like signal promotes asymmetric divisions producing mesenchyme and muscle precursors. In this process, daughter blastomeres that face the inducing endoderm assume notochord and mesenchyme fates, respectively.

precursor blastomeres at the 32-cell stage (Erives et al., 1998; Wada and Saiga, 1999), although it is not known whether the PVC promotes *snail* expression. The *snail* protein of *Ciona intestinalis* binds to the negative *cis*-elements of the *Brachyury* gene to suppresses *Brachyury* expression in the posterior marginal zone (Corbo et al., 1997; Fujiwara et al., 1998). The ascidian *Brachyury* seems to be a master control gene for notochord differentiation (Yasuo and Satoh, 1998). Therefore, the PVC may promote *snail* expression, and then *snail* suppresses *Brachyury* expression, inhibiting notochord formation in the posterior marginal zone.

bFGF in mesoderm formation

The induction of notochord and mesenchyme in ascidian embryos is an example of cell-cell signaling involving the interaction between inducing cells and responding cells. Ascidian mesoderm formation provides a simple system for studying mesoderm induction in chordate embryos. In vertebrate embryos, FGFs function as differentiation factors and play key roles in mesoderm induction (Slack, 1994; Yamaguchi et al., 1995). FGFs can induce muscle, mesenchyme, lateral plate mesoderm and blood islands in *Xenopus*. Inhibition of FGF signaling by the truncated FGF receptor resulted in embryos with reduced mesoderm (Amaya et al., 1991; Yamaguchi et al., 1994; Griffin et al., 1995). FGF signaling is also involved in mesoderm formation during ascidian embryogenesis. Exogenous bFGF induced mesenchyme as well as notochord differentiation.

The positive-feedback regulatory loop between *Xbra* and FGF signaling is necessary for mesodermal maintenance in *Xenopus* embryos (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). By contrast, in ascidians, such a feedback loop is probably not present in the processes of mesoderm induction. Formation of notochord and mesenchyme cells successfully occurred when each precursor was pulse-treated with bFGF for only 10 minutes during the 32-cell stage (Nakatani et al., 1996, and the present study). Sensitivity to bFGF was then quickly lost during the 64-cell stage. At this stage, expression of *Brachyury* starts in the notochord precursor blastomeres (Yasuo and Satoh, 1993, 1994). Even if *Brachyury* up-regulates FGF expression, the blastomeres have already lost their responsiveness after the 64-cell stage. Thus, it seems that FGF signaling in ascidians is involved in the initial phase of the processes of induction but not in the positive-feedback or maintenance processes.

Presumptive notochord and mesenchyme blastomeres are induced to develop into notochord and mesenchyme cells, respectively, by bFGF. This confirms that the same signaling molecule is able to induce both kinds of mesodermal tissue, and that there is a difference in responsiveness between the two kinds of responding blastomeres. Both mesenchyme and notochord precursor blastomeres had a similar profile with regard to induction by bFGF in terms of dose dependency, sensitive period and minimal duration of treatment required for induction (Nakatani et al., 1996, and the present study). It is probable that both mesenchyme and notochord precursors utilize the same receptor molecule, and the intracellular signal transduction pathway may be common to some extent.

Suppression of muscle fate by bFGF

The developmental fate of the B6.2 and B6.4 blastomeres of 32-cell embryos is not yet restricted, and they give rise to both muscle and mesenchyme in normal development (Nishida, 1987; Fig. 1D). When isolated from early 32-cell embryos, these presumptive mesenchyme-muscle blastomeres fail to develop into mesenchyme cells, and all descendants differentiate into muscle cells (Kim and Nishida, 1999). It was suggested that a signal from the adjacent endoderm precursors during the 32- and 64-cell stages suppressed muscle fate and promoted mesenchyme fate in the blastomeres. The present study showed that bFGF indeed suppressed muscle fate and promoted mesenchyme fate (Table 2). Therefore, the same bFGF-like molecule originating from endoderm mediates both inhibition of muscle differentiation and induction of mesenchyme formation.

Asymmetric division and the direction of inductive influence

When the presumptive mesenchyme-muscle blastomeres were

isolated at the early 32-cell stage, all the descendants developed into muscle cells. In contrast, when they were subjected to induction by bFGF over the entire cell surface, both daughter cells assumed the mesenchyme fate (Table 2). During normal embryogenesis, daughter cells closer to the vegetal pole definitely assume the mesenchyme fate (Fig. 9A,B). Since endoderm blastomeres are inducers, the inductive influence is received from the vegetal side of the mesenchyme-muscle precursor blastomeres. Such directed induction would cause the sibling that is closer to the inducer blastomeres to assume a mesenchyme fate. However, we cannot completely eliminate the possibility that cytoplasmic differences might exist endogenously in the mesenchyme-muscle precursor blastomeres at the 32-cell stage.

Detailed temporal analysis involving isolation of mesenchyme-lineage blastomeres at various times during the 32-cell stage showed that the blastomeres start to acquire the ability for autonomous mesenchyme formation during the 32-cell stage (Kim and Nishida, 1999). The present results indicated that the blastomeres showed maximum sensitivity to bFGF during the 32-cell stage. Pulse treatment with bFGF during the 32-cell stage was enough to induce mesenchyme formation. Therefore, some processes of mesenchyme specification related to cellular interaction occur during the 32-cell stage preceding fate restriction. Directed inductive influences polarize the mother cells and cause asymmetric cell divisions, so that only one of the daughter cells acquires the ability to give rise to mesenchyme cells. Presumably, FGF signaling causes localized changes in the mother cells. Similar observations have already been reported in the induction of notochord (Nakatani et al., 1996). Therefore, directed induction and asymmetric divisions may operate in both the anterior and posterior marginal zones in ascidian embryos.

Moreover, similar examples have also been suggested in the development of sensory pigment cells and secondary muscle cells in ascidian embryos (Nishida, 1996). Asymmetric division caused by directed signaling has also been reported in *Caenorhabditis elegans* (Goldstein, 1992, 1995). During the early cleavage stage, the EMS blastomere of the 4-cell embryo divides into the E and MS blastomeres. The progeny of the E blastomere are fated to gut, whereas the progeny of the MS blastomere contribute to the pharynx, body-wall muscle and neurons. Induction by the posterior P2 blastomere at the 4-cell stage is required for alternative cell fates. In the absence of induction, both daughters of the EMS blastomere assume an MS fate. In this case, Wnt signaling is involved (Thorpe et al., 1997; Rocheleau et al., 1997). Thus, directed signaling and asymmetric divisions that produce two distinct cells play a fundamental role in generating different types of embryonic cell, especially in animals whose embryos consist of a small number of cells.

In conclusion, analyses of mesodermal patterning in ascidian embryos lead to significantly different concepts from those in amphibians. First, mesoderm in amphibians is patterned by influence from the organizer, such as antagonism of BMP and Wnt signaling, whereas in ascidian embryos maternal intrinsic factors in the PVC contribute to differences in mesodermal tissues. Secondly, in amphibians dorsal determinants are segregated by cortical rotation to the future notochord side, whereas in ascidians important maternal factors are segregated to the PVC, the side opposite the future notochord-forming region. Thirdly, asymmetric divisions play crucial roles in cell fate choice in the marginal zone of ascidian embryos.

We thank Dr T. Nishikata (Konan University) for providing the Not-1 and Mu-2 monoclonal antibodies, Dr N. Satoh (Kyoto University) for providing *HrMA4* cDNA, and Dr M. Asashima (Tokyo University) for providing human recombinant activin A. We also thank members of the Asamushi Marine Biological Station and the Otsuchi Marine Research Center for supply of *Halocynthia*, and the Misaki Marine Biological Laboratory for maintaining *Halocynthia*. Thanks are also extended to members of our laboratory for useful discussions. G.J.K. is supported by a post-doctoral fellowship from the Japan Society for the Promotion of Science (JSPS) for foreign researchers with a research grant (98503). A. Y. is supported by a pre-doctoral fellowship from the JSPS for young scientists with a research grant (5057). This work was supported by the 'Research for the Future' Program from the JSPS (96L00404) and by the HFSP organization (RG-299/98) to H. N.

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