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Basic fibroblast growth factor induces notochord formation and the expression of *As-T*, a *Brachyury* homolog, during ascidian embryogenesis

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SUMMARY

The tadpole larva of an ascidian develops 40 notochord cells in the center of its tail. Most of the notochord cells originate from the A-line precursors, among which inductive interactions are required for the subsequent differentiation of notochord. The presumptive-endoderm blastomeres or presumptive-notochord blastomeres themselves are inducers of notochord formation. Notochord induction takes place during the 32-cell stage. In amphibia, mesoderm induction is thought to be mediated by several growth factors, for example, activins and basic fibroblast growth factor (bFGF). In the ascidian, *Halocynthia roretzi*, treatment with bFGF of presumptive-notochord blastomeres that had been isolated at the early 32-cell stage promoted the formation of notochord at a low concentration of bFGF (0.02 ng/ml), while activin failed to induce notochord differentiation. The effect of bFGF reached a maximum at the end of the 32-cell stage and rapidly faded at the beginning of the subsequent cleavage, the time for full induction of notochord being at least 20 minutes.

The expression of *As-T*, a previously isolated ascidian homolog of the mouse *Brachyury (T)* gene, starts at the 64-cell stage and is detectable exclusively in the presumptive-notochord blastomeres. The present study showed that presumptive-notochord blastomeres, isolated at the early 32-cell stage, neither differentiated into notochord nor expressed the *As-T* gene. However, when the presumptive-notochord blastomeres were coisolated or recombined with inducer blastomeres, transcripts of *As-T* were detected. When presumptive-notochord blastomeres were treated with bFGF, the expression of the *As-T* gene was also detected. These results suggest that inductive interaction is required for the expression of the *As-T* gene and that the expression of the *As-T* gene is closely correlated with the determined state of the notochord-precursor cells.

Key words: ascidian embryogenesis, notochord differentiation, induction, bFGF, *As-T*

INTRODUCTION

Full details of cell lineage up to the tadpole stage of ascidians have been described (Conklin, 1905; Nishida, 1987) and determinative mechanisms responsible for the developmental fates of cells have been intensively studied (for reviews, see Venuti and Jeffery, 1989; Meedel, 1992; Nishida, 1992a; Satoh, 1994). The notochord of the ascidian embryo consists of 40 cells, aligned longitudinally in a single row along the midline of the tail. 32 of the 40 notochord cells from the anterior to the middle part of the tail are derived from the eight primordial blastomeres of the A-line in the 110-cell embryo, while the other 8 posterior cells originate from the two primordial blastomeres of the B-line in the 110-cell embryo (Fig. 1C,D,I). These ten primordial blastomeres differentiate into 40 notochord cells after two subsequent cell divisions (Fig. 1I). Thus, the two distinct lineages of A-line and B-line precursors contribute to the notochord cells of the tadpole larva (Nishida, 1987).

In the case of A-line precursors, cellular interactions are necessary for the fate specification of notochord (Nakatani and Nishida, 1994). Presumptive-notochord blastomeres of the A-line that are isolated at the early 32-cell embryo do not differentiate into notochord. However, when these presumptive-notochord blastomeres at the 32-cell stage are coisolated or recombined with the vegetal blastomeres, which are the presumptive-endoderm blastomeres and the presumptive-notochord blastomeres themselves, the formation of notochord is observed. These results indicate that the A-line notochord cells arise as a consequence of inductive influences from the blastomeres of the vegetal hemisphere. Since for B-line precursors, in contrast, no combination of blastomeres results in the differentiation of notochord, it has been suggested that the mechanism for determination of notochord might differ between the A-line and the B-line precursors.

The formation of mesoderm in the early amphibian embryo is thought to occur as a result of inductive interactions during which a signal(s) from the vegetal hemisphere of the embryo acts on the overlying animal regions (Nieuwkoop, 1969, 1973). Several growth factors have been identified that are able to induce formation of mesoderm tissues in animal caps (for review, see Smith, 1989). Two factors, bFGF and activin, can

induce mesoderm of various types in such animal-cap assays. Both bFGF and low concentrations of activin can efficiently induce ventrolateral mesoderm, such as mesenchyme and muscle, while high concentrations of activin are capable of inducing dorsal mesoderm, such as notochord (Slack et al., 1987; Green et al., 1990a,b; Sokol et al., 1990; Ariizumi et al., 1991). In amphibians, animal cap cells treated with bFGF do not differentiate into notochord.

The *Brachyury (T)* gene is required in a cell-autonomous manner for the formation of mesoderm in the mouse (Herrmann et al., 1990; Rashbass et al., 1991). The homologs of the mouse *T* gene have been cloned from *Xenopus* (Smith et al., 1991), zebrafish (Schulte-Merker et al., 1992) and chick (Kispert et al., 1995). In these vertebrates, the expression of the *T* gene begins in mesoderm cells and then becomes restricted to notochord. In ascidians, the homolog of *Brachyury*, namely, *As-T*, was cloned by Yasuo and Satoh (1993). The expression of *As-T* is first detected, at the 64-cell stage, exclusively in the presumptive-notochord blastomeres of the A-line. At the 110-cell stage, expression is detected in blastomeres of both the A-line and the B-line. By contrast, none of the blastomeres of the other lineages expresses *As-T* at detectable levels (Fig. 1A-C,E-G). In the A-line precursors, the timing of the expression of *As-T* is consistent with the time of completion of induction of notochord, after which the presumptive-notochord blastomeres can develop autonomously in isolation. This pattern of expression is hence of interest with respect to the involvement of inductive interactions in the expression of *As-T*.

In this study, we found that bFGF, but not activin, had notochord-inducing activity when applied to presumptive-notochord blastomeres isolated at the 32-cell stage. We also found that inductive interactions were indeed required for the expression of *As-T*. The expression of *As-T* was also induced by treatment with bFGF.

MATERIALS AND METHODS

Embryos

Naturally spawned eggs of *Halocynthia roretzi* were artificially fertilized. Fertilized eggs were raised in Millipore-filtered (pore size, 0.45 μm) seawater that contained 50 μg/ml streptomycin sulfate and 50 μg/ml kanamycin sulfate (MFSW) at 13°C. At 13°C, tadpole larvae hatched about 35 hours after fertilization.

Isolation and recombination of blastomeres

Fertilized eggs were manually dechorionated with sharpened tungsten needles and reared in 0.9% agar-coated plastic dishes that contained MFSW. Identified blastomeres were isolated from embryos with a fine glass needle under a stereomicroscope (SZH-10; Olympus, Japan). Isolated blastomeres were cultured separately as partial embryos.

The procedures for recombination of isolated blastomeres have been described previously (Nakatani and Nishida, 1994). In brief, at the early 32-cell stage, a presumptive-notochord blastomere and an inducer blastomere were separately isolated from embryos. They were induced to adhere firmly to each other by treatment with 30% (w/v) polyethylene glycol (PEG) in water. The recombined blastomeres were immediately transferred to MFSW. Cell fusion never occurred after treatment with PEG only, unless some stimulus such as an electric pulse was employed (Nishida, 1992b).

Treatment with bFGF or activin

At the early 32-cell stage, presumptive-notochord blastomeres were isolated and cultured in MFSW that contained 0.1% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) with bFGF or activin A. Recombinant human bFGF was purchased from Amersham

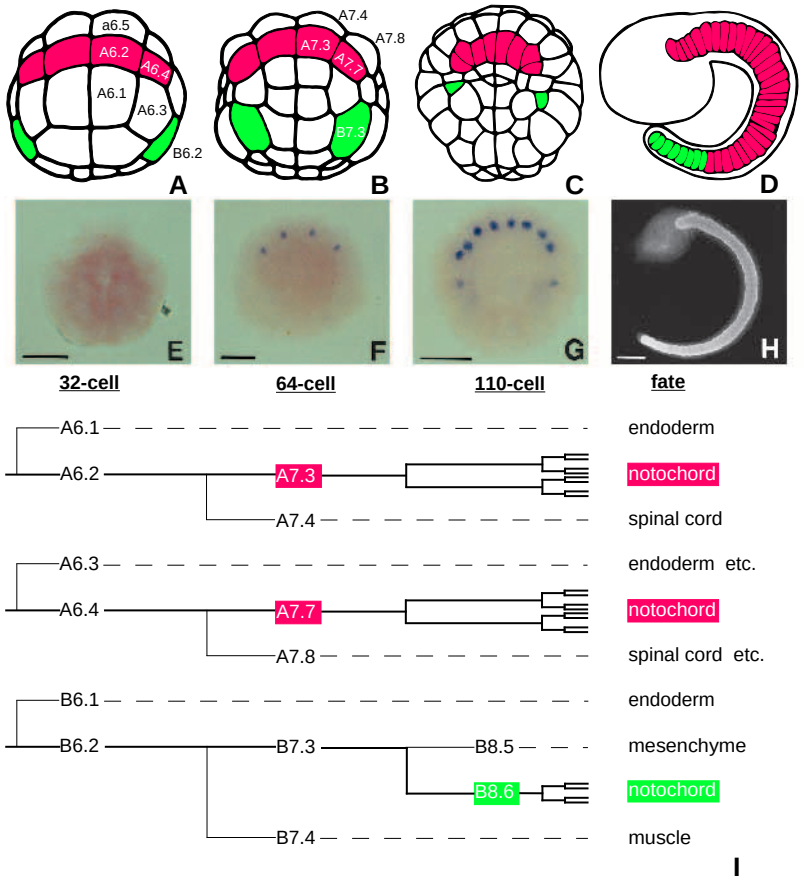

Fig. 1. (A-D) Diagrams showing the lineage of notochord cells. The nomenclature of blastomeres is based on Conklin's description (1905). The A-line precursors are shown in red and the B-line precursors in green. (E-G) Expression of the *As-T* gene, as revealed by whole-mount in situ hybridization with a digoxigenin-labeled antisense probe. (A,E) The 32-cell embryo, vegetal view. No expression of *As-T* is detectable in E. Anterior is up and posterior is down. (B,F) The 64-cell embryo, vegetal view. Hybridization signals are visible in the nuclei of two pairs of A-line primordial-notochord cells (A7.3 and A7.7 pairs) in F. (C,G) The 110-cell embryo, vegetal view. Expression is detectable in four pairs of A-line primordial-notochord cells and in one pair of B-line primordial-notochord cells (B8.6 pairs). (D) The tailbud embryo, lateral view. Of 40 notochord cells, 32 cells in the anterior and middle parts of the tail are derived from the A-line blastomeres (red), while the other 8 cells in the posterior region are derived from the B-line blastomeres (green). (H) Expression of the Not-1 antigen in the tailbud embryo. At this stage, staining is strictly specific to the surfaces of notochord cells. (I) Lineages of notochord cells are indicated by thick lines. Because the fates are bilaterally symmetrical, only half of the embryo is shown. The primordial-notochord blastomeres, whose developmental fate is restricted exclusively to notochord, are indicated by colored squares. Scale bars represent 100 μm in E through G and H.

(England). Recombinant human activin A was a gift from Dr M. Asashima (University of Tokyo, Japan).

Immunohistochemical staining with monoclonal antibodies

The monoclonal antibody 5F1D5 recognizes a notochord-specific antigen, Not-1 (Nishikata and Satoh, 1990). At the tailbud stage (20 hours), this antibody is strictly specific to notochord cells (Fig. 1H). Therefore, specimens were fixed and immunostained at the tailbud stage (Nakatani and Nishida, 1994). The monoclonal antibody Mu-2 binds to the heavy chain of ascidian myosin and specifically recognizes differentiated muscle cells (Nishikata et al., 1987: Makabe and Satoh, 1989). Indirect immunochemical staining was carried out by the standard methods using fluorescein isothiocyanate-conjugated (FITC-conjugated) second antibodies. These tissue-specific antibodies were kindly provided by Dr T. Nishikata (Konan University, Japan).

In situ hybridization with antisense RNA

In situ hybridization was performed with a digoxigenin-labeled antisense RNA probe as described previously (Yasuo and Satoh, 1994). In brief, all specimens were cultured until the controls reached the 110-cell stage. Then embryos were fixed in 4% paraformaldehyde plus 0.5 M NaCl and 0.1 M MOPS, pH 7.5. After they had been washed thoroughly with PBT (phosphate-buffered saline containing 0.1% Tween 20), the fixed specimens were partially digested with 2 μg/ml proteinase K (Sigma) in PBT for 20 minutes at 37°C. They were then postfixed with 4% paraformaldehyde in PBT for 1 hour at room temperature. After prehybridization for 1 hour at 42°C, the specimens were allowed to hybridize with the digoxigenin-labeled (DIG-labeled) antisense probe for about 16 hours at 42°C. The probe was prepared according to the instructions supplied with the kit (DIG RNA Labeling kit; Boehringer Mannheim, Germany). Then specimens were treated with 20 μg/ml RNase A (Sigma). The samples were finally incubated with a 2000-fold diluted solution of alkaline phosphatase-conjugated DIG-specific antibodies in PBT (Boehringer Mannheim, Germany). The reaction catalyzed by alkaline phosphatase was stopped by washing in PBS after 1 to 12 hours.

RESULTS

Notochord is induced by bFGF but not by activin

When presumptive-notochord blastomeres are isolated from early 32-cell embryos and then allowed to develop as partial embryos in Millipore-filtered seawater (MFSW), they do not express any features of notochord. For A-line precursors, formation of notochord requires inductive signals from vegetal blastomeres. To investigate the nature of notochord induction, presumptive-notochord blastomeres at the early 32-cell stage (A6.2, A6.4 and B6.2 cells) were isolated and cultured in the presence and in the absence of growth factors (activin or bFGF). The concentration of growth factors was varied from 500 ng/ml to 2 ng/ml. Differentiation of notochord was recognized morphologically as well as by monitoring the expression of the Not-1 antigen (Nakatani and Nishida, 1994). The results are summarized in Table 1.

In the control experiment, the A6.2, A6.4 and B6.2 cells isolated from 32-cell embryos were cultured in MFSW that contained only 0.1% BSA. They failed to develop features of notochord (Fig. 2A,D,G,J). When activin was added at various concentrations, A6.2, A6.4 and B6.2 isolates from 32-cell embryos rarely developed into notochord (Fig. 2B,E,H). Although activin is capable of inducing notochord differentiation at 50 ng/ml in *Xenopus* (Ariizumi et al., 1991), notochord formation was not observed in ascidian embryos even at concentrations of activin as high as 500 ng/ml. bFGF is generally known as an inducer of the ventrolateral mesoderm in *Xenopus* embryos. In ascidian embryos, significant differentiation of notochord was observed when the A6.2 and A6.4 isolates were treated with bFGF, even when the concentration of bFGF was only 2 ng/ml (Fig. 2C,F). In partial embryos, there are vacuoles at the ends of the notochord cells, which appear elongated (Fig. 2C, arrow) and express the Not-1 antigen. In most cases, every cell in the partial embryos developed notochord-specific features. This result was unexpected because the developmental fate of the A6.2 and A6.4 cells is not yet restricted exclusively to notochord at this stage and these cells still retain a spinal cord fate also (Fig. 1I). This point will be discussed below. By contrast, none of the B6.2 isolates had notochord-specific features after their exposure to bFGF (Fig. 2I,K).

In amphibians, activin induces mesoderm that includes muscle. Therefore, we examined whether the activin-treated

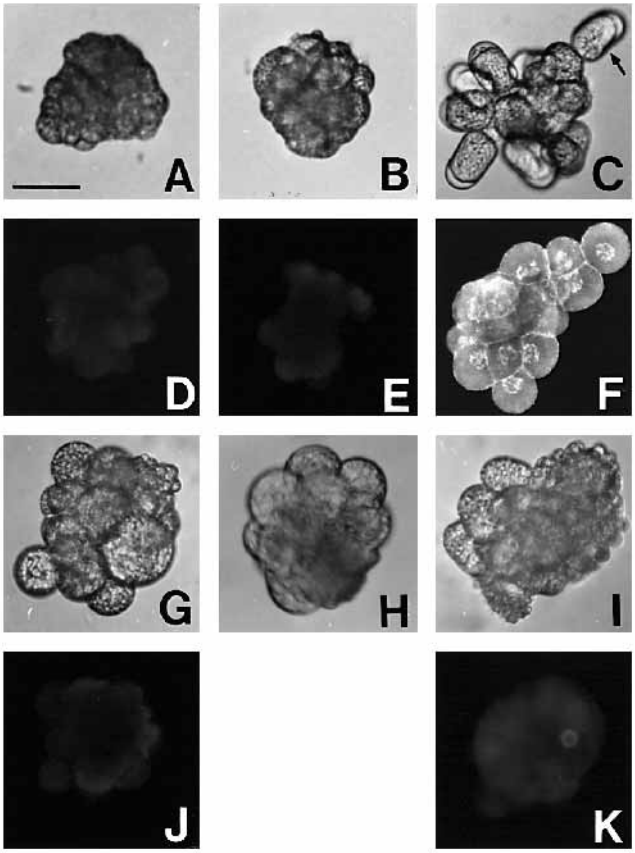

Fig. 2. Expression of notochord-specific features in partial embryos. (A-F) A6.2 partial embryos. (G-K) B6.2 partial embryos. The A6.2 and the B6.2 isolates were cultured in Millipore-filtered seawater (MFSW) that contained only 0.1% BSA (A,D,G,J), 0.1% BSA plus 100 ng/ml activin (B,E,H), or 0.1% BSA plus 2 ng/ml bFGF (C,F,I,K). (A-C,G-I) Morphology of partial embryos. Notochord cells with a vacuole and an elongated profile are indicated by an arrow. The large cells in the B6.2 partial embryos are not notochord cells but muscle cells. (D-F,J,K) Expression of the Not-1 antigen, as visualized by immunofluorescence. Scale bar, 50 μm.

Table 1. Expression of notochord-specific features in isolated blastomeres after treatment with activin and bFGF

Isolated blastomeres	Number of partial embryos with notochord-specific features / Number of embryos examined (%)					
	A6.2		A6.4		B6.2	
	Morph.	Not-1	Morph.	Not-1	Morph.	Not-1
a) activin						
500 ng/ml	0/32 (0%)	0/29 (0%)	2/25 (8%)	0/15 (0%)	0/10 (0%)	N.D.
100 ng/ml	0/32 (0%)	0/29 (0%)	2/25 (8%)	0/13 (0%)	0/10 (0%)	N.D.
20 ng/ml	1/32 (3%)	0/27 (0%)	1/25 (4%)	0/17 (0%)	0/10 (0%)	N.D.
2 ng/ml	0/32 (0%)	0/31 (0%)	1/25 (4%)	0/14 (0%)	0/10 (0%)	N.D.
0 ng/ml	2/31 (6%)	0/31 (0%)	1/21 (5%)	0/16 (0%)	0/10 (0%)	N.D.
b) bFGF						
500 ng/ml	10/10 (100%)	8/8 (100%)	11/11 (100%)	15/16 (94%)	0/10 (0%)	N.D.
100 ng/ml	30/30 (100%)	23/23 (100%)	30/30 (100%)	16/16 (100%)	0/10 (0%)	0/8 (0%)
20 ng/ml	30/30 (100%)	22/22 (100%)	30/30 (100%)	23/23 (100%)	0/10 (0%)	0/9 (0%)
2 ng/ml	30/30 (100%)	24/24 (100%)	28/28 (100%)	16/16 (100%)	0/10 (0%)	0/8 (0%)
0 ng/ml	5/30 (17%)	1/24 (4%)	6/30 (20%)	0/19 (0%)	0/10 (0%)	0/10 (0%)

The presumptive-notochord blastomeres were isolated at the early 32-cell stage and cultured in MFSW plus 0.1% BSA supplemented with various concentrations of activin or bFGF. Expression of notochord-specific features was examined both morphologically (Morph.) and immunohistochemically (Not-1). N.D., Not determined.

blastomeres developed into muscle cells. Muscle differentiation was judged by examining the expression of myosin with the monoclonal antibody Mu-2. No myosin was detected in the A6.2 and A6.4 partial embryos that had been treated with activin at various concentrations. After treatment with bFGF, the A6.2 and A6.4 partial embryos also did not express myosin (data not shown). These results indicate that bFGF and activin do not induce the formation of muscle in the presumptive-notochord blastomeres.

When dechorionated eggs were treated continuously with bFGF or activin from the 1-cell stage until the controls hatched, they developed normally and the tails of larvae had the normal number of notochord cells. No morphological abnormalities were observed in these larvae. To examine the response of cells in the animal hemisphere to treatment with activin and bFGF, presumptive-epidermis blastomeres at the 8-cell stage (a4.2 and b4.2 cells) were isolated and treated with bFGF or activin at various concentrations. The a4.2 and b4.2 partial embryos never developed notochord-specific features after treatment with bFGF or activin. Thus, animal cells in ascidians do not have the capacity to form notochord upon treatment with bFGF.

Dose-response analysis of treatment with bFGF

To examine the dose-response profile for the induction of notochord by bFGF, the concentration of bFGF was reduced to below 2 ng/ml. A6.2 and A6.4 blastomeres were isolated manually and then cultured in MFSW plus 0.1% BSA that contained bFGF at various concentrations (Table 2). At 0.2 ng/ml bFGF, the cells developed notochord-specific morphology and staining for the antigen was detected (Fig. 3A,D); all the cells of a partial embryo developed into notochord in 91-100% of cases. At 0.02 ng/ml bFGF, in most (79%-100%) partial embryos, only some cells had notochord-specific features, while other cells did not (Fig. 3B,E). At 0.002 ng/ml bFGF, the frequency of differentiation of cells to notochord was significantly reduced (Fig. 3C,F). Thus, 0.02 ng/ml was the minimum concentration of bFGF for induction of notochord.

The period at which cells were sensitive to bFGF

To determine the timing of the sensitivity to bFGF of notochord precursor cells, we performed three kinds of experiment. After the 16-cell stage, cleavages become asynchronous. The division of vegetal blastomeres precedes that of animal blastomeres. Blastomeres in the vegetal hemisphere at the 16-cell embryos start the fifth cleavage before most in the animal hemisphere. 30 minutes later, blastomeres in the animal hemisphere divide, with resultant formation of the 32-cell embryo. The relative time designated 0 minutes in Fig. 4 indicates the beginning of the 32-cell stage when all the blastomere have completed the fifth cleavage. The sixth cleavage

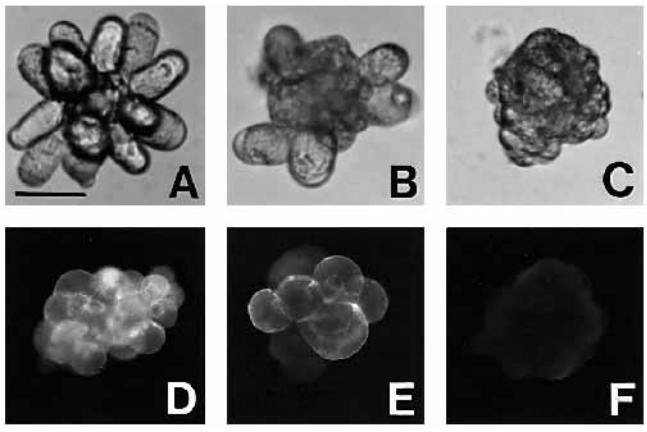

Fig. 3. Photomicrographs of A6.2 partial embryos with notochord-specific features. A6.2 isolates were developed in MFSW plus 0.1% BSA that contained bFGF at 0.2 ng/ml (A,D), 0.02 ng/ml (B,E) and 0.002 ng/ml (C,F). (A-C) Morphology of the partial embryos. (D-F) Expression of the Not-1 antigen, as visualized by immunofluorescence. Scale bar, 50 μm.

Table 2. Expression of notochord-specific features in isolated blastomeres after treatment with bFGF

Isolated blastomeres	Concentration of bFGF		*n*	Notochord formation in embryos: all	partial	none
A6.2	0.2 ng/ml	Morph.	32	100%	0%	0%
		Not-1	22	95%	0%	5%
	0.02 ng/ml	Morph.	30	0%	100%	0%
		Not-1	21	0%	90%	10%
	0.002 ng/ml	Morph.	29	0%	3%	97%
		Not-1	29	0%	0%	100%
A6.4	0.2 ng/ml	Morph.	31	97%	0%	3%
		Not-1	22	91%	9%	0%
	0.02 ng/ml	Morph.	34	0%	100%	0%
		Not-1	24	0%	79%	21%
	0.002 ng/ml	Morph.	27	0%	0%	100%
		Not-1	21	0%	0%	100%

A-line presumptive-notochord blastomeres were isolated from 32-cell embryos and allowed to develop in MFSW plus 0.1% BSA that contained bFGF at 0.2, 0.02 or 0.002 ng/ml. The expression of notochord-specific features was examined both in terms of morphology (Morph.) and in terms of expression of Not-1 (Not-1). all, All the cells of a partial embryo showed notochord-specific features; partial, some cells of a partial embryo developed into notochord; none, none of the cells in a partial embryo differentiated into notochord.

of some of the vegetal blastomeres, including notochord precursors (A6.2 and A6.4), starts at 40 minutes and yields a 44-cell embryo. At the completion of this division, the presumptive-notochord blastomeres acquire developmental autonomy. At 90 minutes, the remaining cells divide and the embryo reaches the 64-cell stage. The A6.2 blastomeres were isolated just after the formation of the A6.2 cells (relative time, −20 minutes in Fig. 4) and treatment with bFGF at 2 ng/ml was initiated at various stages from the 32-cell stage to the 64-cell stage. The formation of notochord cells was determined from cell morphology and by immunohistochemical staining. The results are shown in Fig. 4. When the treatment with bFGF was started before the beginning of division of the A6.2 blastomeres (sixth cleavage; relative time, 40 minutes), they differentiated into notochord. The frequency of notochord formation was significantly reduced during cell division (cell division appears to take 10 minutes or so, but it is difficult to tell precisely when division is completed, by contrast to when division starts). These results suggest that treatment with bFGF must be initiated before the start of the division of A6.2 cells.

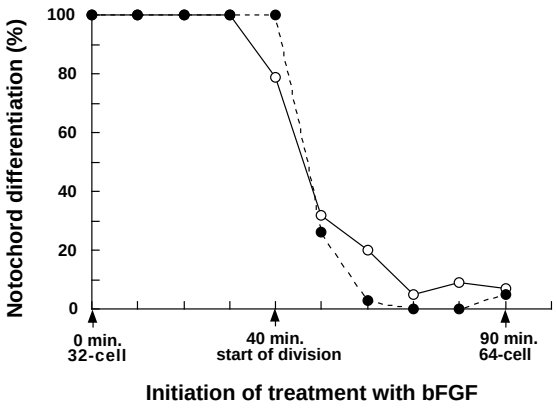

Fig. 4. Results of experiments designed to identify the period when presumptive-notochord (A6.2) blastomeres were sensitive to bFGF. At relative time −20 minutes, the A6.2 blastomeres were isolated and treatment with 2 ng/ml bFGF was started at various stages from the 32-cell stage to the 64-cell stage (0-90 minutes). Notochord differentiation was examined both morphologically (open circles) and immunohistochemically (closed circles). Approximately 25 blastomeres were examined in each experiment.

In the next experiment, cells were pulse-treated with bFGF at 2 ng/ml at various stages (Fig. 5). The duration of the pulse treatment was 10 minutes. The sensitivity to bFGF increased during the 32-cell stage. The sensitivity reached a maximum (50%-60%) within 20-30 minutes and decreased rapidly after the sixth cleavage had started. In the last experiment (Fig. 6), the A6.2 cells were treated with bFGF for 5 minutes to 50 minutes around the period when cells were most sensitive to bFGF. The results indicated that the time required for full induction was approximately 20 minutes.

The expression of *As-T* and notochord induction

A homolog of the mouse *Brachyury (T)* gene in an ascidian has been cloned and designated *As-T* (Yasuo and Satoh, 1993). No expression of *As-T* was detected before the 32-cell stage during normal development (Fig. 1E). At the 64-cell stage, transcripts of *As-T* first become detectable around the nuclei of four A-line primordial-notochord blastomeres (Fig. 1F). At the 110-cell stage, *As-T* is expressed in eight A-line and two B-line primordial-notochord cells (Fig. 1G). After the neurula stage, the signals become less evident and they are undetectable in tadpole larvae. The stage at which the expression of *As-T* is first observed coincides with the stage at which notochord induction is completed. To prove that the expression of *As-T* occurs as a results of notochord induction in ascidian embryos, we cultured A6.2 and A6.4 isolates from the 32-cell stage until the 110-cell stage and then fixed them and examined the expression of *As-T* by in situ hybridization. The results are shown in Table 3. The isolated A6.2 and A6.4 partial embryos did not express detectable transcripts of *As-T* (Fig. 7A). Hybridization signals were detected in the A7.3 and A7.7 isolates from 64-cell embryos when they were examined at the 110-cell stage (Fig. 7B). This result was expected because the expression of *As-T* has already begun by the 64-cell stage.

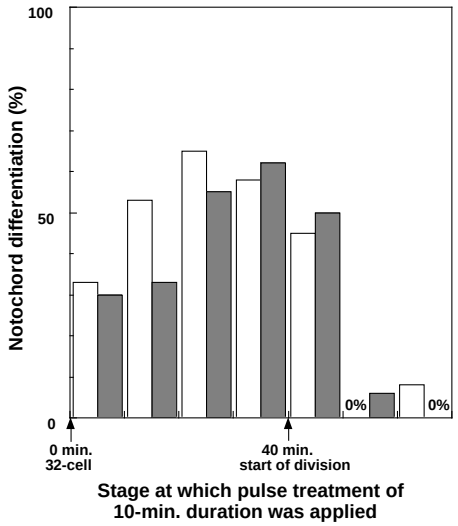

Fig. 5. Histograms showing the percentage of cells that differentiated to notochord in response to pulse treatment of A6.2 blastomeres with 2 ng/ml bFGF. The duration of treatment was10 minutes. Notochord differentiation was monitored by examining morphology (white bars) and the expression of the Not-1 antigen (gray bars). The sensitivity to bFGF increased during the 32-cell stage and decreased sharply after the beginning of the sixth division. Approximately 20 blastomeres were examined in each experiment.

The A6.2 blastomeres were coisolated manually with the adjacent blastomeres from 32-cell embryos, cultured in MFSW as partial embryos and fixed at the 110-cell stage. When an A6.2 blastomere was coisolated with an inducer blastomere, either a presumptive-endoderm (A6.1) blastomere or another presumptive-notochord (A6.4) blastomere, the expression of *As-T* was induced in 100% of cases (Fig. 7C,D). Similar results were obtained when an A6.2 blastomere and an inducer (A6.1 or A6.4) blastomere were isolated separately and immediately recombined by treatment with PEG. Recombination of an A6.2 blastomere with an A6.1 blastomere resulted in the expression of *As-T* in 51% of cases (Fig. 7F). The expression of *As-T* was detected when an A6.2 blastomere and an A6.4 blastomere

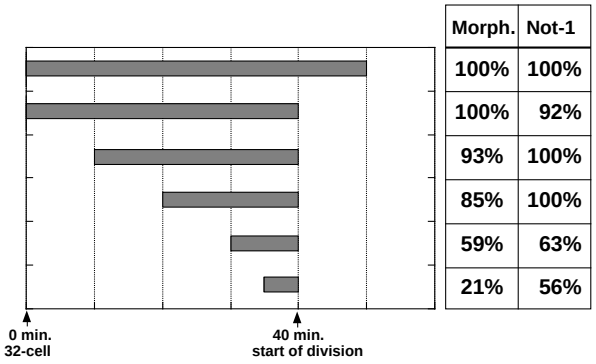

Fig. 6. Effect of pulse treatments with 2 ng/ml bFGF of various durations on A6.2 blastomeres. Bars indicate the duration of treatment with bFGF. The treatment was carried out around the time when the cells were most sensitive to bFGF (relative time, 20-40 minutes). The pulse-treated blastomeres were allowed to develop into partial embryos. Notochord differentiation was monitored in terms of morphology and the expression of the Not-1 antigen. Approximately 15 blastomeres were examined in each experiment.

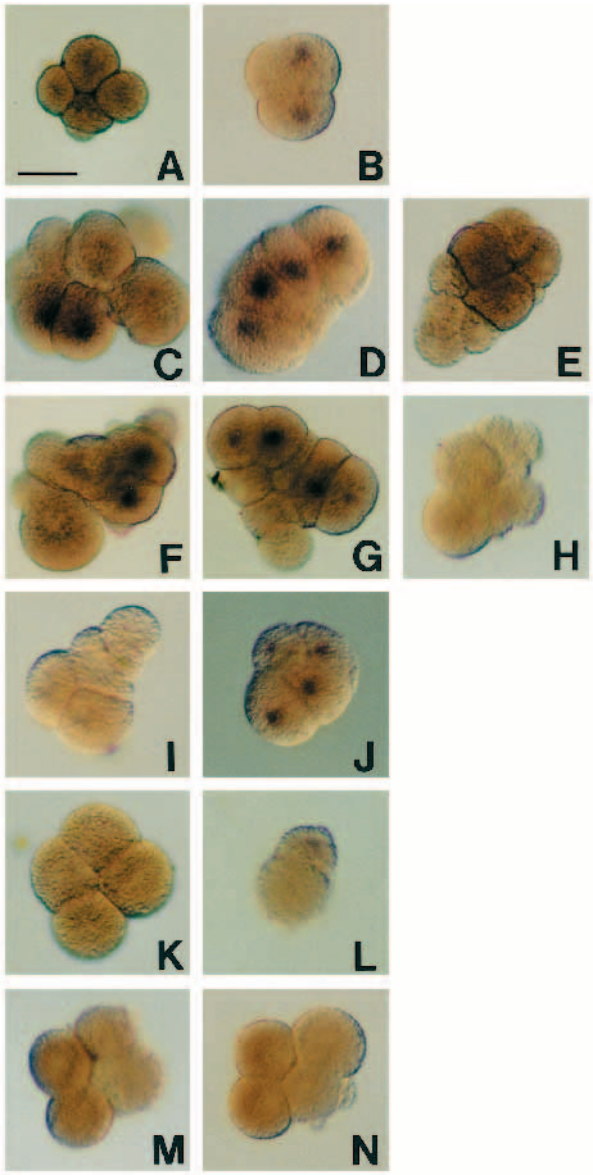

Fig. 7. Photomicrographs of the partial embryos showing the expression of *As-T* transcripts, as visualized by whole-mount in situ hybridization. The partial embryos derived from the A-line presumptive-notochord blastomeres (A-J) and the B-line blastomeres (K-N) are shown. The expression of *As-T* was examined at the 110-cell stage. An A6.2 isolate (A) and an A7.3 or A7.7 isolate (B) were cultured in MFSW. Coisolates of an A6.2 blastomere with an A6.1 blastomere (C), with an A6.4 blastomere (D) and with an a6.5 blastomere (E) were cultured in MFSW. Recombinants of an A6.2 blastomere with an A6.1 blastomere (F), with an A6.4 blastomere (G) and with an a6.5 blastomere (H) were cultured in MFSW. An A6.2 isolate was cultured in MFSW that contained 0.1% BSA (I) and in MFSW plus 0.1% BSA with 0.2 ng/ml bFGF (J). A B6.2 isolate (K) and a B7.3 isolate (L) were cultured in MFSW. A B6.2 isolate was cultured in MFSW plus 0.1% BSA (M) and in MFSW plus 0.1% BSA with 0.2 ng/ml bFGF (N). Scale bar, 50 μm.

Table 3. Expression of the transcripts of *As-T* in blastomeres

Blastomeres	Treatment	Number of partial embryos that expressed *As-T* transcripts / Number of embryos examined	(%)
a) A-line			
A6.2	–	0/22	(0%)
A6.4	–	1/29	(3%)
A7.3, A7.7	–	27/27	(100%)
A6.2+A6.1 (endoderm)	coisolation	33/33	(100%)
A6.2+A6.4 (notochord)	coisolation	31/31	(100%)
A6.2+a6.5 (brain etc)	coisolation	0/36	(0%)
A6.2+A6.1	recombination	19/37	(51%)
A6.2+A6.4	recombination	20/29	(69%)
A6.2+a6.5	recombination	3/24	(13%)
A6.2	0.1% BSA-MFSW	0/21	(0%)
	0.2 ng/ml bFGF	25/25	(100%)
b) B-line			
B6.2	–	0/21	(0%)
B7.3	–	16/19	(84%)
B6.2	0.1% BSA-MFSW	0/26	(0%)
	0.2 ng/ml bFGF	6/29	(21%)
	2 ng/ml bFGF	2/23	(9%)

In the first series of experiments, the presumptive-notochord blastomeres were isolated from 32-cell and 64-cell embryos. In the second series, presumptive-notochord blastomeres and inducer blastomeres (A6.1, presumptive endoderm; and A6.4, presumptive notochord) or animal blastomeres (a6.5, presumptive brain) were coisolated or recombined at the 32-cell stage. In the third series, the presumptive-notochord blastomeres isolated from the 32-cell embryos were cultured in MFSW plus 0.1% BSA with or without bFGF. In all experiments, isolates were allowed to develop until the 110-cell stage and then fixed. The transcripts of *As-T* were detected by in situ hybridization.

were recombined (69% of cases; Fig. 7G). In control experiments, coisolation or recombination of an A6.2 blastomere with an a6.5 (non-inducer) blastomere rarely resulted in expression of transcripts of *As-T* (0 and 13% of the cases, respectively; Fig. 7E,H).

The number of cells that expressed *As-T* in the coisolated and the recombined partial embryos was consistent with the number of notochord-lineage cells predicted from the normal cell lineage. Therefore, it is suggested that the expression of *As-T* was restricted to notochord-precursor cells in these experiments. For example, an A6.2 blastomere divides twice to yield four cells before the 110-cell stage. Of these four cells, two cells are notochord-precursor cells and two cells are spinal-cord precursor cells. By contrast, an A6.1 blastomere generates two endoderm cells after one division prior to the 110-cell stage. Thus the coisolation or recombination of an A6.2 blastomere with an A6.1 blastomere gives rise to six cells, among which two are notochord-precursor cells. In our coisolation experiments, in all 33 cases, the expression of *As-T* was detected in two of the six cells (Fig. 7C). The coisolation or recombination of an A6.2 blastomere with an A6.4 blastomere would be expected to produce eight cells, of which four should be notochord-precursor cells. Indeed, in the embryos derived from coisolates, *As-T* transcripts were detected in four of the eight cells in 24 out of 31 cases (77%) (Fig. 7D). Although the number of cells that expressed transcripts of *As-T* was slightly lower after recombination than after coisolation, the maximum numbers of *As-T*-expressing cells never exceeded the numbers expected from the lineage of notochord cells (Fig. 7F,G).

Treatment with 0.2 ng/ml bFGF also induced the expression of *As-T* as shown in Table 3 and Fig. 7J. Hybridization signals were observed in A6.2 partial embryos that had been cultured with bFGF at 0.2 ng/ml. As mentioned before, bFGF at 0.2 ng/ml induced notochord differentiation in all the descendants of the isolated A6.2 and A6.4 blastomeres (see for example, Fig. 3A,D; Table 2). Similarly, in 20 out of 25 cases (80%), the transcripts of *As-T* were detected in all four cells of the partial embryos (Fig. 7J). By the 110-cell stage, an A6.2 cell has divided into two notochord precursors and two spinal-cord precursors during normal development. Unlike the results of experiments that involved coisolation and recombination, exposure to bFGF led to expression of *As-T* transcripts in all the descendants of the A6.2 blastomeres. This result reflects the morphological features and the expression of the Not-1 antigen, described above.

B6.2 isolates from 32-cell embryos do not develop any features of notochord. No expression of *As-T* was detected in the B6.2 isolates when they were examined at the 110-cell stage (Fig. 7K). Previous studies showed that, from the 64-cell stage, B-line notochord precursors can develop autonomously into notochord cells in isolation (Nakatani and Nishida, 1994). Although *As-T* is not expressed in B7.3 cells at the 64-cell stage during normal embryogenesis (Yasuo and Satoh, 1993), the transcripts of *As-T* were detected in the partial embryos derived from B7.3 cells (Fig. 7L). The B6.2 blastomeres that were treated with bFGF at 2 ng/ml did not differentiate into notochord (Fig. 2I,K; Table 1). Consistent with this observation, the expression of *As-T* was scarcely induced by bFGF at 0.2 ng/ml or at 2 ng/ml (Fig. 7N).

DISCUSSION

Comparisons of notochord induction between ascidian and amphibian embryos

The present study demonstrated that bFGF can induce notochord differentiation in presumptive-notochord blastomeres during ascidian embryogenesis. By contrast, no notochord cells were induced by the treatment with activin. In amphibians, notochord cannot be induced by bFGF but by activin. It is suggested that some aspects of the induction of notochord may be common to ascidians and amphibians but that the molecules involved in the induction appear to be different. However, we also should not ignore the result that injection of *Xenopus* bFGF mRNA results in notochord differentiation in animal cap explants (Kimelman and Maas, 1992).

Two members of the FGF family, bFGF and embryonic FGF (eFGF), are expressed maternally in amphibian eggs and are present at the stage when induction of mesoderm occurs (Kimelman and Kirschner, 1987; Isaacs et al., 1992). These findings suggest that FGF might play an important role during mesoderm induction. However, bFGF does not have the signal sequence that is normally required for secretion of proteins from cells. The mechanism involved in the secretion of bFGF remains unknown. By contrast, eFGF does have a secretory signal sequence and this protein has mesoderm-inducing activity (Isaacs et al., 1992). Recent studies showed that the mesoderm-inducing activity of eFGF is at least 100 times stronger than that of bFGF (Isaacs et al., 1994). Thus, eFGF seems to be the best candidate for a mesoderm-inducing

molecule in vivo. It remains unclear whether fibroblast growth factors can function as endogenous factors during ascidian embryogenesis. Verification of the presence of fibroblast growth factors such as bFGF and eFGF in ascidian embryos is important for future discussions of the mechanism of notochord induction. It is also necessary to prove the presence and characterize the distribution of receptors for such growth factors in ascidian embryos.

bFGF cannot induce notochord formation in the presumptive-epidermis blastomeres

In amphibians, animal cap explants (ectoderm) can be induced to form mesodermal tissues when combined with vegetal explants (endoderm). By contrast, previous experiments involving the recombination of blastomeres suggested that, in ascidians, blastomeres of the animal hemisphere do not have the competence to form notochord in response to the inducer blastomeres. To examine the competence of cells of the animal hemisphere to respond to bFGF, animal (presumptive-epidermis) blastomeres (a4.2 and b4.2 cells) isolated from 8-cell embryos were treated with bFGF. The a4.2 and b4.2 partial embryos did not give rise to notochord cells, a result that suggests that the competence for notochord induction might exist only in the notochord precursor cells in ascidians. The cells of the animal hemisphere contain the determinants of epidermis differentiation, so we can assume that these cells cannot be induced to form notochord.

Sensitive period for notochord induction

In the present study, the sensitivity of presumptive-notochord blastomeres to bFGF reached a maximum before the start of the sixth cleavage. The presumptive-notochord blastomeres lost their ability respond to bFGF after the sixth cleavage (Fig. 4). Results of previous experiments involving recombination of blastomeres suggested that notochord induction has to be initiated before the decompaction of blastomeres during the 32-cell stage and that determination of notochord is completed by the 64-cell stage (Nakatani and Nishida, 1994). The relative time designated 30 minutes in Fig. 4 corresponds the time at which decompaction occurs during the 32-cell stage. Although there is some delay in the period when these cells are sensitive to bFGF as compared to cells after recombination of blastomeres, we demonstrated that the induction of notochord occurred before the sixth cleavage. These results are of particular interest with regard to the cell lineage. The presumptive-notochord blastomeres at the 32-cell stage (A6.2 and A6.4 pairs) are developmentally fated to give rise not only to notochord but also to spinal cord. During the next division, these fates are inherited separately by the two daughter blastomeres and, hence, the fate of one set of daughter blastomeres (A7.3 and A7.7 pairs) is restricted to notochord and that of the other (A7.4 and A7.8 pairs) is restricted to spinal cord (Fig. 1I). Thus, the induction of notochord occurs before the restriction of developmental fate and the induction causes one daughter blastomere to develop into notochord.

The direction of inductive influence and choice of cell fates in the daughter cells

In previous studies that involved coisolation and recombination of a presumptive-notochord blastomere with an inducer blastomere, some of the descendant cells of the A6.2 and A6.4 blastomeres differentiated into notochord while the other cells did not. Similarly, in the present study, for example, after coisolation or recombination of an A6.2 (presumptive-notochord) blastomere with an A6.1 (presumptive-endoderm) blastomere, the expression of *As-T* was detected in two out of the four descendant cells of the A6.2 cell (Fig. 7C,F). These observations coincide with the expectation from cell lineage in normal development. By contrast, when the presumptive-notochord blastomeres isolated from 32-cell embryos were treated with bFGF at 0.2 ng/ml, all the descendant cells developed into notochord, as indicated both by morphological features and the expression of Not-1 (Figs 2C,F and 3A,D). Moreover, the expression of *As-T* was observed in all the descendants of the presumptive-notochord blastomeres that had been treated with bFGF (Fig. 7J). Thus, the treatment of isolated blastomeres with bFGF caused all the descendants to adopt notochord fate. From the results of treatment with bFGF, we propose a hypothesis about induction of notochord. During normal embryogenesis, daughter cells closer to the vegetal pole always assume the notochord fate (Fig. 1A,B). Since endoderm and the adjacent precursors to notochord are inducer blastomeres, the inductive influence is received from the vegetal and lateral sides of the presumptive-notochord blastomeres and not from the animal side. Such directed induction would cause the one sibling that is closer to the inducer blastomeres to assume the notochord fate. When a cell is subject to induction by bFGF over the entire cell surface, both daughter cells assume the notochord fate. Therefore, we suggest that the direction from which the inductive signal is received affects the choice of alternative fates of the daughter cells. However, we cannot completely eliminate the possibility that cytoplasmic differences might exist endogenously in the notochord-lineage cells at the 32-cell stage.

As-T expression is induced by bFGF

The expression of transcripts of *As-T* in the notochord precursor cells is mediated by the induction of notochord. Exogenous bFGF can also induce the expression of *As-T*. It is likely that an FGF-like molecule binds to a receptor to activate a signaling cascade that triggers the expression of *As-T*. It seems that there might be a close relationship between the expression of *As-T* and the determined state of the notochord-precursor cells. It is now necessary to examine whether it is only the expression of *As-T* that is required for establishment of the determined state in the notochord-precursor cells.

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REFERENCES

Ariizumi, T., Moriya, N., Uchiyama, H. and Asashima, M. (1991). Concentration-dependent inducing activity of activin A. *Roux's Arch. Dev. Biol.* **200**, 230-233.

Conklin, E. G. (1905). The organization and cell-lineage of the ascidian egg. *J. Acad. Nat. Sci. (Philadelphia)* **13**, 1-119.

Green, J. B. A. and Smith, J. C. (1990a). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* **347**, 391-394.

Green, J. B. A., Howes, G., Symes, K., Cooke, J. and Smith, J. C. (1990b). The biological effects of XTC-MIF: quantitative comparison with *Xenopus* bFGF. *Development* **108**, 173-183.

Herrmann, B. G., Labeit, S., Poustka, A., King, T. R. and Lehrach, H. (1990). Cloning of the*T* gene required in mesoderm formation in the mouse. *Nature* **343**, 617-622.

Issacs, H. V., Tannahill, D. and Slack, J. M. W. (1992). Expression of a novel FGF in the *Xenopus* embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. *Development* **114**, 711-720.

Issacs, H. V., Pownall, M. E. and Slack, J. M. W. (1994). eFGF regulates *Xbra* expression during *Xenopus* gastrulation. *EMBO J.* **13**, 4469-4481.

Kimelman, D. and Kirschner, M. (1987). Synergistic induction of mesoderm by FGF and TGF-ß and the identification of an mRNA coding for FGF in the early Xenopus embryo. *Cell* **51**, 869-877.

Kimelman, D. and Maas, A. (1992). Induction of dorsal and ventral mesoderm by ectopically expressed *Xenopus* basic fibroblast growth factor. *Development* **114**, 261-269.

Kispert, A., Ortner, H., Cooke, J. and Herrmann, B. G. (1995). The chick *Brachyury* gene: developmental expression pattern and response to axial induction by localized activin. *Dev. Biol.* **168**, 406-415.

Makabe, K. W. and Satoh, N. (1989). Temporal expression of myosin heavy chain gene during ascidian embryogenesis. *Dev. Growth Differ.* **31**, 71-77.

Meedel, T. H. (1992). Development of the ascidian embryo: cell fate specification by autonomous and inductive process. In *Morphogenesis* (ed. E. F. Rossomando and S. Alexander), pp. 263-317. New York: Dekker Inc.

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

Nieuwkoop, P. D. (1969). The formation of mesoderm in Urodelean amphibians. I. Induction by the endoderm. *Wilhelm Roux's Arch. Entwicklungsmech. Org.* **162**, 341-373.

Nieuwkoop, P. D. (1973). The 'organization centre' of the amphibian embryo, its origin, spatial organization and morphogenetic action. *Adv. Morphol.* **10**, 1-39.

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. (1992a). Determination of developmental fates of blastomeres in ascidian embryos. *Dev. Growth Differ.* **34**, 253-262.

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

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

Nishikata, T., Mita-Miyazawa, I., Deno, T. and Satoh, N. (1987). Muscle cell differentiation in ascidian embryos analysed with a tissue-specific monoclonal antibody. *Development* **99**, 163-171.

Rashbass, P., Cooke, L. A., Herrmann, B. G. and Beddington, R. S. P. (1991). A cell autonomous function of *Brachyury* in *T/T* embryonic stem cell chimaeras. *Nature* **353**, 348-351.

Satoh, N. (1994). Developmental strategies of solitary ascidians. In *Developmental Biology of Ascidians.* pp. 82-131. New York: Cambridge University Press.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nüsslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse*T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.

Slack, J. M. W., Darlington, B. G., Heath, J. K. and Godsave, S. F. (1987). Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature* **326**, 197-200.

Smith, J. C. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. *Development* **105**, 665-667.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Herrmann, B. G. (1991). Expression of a Xenopus homolog of *Brachyury (T)* is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Sokol, S., Wong, G. G. and Melton, D. A. (1990). A mouse macrophage factor induces head structures and organizes a body axis in *Xenopus. Science* **249**, 561-564.

Venuti, J. M. and Jeffery, W. R. (1989). Cell lineage and determination of cell fate in ascidian embryos. *Int. J. Dev. Biol.* **33**, 197-212.

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

Yasuo, H. and Satoh, N. (1994). An ascidian homolog of the mouse *Brachyury (T)* gene is expressed exclusively in notochord cells at the fate restricted stage. *Dev. Growth Differ.* **36**, 9-18.

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