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The BMP signaling pathway is required together with the FGF pathway for notochord induction in the ascidian embryo

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SUMMARY

The 40 notochord cells of the ascidian tadpole invariably arise from two different lineages: the primary (A-line) and the secondary (B-line) lineages. It has been shown that the primary notochord cells are induced by presumptive endoderm blastomeres between the 24-cell and the 64-cell stage. Signaling through the fibroblast growth factor (FGF) pathway is required for this induction. We have investigated the role of the bone morphogenetic protein (BMP) pathway in ascidian notochord formation. *HrBMPb* (the ascidian *BMP2/4* homologue) is expressed in the anterior endoderm at the 44-cell stage before the completion of notochord induction. The BMP antagonist *Hrchordin* is expressed in a complementary manner in all surrounding blastomeres and appears to be a positive target of the BMP pathway. Unexpectedly, *chordin* overexpression reduced formation of both primary and secondary notochord. Conversely, primary notochord precursors isolated prior to induction formed notochord in presence of BMP-4 protein. While bFGF protein had a similar activity, notochord precursors showed a different time window of competence to respond to BMP-4 and bFGF. Our data are consistent with bFGF acting from the 24-cell stage, while BMP-4 acts during the 44-cell stage. However, active FGF signaling was also required for induction by BMP-4. In the secondary lineage, notochord specification also required two inducing signals: an FGF signal from anterior and posterior endoderm from the 24-cell stage and a BMP signal from anterior endoderm during the 44-cell stage.

Key words: Notochord induction, BMP, Chordin, bFGF, Ascidian embryo, Asymmetric division, *Halocynthia roretzi*

INTRODUCTION

In ascidian embryos, mesoderm formation relies on both inheritance of maternal determinants and cell-cell interactions (Nishida, 1997). Muscle forms essentially in a cell-autonomous manner and the zinc-finger transcription factor *macho-1* is a key maternal regulator (Nishida and Sawada, 2001). In contrast, the other mesodermal tissues require cell-cell interactions. Notochord and mesenchyme formation has been extensively studied. They are both induced by the endoderm precursors between the 24-cell stage and the 64-cell stage (Fig. 1; Nakatani and Nishida, 1994; Kim and Nishida, 1999). In both cases, basic fibroblast growth factor (bFGF) can replace the endogenous endodermal signal (Nakatani et al., 1996; Kim et al., 2000). Moreover, the protein Ras, which acts downstream of FGF, has been shown to be required for notochord formation in vivo (Nakatani and Nishida, 1997). These studies have led to the proposal that endodermal precursors secrete a bFGF-like signal at the 32-cell stage. The type of mesoderm induced depends on the responsiveness of mesoderm precursors. The mesenchyme precursors inherit posterior vegetal cytoplasm (PVC) of the egg and differentiate into mesenchyme when induction occurs, while the notochord precursors, which do not contain the PVC, differentiate into notochord (Kim et al., 2000).

In the ascidian larva, the notochord is composed of 40 cells. The 32 anterior cells derive from the anterior lineage (A-line) and are termed primary notochord. The eight posterior cells compose the secondary notochord or B-line notochord (Nishida, 1987; Fig. 1). Most studies described above have focused on the primary notochord, and formation of the secondary lineage is much less understood. It is known that the secondary notochord formation requires cell-cell interactions, but the identity of the inducing blastomeres and the nature of the inducing molecules are not known (Nakatani and Nishida, 1994; Nakatani et al., 1996).

In the present study, we provide evidence for the role of the bone morphogenetic protein (BMP) pathway in notochord induction. First, overexpression of the BMP antagonist *chordin* prevented notochord development. Second, BMP-4 was able to induce primary notochord formation from isolated notochord precursors. We could also address the temporal requirement for notochord induction by bFGF and BMP-4. Although isolated notochord precursors responded to bFGF during the 32-cell stage, they responded to BMP-4 during the 44-cell stage, i.e. when *HrBMPb* transcripts were first detected in the anterior

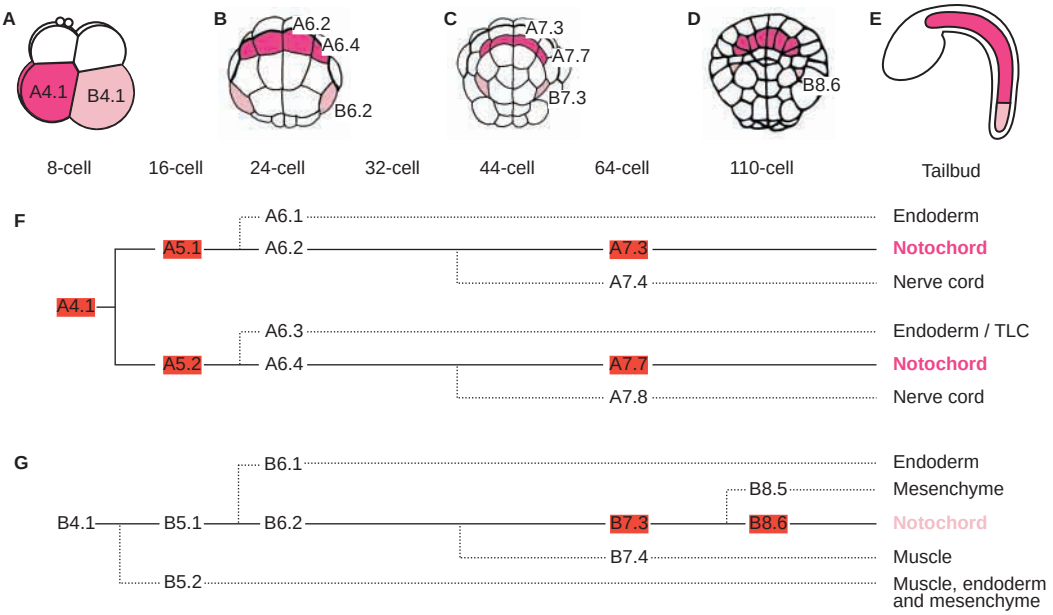

Fig. 1. Schematic representation of the notochord lineage. Primary notochord precursors are indicated in dark pink and secondary notochord precursors in light pink. (A) Eight-cell stage embryo. Lateral view. Anterior is towards the left, animal is upwards. (B) 24-cell stage. (C) 44-cell stage. (D) 110-cell stage. Vegetal view, anterior is upwards in B-D. (E) Mid-tailbud. The 32 anterior cells constitute the primary notochord, while the eight posterior cells form the secondary notochord. (F) Lineage tree of the primary notochord starting from the eight-cell stage. (G) Lineage tree of the secondary notochord. Red rectangles correspond to blastomeres that develop into notochord after isolation.

endoderm. Interestingly, notochord induction by BMP-4 required active FGF signaling. In a similar manner, bFGF and BMP-4 acted sequentially to induce secondary notochord. The inducing blastomeres were identified and enabled us to draw a common scenario for induction of both primary and secondary lineages. From the 24-cell stage to the 44-cell stage, active FGF signaling would be a prerequisite for enabling the BMP pathway to complete notochord induction between the 44-cell stage and the 64-cell stage.

MATERIALS AND METHODS

Animals and embryos

Naturally spawned eggs of the Japanese ascidian *Halocynthia roretzi* were artificially fertilized with a suspension of non-self sperm and raised in Millipore-filtered seawater containing 50 μg/ml streptomycin sulfate and 50 μg/ml kanamycin sulfate at 13°C. Tadpole larvae hatch approximately 35 hours after fertilization. Embryo staging was carried out according to Nishida (Nishida, 1987). After the 16-cell stage, cleavages are asynchronous between vegetal and animal cells, the vegetal cells dividing first. The 24-cell stage corresponds to the completion of the fifth cleavage of vegetal cells, the 32-cell stage to the fifth cleavage of animal cells, the 44-cell stage to the sixth cleavage of vegetal cells, the 64-cell stage to the sixth cleavage of animal cells (Fig. 1). For blastomere isolation, the vitelline membrane was chemically removed during the first cell cycle as previously described (Mita-Miyazawa et al., 1985). Embryos were cultured on 1% agar-coated dishes until the desired stage when blastomeres were isolated with a fine glass needle. Isolated blastomeres were treated with 5 ng/ml recombinant human bFGF protein (Amersham) or 50 ng/ml recombinant human BMP-4 protein (R&D Systems) in seawater containing 0.1% bovine serum albumin (BSA) and cultured separately. As a control, blastomeres were cultured in 0.1% BSA-seawater. Inhibition of cleavage and morphogenesis was carried out using 2 μg/ml cytochalasin B, an inhibitor of actin polymerization, as described by Whittaker (Whittaker, 1973). The FGF signaling pathway was blocked with two specific inhibitors: FGF receptor 1 inhibitor (SU5402) and MEK-1 inhibitor (U0126) (Calbiochem). Both were used at 2 μM, a concentration sufficient to prevent notochord induction.

Injection of synthetic mRNA

mRNA was synthesized according to the manufacturer protocol (mMessage mMachine, Ambion) with T3 polymerase for pBSRN3Hrchordin, pBSRN3HrBMPb and pBSRN3XlnogginΔ3′. Ascidian eggs were injected with mRNAs during the second phase of ooplasmic segregation before the first cleavage (Miya et al., 1997).

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was performed as described previously (Miya et al., 1997) except that the DIG-probes were not hydrolyzed by alkaline treatment. The Not-1 monoclonal antibody specifically recognizes notochord cells at the mid-tailbud stage (Nishikata and Satoh, 1990). The Mu-2 monoclonal antibody recognizes the myosin heavy chain in tail muscle cells of larvae (Nishikata et al., 1987). Samples were fixed in methanol for 10 minutes at –20°C and stained by indirect immunofluorescence with an Alexa 488-conjugated secondary antibody (Molecular Probes).

RESULTS

chordin reduced notochord formation

The palps and sensory pigment cells are affected in embryos injected with *Hrchordin* mRNA (Darras and Nishida, 2001). We provide evidence that *chordin* overexpression reduces notochord formation. We found that a large number of embryos injected with *Hrchordin* mRNA exhibited a shortened tail at tailbud and larval stages (not shown). To determine if this phenotype was caused by a lack of notochord cells, or by a defect in tail elongation, we stained injected embryos fixed at the mid-tailbud stage with the notochord specific Not-1 antibody. In intact embryos, 40 notochord cells were stained

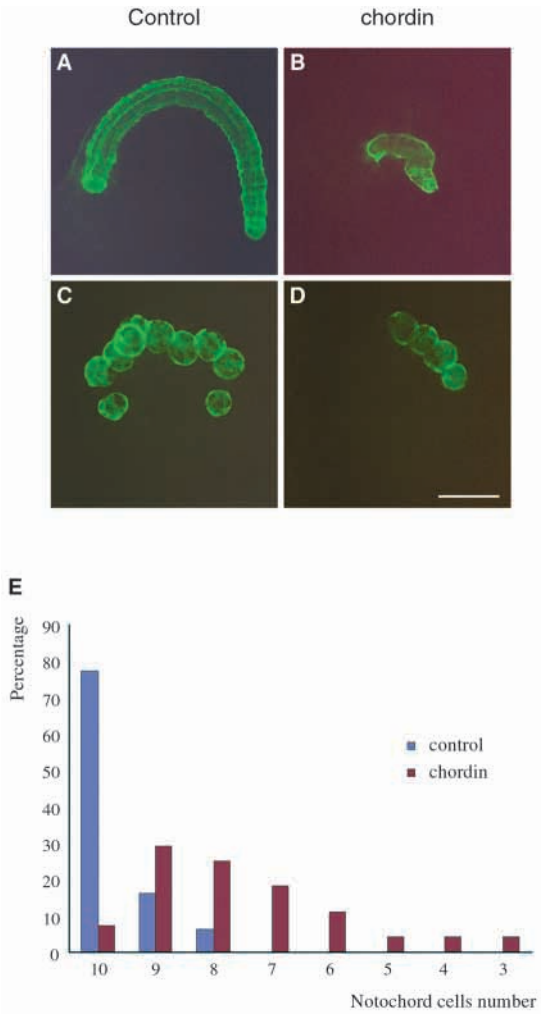

Fig. 2. *chordin* reduces notochord formation. (A-D) Immunostaining for Not-1, a notochord-specific antigen. (A) Control mid-tailbud. 40 cells are stained. (B) 17 cells are stained after injection of 200 pg of *chordin* mRNA. (C) When cleavage was arrested at the 110-cell stage, 10 notochord cells were eventually stained at the mid-tailbud stage. (D) Only four cells are positive after injection of 200 pg of *chordin*. Scale bar: 100 μm. (E) Number of Not-1-positive cells per embryo in cleavage arrested control embryos (blue, total number of embryos examined; *n*=31) and *chordin*-injected embryos (purple, *n*=28).

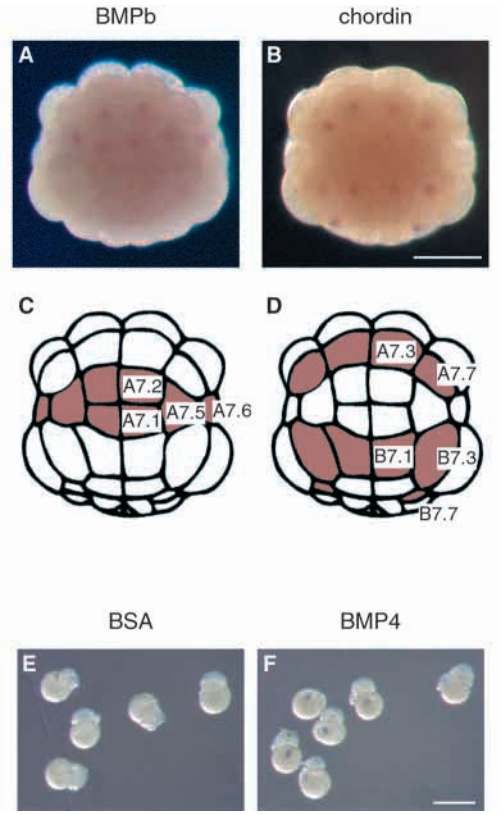

Fig. 3. Expression pattern and its schematic representation of *BMPb* (A,C) and *chordin* (B,D) at the 64-cell stage. (E) When A6.2/A6.4 were isolated at the 24-cell stage, they did not express *chordin* at the 64-cell stage. (F) BMP-4 induced *chordin* expression in A6.2/A6.4 isolated blastomeres. Scale bar: 100 μm.

(Fig. 2A). However, fewer cells were stained in injected embryos (Fig. 2B), ranging from 14 to 35 cells per embryo (150 pg of mRNA: 54% of embryos had reduced number of notochord cells, *n*=26; 200 pg: 63%, *n*=15). This phenotype was never observed in embryos injected with *lacZ* mRNA (*n*=99). In order to better visualize the effect of *chordin* injection, embryos were cleavage arrested by treatment with cytochalasin B from the 110-cell stage (beginning of gastrulation). In normal embryos, 10 blastomeres are fated to form notochord at the 110-cell stage (Fig. 1D). When embryos were cleavage-arrested at the 110-cell stage and cultured until the mid-tailbud stage, the same 10 blastomeres expressed Not-1 antigen in most cases, as reported by Nishikata and Satoh (Nishikata and Satoh, 1990; Fig. 2C,E; *n*=31). In *chordin*-injected embryos the number of positive cells was reduced (Fig. 2D,E; *n*=28). This partial effect on notochord formation is probably due to incomplete partitioning of injected mRNA, as *lacZ* mRNA injection into eggs often resulted in partial staining of embryos at latter stages. These results were confirmed by examining the earlier marker *As-T*, the ascidian homolog of *Brachyury*, which is expressed exclusively in notochord precursors after the 64-cell stage, and is sufficient for triggering notochord differentiation (Yasuo and Satoh, 1993; Yasuo and Satoh, 1994; Yasuo and Satoh, 1998). In intact embryos, 10 notochord precursors expressed *As-T* at the 110-cell stage, while the number of *As-T* positive cells was reduced in *chordin*-injected embryos in the same proportion as for Not-1 (79% of embryos with a partial loss of *As-T* staining, *n*=14, 200 pg of mRNA, data not shown). Similar results were obtained upon overexpression of *Xenopus noggin*, which encodes another BMP antagonist structurally unrelated to Chordin (Smith and Harland, 1992). *noggin*-injected embryos developed a shortened tail, with a reduced number of notochord cells (data not shown). These results strongly suggest that *chordin* or *noggin* overexpression reduces notochord formation, and consequently that a secreted BMP is required for notochord specification.

To elucidate the role of BMP signaling in notochord induction, we examined the expression of *HrBMPb* (the ascidian *BMP2/4* homolog; Miya et al., 1997) and *Hrchordin* at the early cleavage stages. We first detected *BMPb* expression at the 44-cell in vegetal blastomeres (not shown). The pattern of expression remains unchanged at the 64-cell stage as vegetal

Table 1. Induction of A-line primary notochord by BMP-4

Isolated blastomere	Treatment	*n*	Not-1
(A) Isolation at the 24-cell stage			
A6.2/A6.4	BSA alone	77	5%
	BMP-4	132	89%
	BMP-4 + U0126	60	2%
	BMP-4 + SU5402	31	13%
A6.2/A6.4	BSA alone	29	31%
	BMP-4 up to the 44-cell stage	26	35%
	BMP-4 from the 44-cell stage	30	90%
(B) Isolation at the 44-cell stage			
A7.3/A7.7	BSA alone	43	5%
presumptive	BMP-4	17	94%
notochord	BMP-4 up to the 68-cell stage	30	80%
	BMP-4 + U0126 up to the 68-cell stage	13	0%
	bFGF up to the 68-cell stage	21	81%
A7.4/A7.8	BSA alone	51	0%
presumptive	BMP-4 up to the 68-cell stage	34	0%
nerve cord	bFGF up to the 68-cell stage	46	0%
	BMP-4 + bFGF up to the 68-cell stage	29	3%

blastomeres do not divide. *BMPb* was expressed in anterior endoderm precursors (A7.1, A7.2 and A7.5 blastomeres) and trunk lateral cells (TLC) precursors (A7.6 blastomere) (Fig. 3A,C). We also detected *chordin* transcripts first at the 44-cell stage, and in the same blastomeres at the 64-cell stage, A7.3 and A7.7 blastomeres (primary notochord precursors), B7.3 and B7.7 blastomeres (secondary notochord and mesenchyme precursors), and B7.1 blastomere (posterior endoderm; Fig. 3B,D). The expression of either gene was not detected at the 32-cell stage or earlier. Thus, expression of *BMPb* and *chordin* starts at the 44-cell stage, when notochord induction is not yet completed (see below), in complementary domains with *chordin* transcripts being found around *BMPb*-expressing cells. Next, we tested the possibility that *chordin* expression could depend on BMP activity. When A6.2 and A6.4 blastomeres (primary notochord precursors; Fig. 1B,F) were isolated at the 24-cell stage and cultured up to the 64-cell stage, they rarely expressed *chordin* (9%, *n*=23; Fig. 3E). By contrast, treatment of the isolated blastomeres with BMP-4 protein induced robust *chordin* expression (95%, *n*=21; Fig. 3F). Interestingly, our results suggest that BMP signaling activates the expression of its own antagonist *chordin*.

Induction of primary notochord by BMP-4

The results described in the previous section suggested that BMP is required for notochord formation. We then examined whether BMP could induce ectopic notochord. We injected mRNA encoding BMPb and analyzed notochord formation with the Not-1 antibody. Even at high doses (100 pg of mRNA), there was no ectopic notochord formation (not shown). We therefore asked whether BMP could induce isolated notochord precursors to form notochord (Table 1A; Fig. 4). Notochord induction takes place between the 24-cell stage and the 64-cell stage. Primary notochord precursors isolated at the 24-cell stage do not form notochord, while precursors isolated at the 64-cell stage form notochord autonomously (Fig. 1F; Nakatani and Nishida, 1994). At the 24-cell stage there are two pairs of blastomeres fated to form primary notochord, A6.2 and A6.4 (Fig. 1B,F). We isolated A6.2 and A6.4 blastomeres and treated them with BMP-4 protein at the 24-cell stage. After treatment with BSA seawater,

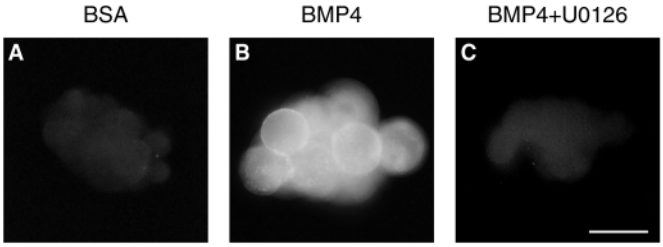

Fig. 4. BMP-4 induced notochord in the primary lineage. Immunofluorescence for Not-1 antigen. (A) Notochord precursors isolated at the 24-cell stage did not form notochord. (B) Treatment with BMP-4 induced notochord formation. (C) U0126 blocked notochord induction by BMP-4. Scale bar: 50 μm.

they did not express the Not-1 antigen, as observed by Nakatani and Nishida (Nakatani and Nishida, 1994; Fig. 4A). By contrast, when the isolates were treated with BMP-4 protein, they developed into notochord (Fig. 4B). In most cases, all cells within a partial embryo were positive for Not-1 staining. Because A6.2 and A6.4 blastomeres are fated to form both notochord and nerve cord (Fig. 1F), this observation suggested that nerve cord precursor also assumed a notochord fate in presence of BMP-4. As we found that A6.2 and A6.4 blastomeres showed an identical response, we did not discriminate between these blastomeres in Table 1 and in the following experiments.

We then determined the period that was sensitive to BMP-4 treatment (Table 1A; Fig. 5). When A6.2 and A6.4 are isolated and treated with BMP-4 from the 24-cell stage to the next division (equivalent of the 44-cell stage) notochord formation was not observed in most cases. By contrast, notochord formation was induced when BMP-4 was added to A6.2/A6.4 blastomeres after their first cleavage in culture. Thus, the competence of isolated blastomeres to respond to BMP-4 appears when *BMPb* expression becomes detectable in the anterior endoderm of whole embryos (Fig. 3A,C).

At the 44-cell stage, notochord and nerve cord lineages separate and become fate restricted. A6.2 and A6.4 divide into A7.3 + A7.4 and A7.7 + A7.8, respectively (Fig. 1F). When notochord precursors (A7.3 and A7.7) were isolated at the 44-cell stage, they did not develop into notochord, indicating that notochord induction is not yet completed at the 44-cell stage (Table 1B; Minokawa et al., 2001). When the same blastomeres are isolated at the 64-cell stage (about 50 minutes later), they autonomously differentiate into notochord as previously reported (Nakatani and Nishida, 1994). The notochord-inducing signals emitted during this period could be replaced by BMP-4 protein treatment (Table 1B; Fig. 5). However, the nerve cord precursors (A7.4 and A7.8 blastomeres) isolated at the 44-cell stage did not differentiate into notochord, regardless of the presence or not of BMP-4 protein (Table 1B).

Primary notochord induction by BMP-4 requires FGF signaling

bFGF is also a potent primary notochord inducer (Nakatani et al., 1996). The sensitive period to bFGF ends when isolated notochord precursors divide at the 44-cell stage (Nakatani et al., 1996). Therefore, notochord precursors have different sensitive periods to bFGF and BMP-4 treatment. We examined the relationship of these two signaling pathways with drugs

that specifically block the FGF pathway: inhibitor of FGF receptor 1 (SU5402) and inhibitor of MEK-1 (U0126). These inhibitors blocked notochord formation when applied to whole embryos from the 24-cell stage to the 64-cell stage as revealed by the loss of *As-T* expression (SU5402: 3%, *n*=62; U0126: 0%, *n*=54) and Not-1 staining (SU5402: 0%, *n*=22; U0126: 9%, *n*=22; in G. J. Kim and H. N., unpublished). Then we isolated A6.2/A6.4 blastomeres and treated them with BMP-4 in the presence of SU5402 or U0126. In both cases, notochord induction by BMP-4 was suppressed (Figs 4C, 5; Table 1A). Similarly in A7.3/A7.7 notochord precursors isolated at the 44-cell stage, inhibition of the FGF pathway prevented induction of notochord by BMP-4 (Fig. 5; Table 1B). Thus, induction of notochord by BMP-4 required a functional FGF signaling pathway.

An asymmetric division separates the notochord and nerve cord lineages

We examined the competence of notochord and nerve cord precursors to bFGF treatment at the 44-cell stage. Unexpectedly, bFGF treatment induced the notochord precursors isolated at the 44-cell stage to form notochord (Table 1B; Fig. 5). However, the mother cells of these blastomeres that are isolated at the 24-cell stage and cultured in isolation do not respond to bFGF after the 44-cell stage (Nakatani et al., 1996). Nerve cord precursors isolated at the 44-cell stage never formed notochord after treatment with bFGF, BMP-4 or a combination of both. When a notochord/nerve cord precursor was isolated at the 24-cell stage and treated immediately with either BMP-4 or bFGF, all cells within a partial embryo differentiated into notochord (present study and Nakatani et al., 1996). However, when the daughters of this blastomere were isolated after their division at the 44-cell stage, only the notochord precursor was competent to form notochord. These results suggest that the competence to form notochord is restricted to only one of the two daughter cells (the notochord precursors) through an asymmetric division.

The combination of bFGF and BMP-4 promotes B-line notochord induction

chordin overexpression reduced the formation of both primary (A-line) and secondary notochord (B-line). Therefore, we investigated the role of the BMP pathway in secondary notochord formation, as its mechanism of induction is poorly understood. We first tested if BMP could be a B-line notochord inducer. Contrary to A4.1 blastomeres cultured alone, when B4.1 blastomeres were isolated at the eight-cell stage notochord formation did not occur (Table 2; Nakatani and Nishida, 1994). Treatment of B4.1 with BMP-4 induced notochord formation. A similar result was obtained with B5.1 isolates, taken from 16-cell stage embryos. In contrast, BMP-4 was unable to induce notochord in B6.2 blastomeres isolated at the 24-cell stage (Fig. 6B,F; Table 2).

The 24-cell stage corresponds to the time when endoderm and mesoderm fates separate (Fig. 1G). B6.2 blastomere is fated to form primary muscle, mesenchyme and secondary notochord. All descendant cells of a B6.2 partial embryo develop into muscle, probably because of the inheritance of maternal muscle determinants. Endoderm is known to induce mesenchyme fate in the B6.2 blastomere by repressing muscle formation (Kim and Nishida, 1999). The endodermal mesenchyme-inducing signal can be substituted for with bFGF (Kim et al., 2000). Thus, we postulated that the inability of B6.2 to respond to BMP-4 could be attributed to the absence of posterior endoderm (B6.1 blastomere; Fig. 1G). To test this hypothesis, we tried to substitute the endoderm signal with bFGF, and treated B6.2 blastomeres with bFGF, BMP-4 or a combination of both. We confirmed that when B6.2 is isolated and cultured in isolation, all cells form muscle (97%, *n*=29). After treatment with bFGF alone, notochord formation did not occur (Fig. 6C,G, Table 2; Nakatani et al., 1996), but all cells were converted into mesenchyme (recognized by their morphology in Fig. 6C) as reported by Kim et al. (Kim et al., 2000). BMP-4 treatment did not induce B6.2 blastomeres to form notochord either. When treated with bFGF and BMP-4 together, notochord was induced in 61% of the cases (Fig. 6D,H; Table 2). Treatment of B6.2 first with bFGF up to the 44-cell stage, and then with BMP-4 also resulted in notochord formation in 70% of the cases (Table 2).

B7.3 blastomeres isolated at the 44-cell stage did not form notochord (Table 3), while they autonomously formed notochord when isolated at the 64-cell stage (Table 3; Nakatani and Nishida, 1994). Thus, signals might be emitted between the 44-cell and the 64-cell stages to complete notochord induction. BMP-4 alone can replace these signals because it induced notochord in B7.3 blastomeres isolated at the 44-cell stage (Table 3), probably because B7.3 already received an FGF-like endodermal signal during the 32-cell stage.

B-line notochord fate was correlated with the unequal cleavage of B7.3 that normally occurs between the 64-cell and the 110-cell stages to produce a large B8.5 mesenchyme precursor and a small B8.6 blastomere that gives rise to four notochord cells (Fig. 1C,D,G). This unequal cleavage occurred when B7.3 was isolated at the 64-cell stage after induction (Fig. 6K; Table 3) The ratio between volumes of larger and smaller blastomeres was 3.1 (*n*=3). By contrast, the cleavage was equal when B7.3 was isolated at the 44-cell stage (Fig. 6I; Table 3;

Table 2. Induction of B-line secondary notochord by a combination of bFGF and BMP-4

Isolated blastomere	Treatment	*n*	Not-1
B4.1	BSA alone	44	9%
B4.1	BMP-4	45	75%
B5.1	BSA alone	24	4%
B5.1	BMP-4	22	45%
B6.2	BSA alone	33	0%
B6.2	BMP-4	49	4%
B6.2	bFGF	13	15%
B6.2	BMP-4 + bFGF	41	61%
B6.2	bFGF up to the 44-cell stage, then BMP-4	20	70%

Table 3. Induction of B-line secondary notochord by BMP-4

Isolated blastomere	*n*	Unequal cleavage	*n*	Not-1	Four cells
B7.3 at the 44-cell stage	67	9%	51	0%	-
B7.3 at the 44-cell stage + BMP-4	52	60%	74	62%	49%
B7.3 at the 64-cell stage	50	96%	45	89%	87%

ratio=1.2, *n*=3). BMP-4 treatment triggered such an unequal cleavage of B7.3 isolated at the 44-cell stage (Fig. 6J; Table 3; ratio=1.9, *n*=3), although it was not as pronounced as the unequal cleavage seen after isolation at the 64-cell stage. The frequency of unequal cleavages correlated with the frequency of notochord formation (Table 3). It is likely that this unequal cleavage is of biological significance. B7.3 blastomere gives rise to four notochord cells during normal development (Fig. 1). In most cases we observed formation of four Not-1-positive cells in the partial embryos as shown in Fig. 6H (B6.2 + bFGF + BMP-4: 46% of the positive partial embryos with four cells, 20% with more than 4 cells; B7.3 + BMP-4: 49% with four cells, 20% with more than 4 cells; B7.3 isolated at the 64-cell stage: 87% with four cells, 0% with more than 4 cells; Table 3). Taken together, our results suggest that B-line notochord is induced by two signals (FGF and BMP) that are temporally separated.

B-line notochord is induced by two signals spatially separated

We tried to see if we could attribute each signal to a defined blastomere neighboring the secondary notochord precursor. At the 24-cell stage, the B6.2 blastomere is surrounded by precursors of anterior endoderm/TLC (A6.3), posterior endoderm (B6.1), muscle/mesenchyme (B6.4) and ectoderm (b5.3) (Fig. 7A). None of the possible co-isolations of two blastomeres (B6.2+B6.1, B6.2+A6.3) resulted in notochord formation (Fig. 7A; Nakatani and Nishida, 1994). However, co-isolation of the three blastomeres (B6.2+B6.1+A6.3) promoted notochord differentiation. This induction was unlikely to be due to a mass effect, as isolations of three blastomeres or more, excluding the A6.3 blastomeres, did not promote notochord formation (B6.2+B6.1+b5.3, B6.2+B6.1+B6.3+B6.4, B6.2+B6.1+B6.3+B6.4+b5.3+b5.4) (Fig. 7A). The signal from the A6.3 blastomere could be substituted by BMP-4 and the signal from B6.1 by bFGF, although with a weaker efficiency (Fig. 7A). We then performed the same type of co-isolations at the 44-cell stage (Fig. 7B). We found that, at this stage, the posterior endoderm precursors were no longer required. The co-isolation of B7.3 notochord precursor with either of the A6.3 descendants (B7.3+A7.5, B7.3+A7.6)

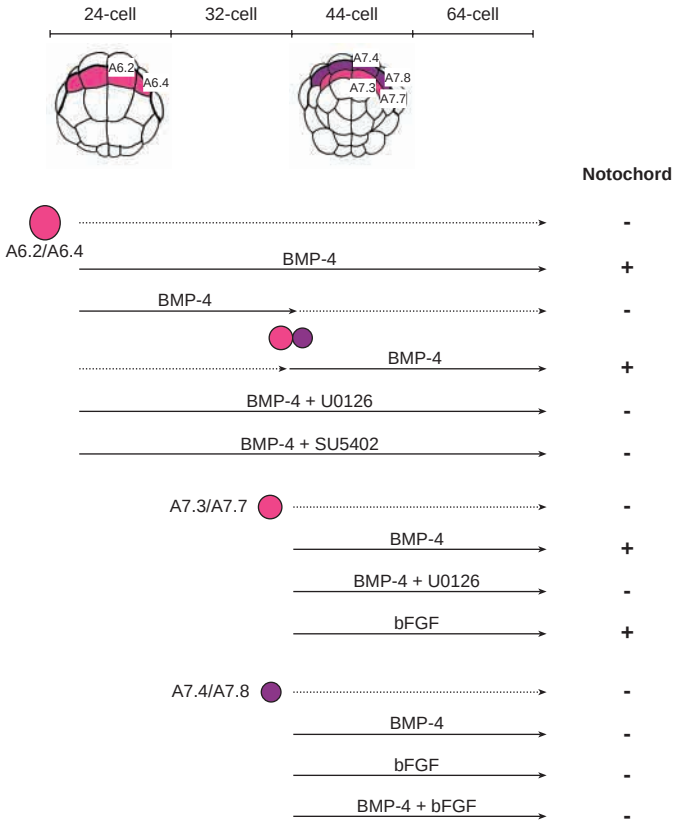

Fig. 5. Treatment of isolated primary notochord precursors with BMP, FGF, U0126 (MEK inhibitor) and SU5402 (FGFR1 inhibitor) starting from 24- and 44-cell stages. The formation of notochord evaluated by immunostaining for Not-1 antigen is indicated on the right-hand side.

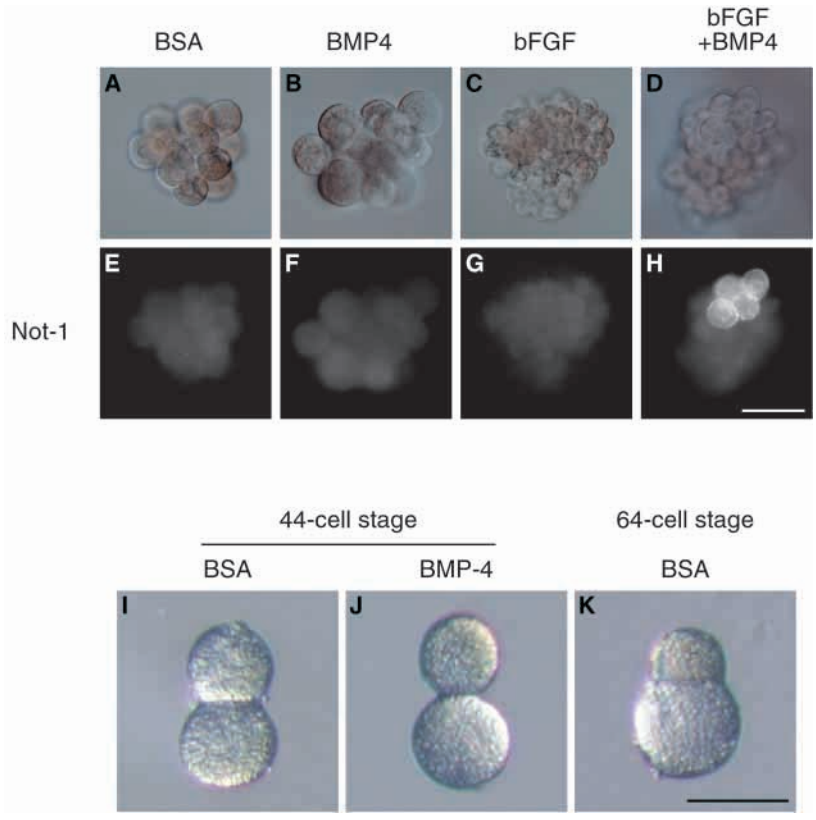

Fig. 6. B-line secondary notochord induction.
(A-H) bFGF and BMP-4 cooperate to induce B-line notochord. (A-D) Bright field view.
(E-H) Immunofluorescence for Not-1 antigen.
(A,E) BSA-treated control B6.2 blastomere. Upon this treatment, all constituent cells develop into large muscle cells (Kim and Nishida, 1999). (B,F) BMP-4 protein treatment. (C,G) bFGF protein treatment. Upon this treatment, all constituent cells develop into small mesenchyme cells (Kim et al., 2000).
(D,H) Simultaneous treatment with bFGF and BMP-4 proteins. Four notochord cells were induced in the partial embryo. (I-K) Unequal cleavage of B7.3. When B7.3 was isolated at the 44-cell stage, the cleavage was equal and produced daughter cells of similar size (I). Treatment with BMP-4 protein provoked a slightly unequal cleavage (J), similar to that observed when B7.3 was isolated at the late 64-cell stage (K). Scale bars: 50 μm.

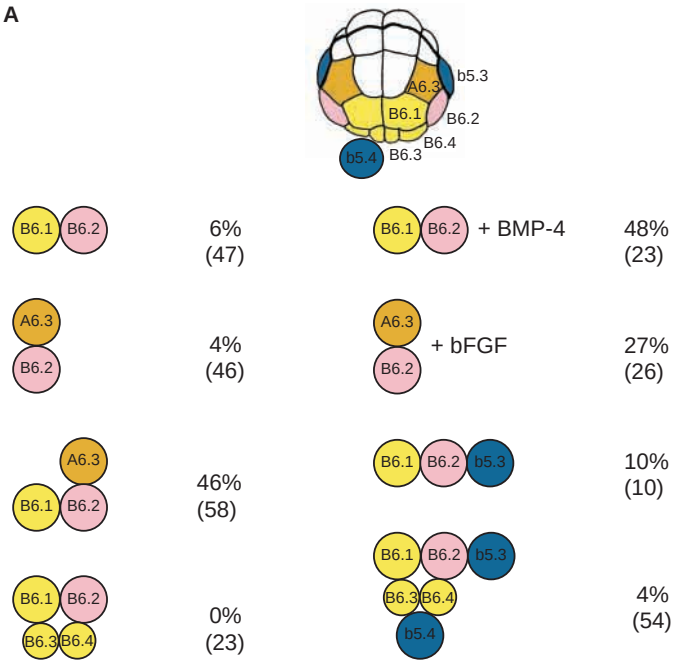

Fig. 7. Co-isolation experiments. Schematic representation of the B-line notochord precursor and its neighbors at the 24-cell stage (A) and at the 44-cell stage (B) with vegetal views of whole embryos at the top. The b5.4 presumptive epidermis blastomere indicated outside diagram of the top figure is a cell in the animal hemisphere. Each drawing represents a given type of co-isolation experiment. The occurrence of notochord formation is shown as percentage of resulting partial embryos positive for Not-1 antigen. The number of partial embryos is indicated in parentheses.

or with both daughter cells (B7.3+A7.5+A7.6) was sufficient to induce notochord formation, although the A7.6 blastomere appeared to be more efficient (Fig. 7B). The results of these co-isolation experiments are in agreement with those of bFGF and BMP-4 treatment experiments, and allow us to propose the following model. B-line notochord is induced by two signals. The first signal, which emanates from the posterior endoderm precursor (B6.1), acts from the 24-cell stage to the 44-cell stage and can be substituted by bFGF. The second signal, which emanates from the anterior endoderm and TLC precursors (A7.5 and A7.6), acts from the 44-cell stage and can be substituted by BMP-4.

DISCUSSION

A common mechanism for A-line and B-line notochord formation

Notochord precursors do not differentiate into notochord when they are isolated before induction at the 24-cell stage (Nakatani and Nishida, 1994). In this study, we showed that BMP-4 is able to induce notochord precursors to adopt a notochord fate. bFGF has previously been shown to bear the same inducing activity (Nakatani et al., 1996), although no FGF has been cloned to date in ascidians. However, our study reveals differential temporal requirement for FGF and BMP in notochord precursors. If A-line precursors are isolated at the 24-cell stage, treatment with bFGF up to the next cleavage (44-

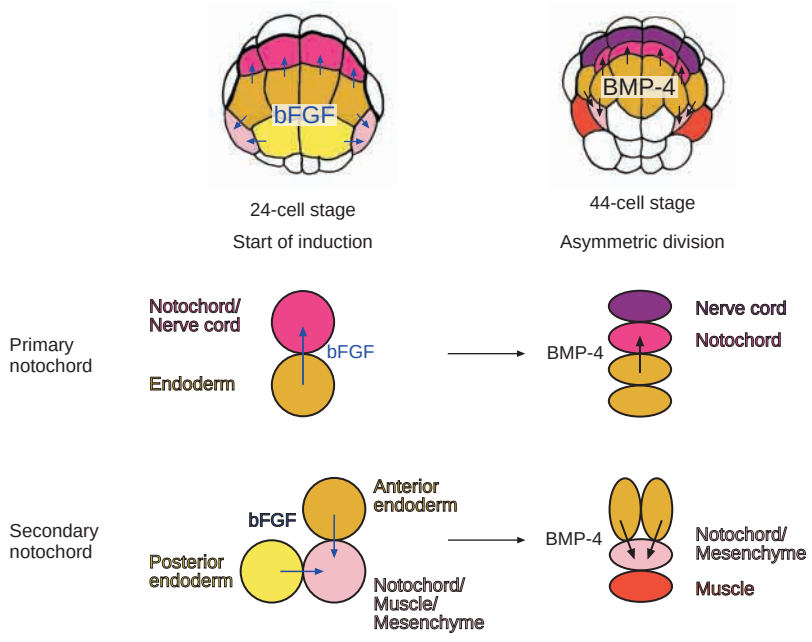

Fig. 8. Model for notochord induction in ascidian embryos. At the 24-cell stage, a bFGF-like signal is secreted from all endodermal precursors. Then at the 44-cell stage, *BMPb* is expressed in the anterior endoderm and completes the induction in combination with bFGF-like signal.

cell stage) is sufficient to trigger notochord differentiation (Nakatani et al., 1996). Although BMP-4 was not sufficient during this time window, it was potent after the 44-cell stage. The same temporal requirement was observed for B-line notochord precursors. Moreover, BMP-4 alone was able to induce both A-line and B-line notochord precursors isolated at the 44-cell stage. These results suggest that these two signaling molecules, secreted from the endoderm, have a different temporal action during embryogenesis. From the 24-cell stage, all endoderm precursors seem to secrete a bFGF-like signal (Kim et al., 2000). Then, *BMPb*, expressed in the anterior endoderm (Fig. 3), acts from the 44-cell stage to the 64-cell stage when primary and secondary notochord precursors are specified (Fig. 8). Therefore, it appears that both A-line and B-line notochord share common mechanisms of induction, contrary to what was thought previously. These similarities are supported by the analysis of the *Brachyury* promoter in the two ascidian species *Halocynthia roretzi* and *Ciona intestinalis*, as no separate regulatory element for the A-line or the B-line notochord was found. Minimal promoters in both species could drive expression of reporter genes in both lineages (Corbo et al., 1997; Takahashi et al., 1999).

The relationship between BMP and FGF pathways in notochord formation

In this study, we showed that the BMP pathway is required for notochord induction, by blocking its activity with Chordin and Noggin. Previous studies have demonstrated that Ras, which is thought to transduce FGF signal, is also required (Nakatani and Nishida, 1997). Therefore, in normal embryos, both FGF and BMP are required to complete notochord induction, although treatment of isolated precursors with either FGF or BMP, probably at higher dose than in normal embryos, is sufficient for notochord induction. Here, we propose that two distinct steps are involved in notochord induction, as shown in Fig. 8.

First step: acquisition of competence

The first step occurs at the 32-cell stage. We showed that, in B-line notochord, BMP-4 could not induce notochord if the presumptive notochord blastomere has not received a bFGF-like signal first (Figs 6, 7). However, at the 44-cell stage, BMP-4 alone or anterior endoderm is sufficient to induce B7.3 to form notochord (Table 3; Fig. 7). In the primary notochord, we did not address this question directly. However, the ability of A-line notochord precursors to induce each other (Nakatani and Nishida, 1994) suggests that they possess an autonomous FGF secretion. Thus, we propose that a bFGF-like signal is required during the 32-cell stage to make notochord precursors competent to respond to BMP-4. Moreover, in the primary lineage at the 44-cell stage, the competence to form notochord is restricted only to notochord precursors through an asymmetric division that separates notochord and nerve cord lineages. FGF-like molecules secreted from endoderm blastomeres that are located next to the notochord precursors are likely to control this asymmetric division. Owing to this restriction of competence at the 44-cell stage, the secreted molecules that complete notochord induction should be considered as permissive signals.

Second step: completion of induction

At the 44-cell stage, treatment with BMP-4 alone was sufficient to induce notochord in both A-line and B-line precursors that are not yet specified. This indicates the involvement of BMP in the second step. However, blocking the FGF pathway blocks this effect at least in the primary lineage. Several explanations are possible (as follows).

The requirement of the FGF signaling at the 44-cell stage, as shown by the inhibition of BMP-4 action by U0126, suggests that MEK activity is still needed at the 44-cell stage. One possibility is that it takes some time to complete intracellular signal after FGF signal has been received at the cell surface during the 32-cell stage. Another possibility might be that secreted FGF molecules act at the cell surface during the 44-cell stage. This might be explained by the presence of a certain amount of FGF molecules on the notochord precursor surface, as FGF protein is known to bind to the cell surface heparan sulfate proteoglycans (Ornitz, 2000). The bound FGF might not be sufficient to complete notochord induction, but might be required at the 44-cell stage when BMP acts. An additive effect of FGF and BMP would then be sufficient to promote notochord induction (Fig. 8). Alternatively, BMP-4 might promote the immediate autocrine secretion of FGF from the notochord blastomere at the 44-cell stage, and this secreted FGF might achieve notochord specification.

The canonical BMP pathway involves activation of Smad proteins through the serine-threonine kinase activity of BMP receptors (for a review, see Kretschmar and Massagué, 1998). However, there is evidence for a crosstalk between the Ras/MAPK pathway and the transforming growth factor β (TGFβ) pathway. Smad activity has been shown to be regulated via phosphorylation by protein kinases downstream of MEK-1 (de Caestecker et al., 1998; Massague and Chen, 2000 and references therein). It has also been shown that TGFβ action could be transduced through the Ras/MAPK pathway (reviewed by Mulder, 2000). Therefore, it is possible that, in our case, the BMP and FGF pathways also converge to the regulation of the signaling mediators of the BMP pathway, namely Smad 1,5 or 8, although there is no direct evidence for this in ascidian embryos.

Comparison with vertebrate mesoderm formation

In vertebrate embryos, FGF and TGFβ superfamily growth factors have been shown to be required for mesoderm formation. Both types of molecules can induce mesoderm in vitro (Slack, 1994), but the inducing signals emitted from the endoderm are likely to be TGFβ of the activin/Nodal subfamilies (Kimelman and Griffin, 2000). However, the actual nature of inducers, especially notochord inducers, is not known. In ascidians, while bFGF is a potent notochord inducer, activin is not (Nakatani et al., 1996). Here, we have shown that both BMP and FGF are required for notochord formation, and we would like to compare this observation with mesoderm formation in vertebrates.

In vertebrates, an early role for FGF concerns the competence to respond to TGFβ-like signals (Cornell et al., 1995). As we described, a similar situation may be found in ascidians, although a main divergence is observed: BMP rather than activin/Nodal would act as a notochord inducer. Moreover, in the case of A-line notochord induction, while a 10 minute pulse of bFGF is sufficient during the 32-cell stage to trigger notochord differentiation (Nakatani et al., 1996),

FGF signal during the same period does not appear to be sufficient in vivo, as blastomeres isolated at the 44-cell stage (about 60 minutes after the beginning of the 24-cell stage) did not form notochord. We propose that the early action of FGF, during the 32-cell stage, is to make cells competent to respond to BMP, similar to the early role of FGF in *Xenopus*.

In *Xenopus*, *eFGF* acts within a positive regulatory loop with *Brachyury*, which is necessary for notochord formation during gastrulation (Schulte-Merker and Smith, 1995; Casey et al., 1998). In ascidian, notochord precursors are determined between the 44-cell and the 64-cell stage, as they autonomously differentiate into notochord when they are isolated about 20-30 minutes after the beginning of the 44-cell stage. This timing coincides precisely with the initiation of *As-T* expression revealed by in situ hybridization (not shown). Moreover, blocking FGF signaling from this stage no longer inhibits notochord formation (G. J. Kim and H. N., unpublished). These observations suggest that a positive feedback loop between *FGF* and *As-T* is unlikely to take place in ascidians (see also Kim et al., 2000).

In *Xenopus* embryos, *chordin* has been shown to induce a secondary axis while *BMP-2* or *BMP-4* overexpression suppresses axis and notochord formation (Sasai et al., 1994; reviewed by Dale and Jones, 1999). Loss-of-function experiments in mouse and zebrafish show that blocking BMP pathway is necessary for proper notochord formation, however, the notochord induction by itself is not affected in embryos mutant for BMP antagonists (Hammerschmidt et al., 1996; Schulte-Merker et al., 1997; Bachiller et al., 2000). We were surprised to observe that the effect of Chordin and BMP in ascidian appeared to be opposite to their effect in vertebrates. In mesoderm of vertebrate embryos, *BMP-2* and *BMP-4* are involved in dorsoventral patterning through a gradient of activity (Dosch et al., 1997) but not in mesoderm formation itself. In contrast, mesoderm patterning in ascidians, i.e. the definition of major mesodermal tissues that are notochord, mesenchyme and muscle, is mainly controlled by localized cytoplasmic factors (Kim et al., 2000). The secreted growth factors then act rather as permissive than instructive factors.

chordin as a positive target of the BMP pathway

chordin expression was initiated at the 44-cell stage in all vegetal blastomeres surrounding the anterior endoderm expressing *BMPb*. We showed that *chordin* might be a positive target of the BMP pathway in the A-line notochord precursors (Fig. 3). Therefore, it appears that the BMP pathway activates its own antagonist. It has been proposed that *noggin* and other inhibitors of the BMP pathway are involved in negative feedback regulatory loops (reviewed by Massagué and Chen, 2000). One could consider that *chordin* activation is important for restricting the range of BMPb action, for example, by preventing the nerve cord precursors from receiving the BMP signal and forming notochord. But this hypothesis is unlikely, as we have shown that nerve cord precursors are no longer competent to respond to BMP-4 in isolation at the 44-cell stage. Chordin binds directly to BMP protein (Piccolo et al., 1996) and consequently, it might prevent BMP from diffusing away, creating a high concentration of BMP close to the Chordin-secreting cells. Therefore, in ascidians, *chordin* might be involved in generating the highest BMPb concentration close to the notochord precursors. The significance of *chordin* expression during this period of development will require further experiments.

Unequal cleavage of B7.3 blastomere

As mentioned above, the primary and secondary notochord inductions share common mechanisms. But some differences were also noted. First, the precursor of primary notochord isolated at the 24-cell stage could be induced to form notochord cells by treatment with either FGF or BMP. In contrast, similar treatment of the precursor of secondary notochord isolated at the 24-cell stage did not result in notochord formation. Second, B7.3 blastomere at the 64-cell stage, which is when this cell acquires cell autonomy to form notochord, is still fated to form mesenchyme and secondary notochord. The next division separates these two fates so that B8.6 (smaller daughter cell; Fig, 1C,D) is restricted to form notochord.

B7.3 blastomeres isolated at the 44-cell stage divided equally and never formed notochord. When these blastomeres were treated with BMP-4 or isolated at the 64-cell stage, they divided unequally and formed four notochord cells in most cases. The unequal cleavage of B7.3 is likely to be an intrinsic property of this cell when it receives an extracellular signal; because it is difficult to imagine how BMP-4 protein, dissolved in seawater and thus present all around the cell surface, could behave as an asymmetric clue to control an unequal cleavage. BMP should be considered a permissive signal for unequal cleavage, rather than an instructive molecule that polarizes the cell. Therefore, BMP signaling might not be the sole mechanism controlling notochord specification and unequal cleavage of B7.3 blastomere in this case; something else is also involved.

In this study, we have described the involvement of the FGF and the BMP pathways in ascidian notochord induction. *chordin* overexpression reduced formation of both primary and secondary notochord, indicating that BMP signaling is required for induction of notochord. Two distinct steps in notochord induction can be distinguished. During the 32-cell stage, FGF would act as a competence factor to allow BMP to complete notochord induction from the 44-cell stage. This induction by BMP, however, requires active FGF signaling. By comparison with vertebrates embryogenesis, these results may appear surprising. While the BMP pathway is undoubtedly involved in ventralization of the early vertebrate embryo, we would like to speculate that it might also have a separate function in notochord formation. The present study may open new perspectives in understanding vertebrates mesoderm formation.

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