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Suppression of macho-1-directed muscle fate by FGF and BMP is required for formation of posterior endoderm in ascidian embryos

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SUMMARY

Specification of germ layers is a crucial event in early embryogenesis. In embryos of the ascidian, *Halocynthia roretzi*, endoderm cells originate from two distinct lineages in the vegetal hemisphere. Cell dissociation experiments suggest that cell interactions are required for posterior endoderm formation, which has hitherto been thought to be solely regulated by localized egg cytoplasmic factors. Without cell interaction, every descendant of posterior-vegetal blastomeres, including endoderm precursors, assumed muscle fate. Cell interactions are required for suppression of muscle fate and thereby promote endoderm differentiation in the posterior endoderm precursors. The cell interactions take place at the 16- to 32-cell stage. Inhibition of cell signaling by FGF receptor and MEK inhibitor also supported the requirement of cell interactions. Consistently, FGF was a potent signaling molecule, whose signaling is transduced by MEK-MAPK. By contrast, such cell interactions are not required for formation of the anterior endoderm. Our results suggest that another redundant signaling molecule is also involved in the posterior endoderm formation, which is likely to be mediated by BMP.

Suppression of the function of macho-1, a muscle determinant in ascidian eggs, by antisense oligonucleotide was enough to allow autonomous endoderm specification. Therefore, the cell interactions induce endoderm formation by suppressing the function of macho-1, which is to promote muscle fate. These findings suggest the presence of novel mechanisms that suppress functions of inappropriately distributed maternal determinants via cell interactions after embryogenesis starts. Such cell interactions would restrict the regions where maternal determinants work, and play a key role in marking precise boundaries between precursor cells of different tissue types.

Key words: Ascidian embryos, Endoderm formation, Muscle determinants, Inductive interactions, FGF, BMP

INTRODUCTION

A great deal of experimental evidence has accumulated over the years demonstrating the important role of intercellular interactions in developmental processes, even in animals that have determinate and invariant cell lineages. Ascidian embryogenesis has been regarded as a typical example of 'mosaic development' (Conklin, 1905). However, recent results have revealed that cellular interactions also play important roles in fate specification in ascidian embryos (reviewed by Satoh, 1994; Nishida, 1997; Nishida, 2002). Therefore, ascidian developmental systems exhibit a highly stereotyped cell lineage, yet also use cell interactions like aspects of *Caenorhabditis elegans*. We report that cell interactions are required for posterior endoderm formation of ascidian embryos, which has hitherto been thought to be solely regulated by localized egg cytoplasmic factors.

Endoderm cells are present in the central part of the trunk region of tadpole larvae (Fig. 1A). These cells are homogeneous in appearance and rich in yolk granules, and probably provide the embryos with nutrients. Endoderm cells in *Halocynthia* do not undergo terminal differentiation during larval development, because *Halocynthia* tadpoles do not feed. After metamorphosis, larval endoderm cells mainly give rise to peribranchial epithelium, gill and digestive organs (Hirano and Nishida, 2000). However, during embryogenesis, endoderm cells start some differentiation processes and initiate to express endoderm-specific alkaline phosphatase (ALP) (Minganti, 1954; Whittaker, 1977; Nishida and Kumano, 1997; Kumano and Nishida, 1998). All of the endoderm cells of a larva are derived from the vegetal blastomeres of an eight-cell embryo – namely the anterior A4.1 cell pair and the posterior B4.1 cell pair (Fig. 1A). By contrast, blastomeres of the animal hemisphere do not produce endoderm.

Expression of ALP occurs in partial embryos that are derived from isolated presumptive-endoderm blastomeres (Whittaker, 1990; Nishida, 1992). The expression of ALP can be observed in continuously dissociated embryos (Nishida, 1992). In embryos in which cell division is arrested after cleavage stages, ALP activity appears only in endoderm-lineage cells (Whittaker, 1977). These observations demonstrate the cell autonomy of endoderm differentiation and suggest the

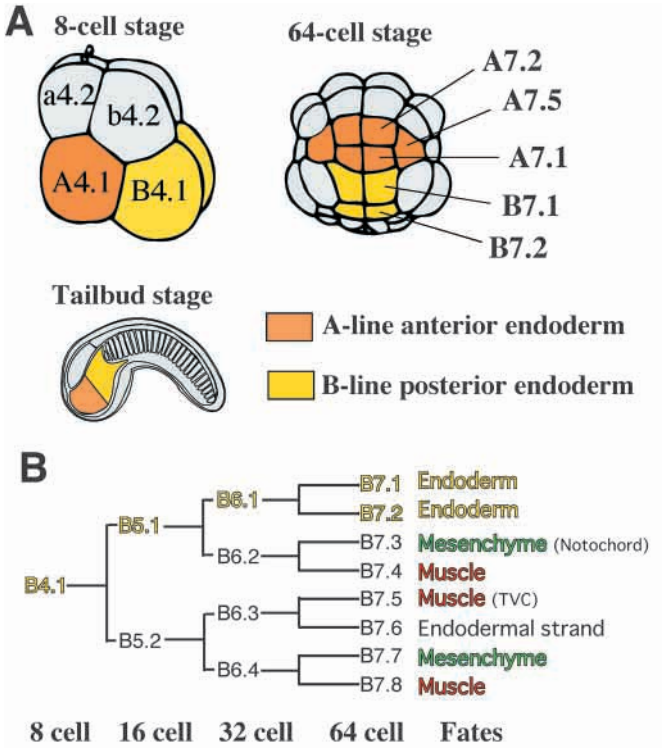

Fig. 1. Cell lineage of endoderm in the ascidian. (A) Positions of the anterior (orange) and posterior (yellow) endoderm and their precursor blastomeres. Eight-cell embryo is lateral view. Anterior is towards the left, animal pole is upwards. Sixty-four-cell embryo is vegetal view. Anterior is upwards. (B) Lineage tree starting from B4.1 posterior-vegetal blastomeres of an eight-cell embryo. Endoderm lineage is yellow. Minor fates are indicated in parentheses.

existence of cytoplasmic determinants that direct the formation of endoderm cells. To demonstrate the presence and localization of endoderm determinants, experiments involving transfer of cytoplasm have been carried out by fusing isolated blastomeres with cytoplasmic fragments that were prepared from various regions of eggs and embryos (Nishida, 1993; Yamada and Nishida, 1996). Endoderm determinants reside in the unfertilized egg, and after ooplasmic segregation, the determinants are present within the entire vegetal hemisphere from which the future endoderm-lineage blastomeres are formed. During cleavages, these determinants are partitioned into endoderm-lineage cells.

Glycogen synthetase kinase 3 (GSK3) and the β-catenin signaling pathway play crucial roles in maternal mechanisms that specify the animal-vegetal axis in sea urchin (Wikramanayake et al., 1998; Emily-Fenouil et al., 1998; Logan et al., 1999). Imai et al. (Imai et al., 2000) have reported a role for β-catenin in the specification of vegetal fate in embryos of the ascidians *Ciona intestinalis* and *C. savignyi*. In this regard, ascidian embryos show similarity to echinoderm embryos. The authors reported preferential β-catenin nuclear localization in the vegetal hemisphere in cleavage stage embryos. When mRNA encoding the stabilized form of β-catenin was injected into eggs, nuclear β-catenin was observed also in the animal hemisphere. In these embryos, most embryonic cells expressed ALP. To inhibit β-catenin function in nuclei, β-catenin was sequestered to a cell adhesion complex by overexpression of cadherin. Nuclear staining with β-catenin antibody was abolished in the entire embryo and ALP expression was lost. These observations indicate that animal-vegetal axis specification is mediated by β-catenin signaling. β-catenin is not localized in eggs and early cleavage stage embryos; so localized endoderm determinants would be molecules that stabilize β-catenin in the vegetal hemisphere.

Early zygotic events during endoderm formation have been well analyzed in nematodes, *Xenopus*, zebrafish and mammals (Hudson et al., 1997; Zaret, 1999; Aoki et al., 2002; Maduro and Rothman, 2002). In ascidian embryos, the expression of a LIM class homeobox gene, *Hrlim*, starts in endoderm precursors at the 32-cell stage, although it is not restricted to endoderm (Wada et al., 1995). In *Ciona savignyi*, the ortholog, *Cs-lhx3*, lies downstream of β-catenin and is responsible for ALP expression in endoderm (Satou et al., 2001). At the 76-cell stage, expression of the *TITF1* homologs of *Ciona intestinalis* and *C. savignyi*, *Cititf1* and *Cs-ttf1*, is initiated exclusively in endoderm precursors. TITF1 is a transcription factor containing an NK-2-like homeodomain (Lazzaro et al., 1991; Kimura et al., 1996). When synthetic mRNA of *Cititf1* and *Cs-ttf1* is injected into eggs, ectopic expression of ALP is promoted in non-endoderm cells (Ristoratore et al., 1999; Satou et al., 2001).

macho-1 mRNA has been identified as a localized maternal muscle determinant within ascidian egg cytoplasm (Nishida and Sawada, 2001). *macho-1* encodes a putative transcription factor that has zinc-finger domain. The presence of macho-1 protein promotes muscle fate. However, macho-1 products are inferred to be also present in mesenchyme precursor cells, and macho-1-directed muscle fate must be suppressed by FGF signaling for proper formation of mesenchyme cells (Kim and Nishida, 1999; Kim et al., 2000). In this study, we demonstrate that muscle fate directed by macho-1 should also be suppressed by cell interactions in the posterior endoderm. These observations suggest the presence of novel mechanisms that suppress functions of inappropriately distributed maternal determinants via cell interactions after embryogenesis starts.

MATERIALS AND METHODS

Animals and embryos

Adults of *Halocynthia roretzi* were purchased from fishermen near the Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, Japan, and near the Asamushi Marine Biological Station, Tohoku University, Aomori, Japan. Naturally spawned eggs were fertilized with a suspension of non-self sperm. Embryos were cultured at 9-13°C. Tadpole larvae hatched after 35 hours of development at 13°C.

Isolation and dissociation of blastomeres

Eggs were manually devitellinated with sharpened tungsten needles, and reared in 0.9% agar-coated plastic dishes filled with sea water. Blastomeres were identified and isolated from embryos by use of a fine glass needle under a stereomicroscope. Isolated blastomeres were cultured separately in agar-coated plastic dishes. Partial

embryos were cultured until control embryos reached hatching stage, and they were then prepared for histochemical and immunohistochemical staining to detect endoderm and muscle formation. For dissociation of embryonic cells, isolated blastomeres were incubated in Ca^{2+}-free artificial sea water (CFSW), such that daughter cells were continuously separated. CFSW consisted of 460 mM NaCl, 9.3 mM KCl, 48 mM $MgSO_4 \cdot 7H_2O$, 6 mM $NaHCO_3$, and 0.2 mM ethylene-bis(oxyethylenenitrilo)-tetraacetic acid (EGTA). Dissociation was monitored at frequent intervals and facilitated by gentle pipetting. Dissociated cells continued to divide at normal rates. After the period of dissociation, the cells were transferred separately to normal sea water and allowed to develop into multicellular partial embryos. In some experiments, cleavage of embryos was permanently arrested by treatment with 2 μg/ml cytochalasin B (Sigma) at the 110-cell stage.

Treatment with growth factors and MEK inhibitor

Isolated or dissociated blastomeres were transferred into sea water that contained 0.1% bovine serum albumin (BSA; Sigma) and 4 ng/ml bFGF protein (Amersham) or 50 ng/ml BMP4 protein (R&D Systems). The concentrations of FGF and BMP are effective enough to induce notochord formation in *Halocynthia* (Nakatani et al., 1996; Darras and Nishida, 2001). In controls, blastomeres were treated with BSA in sea water. To inhibit activation of the FGF-MAPK cascade, embryos were treated with 1.3-2.0 μM SU5402 (Calbiochem) or 2 μM U0126 (Promega) from the eight-cell stage to fixation. SU5402 belongs to a family of FGF signaling inhibitors that bind specifically to the active sites of FGFR kinase domains (Mohammadi et al., 1997). U0126 is an MEK inhibitor that inhibits phosphorylation and thereby activation of MAPK by MEK (Favata et al., 1998). Both inhibitors work well in *Halocynthia* embryos (Kim and Nishida, 2001). In controls, blastomeres were treated with 0.02% DMSO, the solvent of SU5402 and U0126.

Histochemistry, immunohistochemistry and in situ hybridization

To detect endoderm formation, histochemical staining for alkaline phosphatase (ALP) activity was carried out as described by Meedel and Whittaker (Meedel and Whittaker, 1989) with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate. The reaction deposits purple products. Muscle cells were histochemically stained for acetylcholinesterase (AChE) by the method described by Whittaker (Whittaker, 1980) with acetylthiocholine iodide as substrate. The reaction deposits brown products. The monoclonal antibody Mu-2 binds to the heavy chain of ascidian myosin, and is specific for muscle cells in larvae (Nishikata et al., 1987; Makabe and Satoh, 1989). Specimens were stained for indirect immunofluorescence with Mu-2 antibody by using Alexa 488-conjugated secondary antibody (Molecular Probes) or a TSA fluorescein system (PerkinElmer Life Sciences) according to the manufacturer's protocol. Specimens were then mounted in 80% glycerol and examined under an epifluorescence microscope. RNA probes for in situ hybridization of *HrMA4* were prepared with a DIG RNA labeling kit (Boehringer-Mannheim, Germany). *HrMA4* encodes larval muscle actin of *Halocynthia* (Satou et al., 1995).

Morpholino antisense oligonucleotide

The efficiency of morpholino antisense oligonucleotide (MO; Gene Tools), which is complementary to macho-1, has already been tested in *Halocynthia* (K.K. and H.N., unpublished). The MO was 5′-AATTGCAAAACACAAAAATCACACG-3′, which covers the 5′-UTR of macho-1 mRNA (GenBank Accession Number, AB045124). MO was dissolved in water, and ~100-300 pg was injected into each fertilized egg. In control experiments, 300 pg of four-mismatch control oligonucleotide (5′-AATTCCAAATCACAATAATCTCACG-3′) was injected.

RESULTS

Cell interactions are required for formation of the posterior endoderm but not of the anterior endoderm

Endoderm cells in the ascidian larvae are derived from two distinct lineages, A-line and B-line (Fig. 1A). Anterior endoderm of the larva originates from A4.1 anterior-vegetal blastomeres of the eight-cell embryo. B4.1 posterior-vegetal blastomeres give rise to posterior endoderm. To analyze the nature of fate specification in these distinct lineages and to examine the involvement of cell interactions in fate determination, we isolated the A4.1 or B4.1 blastomeres at the eight-cell stage, and the descendant cells were continuously dissociated in Ca^{2+}-free sea water during three successive cleavages up to the 64-cell stage. Then the dissociated cells were transferred to normal sea water to make them develop as multicellular partial embryos. Endoderm formation was evaluated by detecting alkaline phosphatase (ALP) expression (Whittaker, 1977; Whittaker, 1990; Kumano and Nishida, 1998).

In the first series of experiments, isolated blastomeres were collected and dissociated together (Table 1). In dissociation of B4.1 descendants, endoderm formation was significantly reduced. The fate of each descendant of the B4.1 blastomere is shown in Fig. 1B. After three cleavages, two out of eight blastomeres assume endoderm fate in normal embryogenesis (Nishida, 1987). The B7.6 blastomere gives rise to endodermal strand cells of the tip of the tail. But in *Halocynthia*, these cells do not express ALP activity. Therefore, in dissociation experiments, we expected that 25% (two out of eight) of partial embryos would develop into ALP-expressing endoderm cells. However, we observed ALP expression in only 6% of the partial embryos (Table 1; Fig. 2A). In dissociation of A4.1 descendants, endoderm formed in 31% of the partial embryos. This result is comparable with the expectation from the cell lineage of A4.1 (38%, three out of eight).

In the first series of experiments, we could not exclude a possibility that endoderm blastomeres were selectively lost during dissociation, because they are rich in yolk and could be more fragile. Consequently, the proportion of endoderm partial embryos might be underestimated, although we did not observe significant reduction of endoderm in the case of A4.1 dissociation. Therefore, we conducted a second series of

Table 1. Differentiation of endoderm and muscle in cell dissociation experiments

Isolated blastomeres	*n*	Endoderm (ALP)	Muscle (myosin)	Muscle (AchE)
B4.1	217	6% (25%)	91% (38%)	–
B4.1	217	–	–	92% (38%)
A4.1	105	31% (38%)	0% (0%)*	–

Isolated blastomeres were dissociated three times at the 16-, 32- and 64-cell stages and then cultured as partial embryos. ALP and myosin were double stained and simultaneously detected in the same specimens. Expectations from the cell lineage tree (Fig. 1) are indicated in parentheses. *n*, number of partial embryos examined.

*A4.1 blastomeres give rise to secondary muscle cells, but secondary-muscle formation requires cell interactions. The cells are not formed from isolated A4.1 blastomeres.

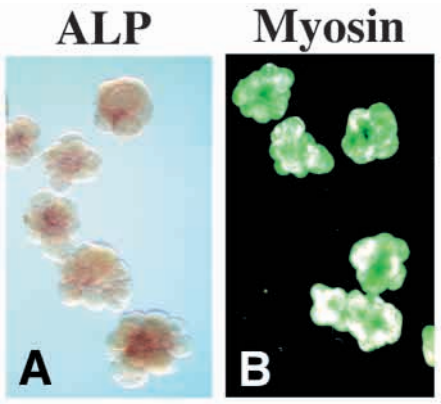

Fig. 2. Dissociation of the descendant cells of the isolated B4.1 blastomeres at the 16-, 32- and 64-cell stages. After dissociation, cells were cultured as partial embryos until hatching stage. Then endoderm formation was detected by expression of alkaline phosphatase (ALP) (A), and muscle formation was monitored by expression of myosin (B). Endoderm formation was suppressed, and every partial embryo developed into muscle.

experiments in which we isolated a single B4.1 or A4.1 blastomere and cultured the dissociated descendants in a single dish to confirm that all of the descendants were maintained without loss up to the end of the experiment. The proportion of endoderm partial embryos was then scored in each dish. Normally, a B4.1 blastomere produces eight partial embryos. However, it turned out that putative B7.6 blastomeres, which are much smaller after three successive unequal cleavages (Hibino et al., 1998), were frequently lost during histochemical staining. The unequal cleavages in normal embryogenesis were faithfully reproduced even in dissociation condition, probably because of the presence of a centrosome-attracting body (Nishikata et al., 1999). Therefore, we recovered seven partial embryos in each experiment. Fifteen sets of experiments were carried out. No partial embryos expressed ALP in any set. These results suggest that cell interactions are required to ensure fate specification of endoderm in the B-line blastomeres.

Suppression of muscle fate is required for posterior endoderm formation

Next, we addressed what kind of tissue type cells the presumptive endoderm blastomeres developed into under dissociation. The major fates of the B4.1 blastomeres of the eight-cell embryos are endoderm, muscle and mesenchyme (Fig. 1B). For mesenchyme formation, inductive cell interaction is required, and presumptive mesenchyme blastomeres assume muscle fate without induction in blastomere-isolation experiments (Kim and Nishida, 1999). Another indicative results is that, in cell dissociation of whole embryos, the proportion of partial embryos composed of muscle increases to one fourth of total partial embryos (Nishida, 1992). Therefore, it is probable that every dissociated B-line (posterior-vegetal) blastomere assume muscle fate, and we examined the possibility.

In massive dissociation of B4.1 blastomeres, 92% of partial embryos showed acetylcholinesterase activity, a muscle-specific enzyme (Whittaker, 1980) and 91% expressed muscle myosin, which was detected by Mu-2 monoclonal antibody (Nishikata et al., 1987) (Table 1; Fig. 2B). The proportion was much higher than expected from the cell lineage (38%; three out of eight), even if mesenchyme cells develop into muscle (totally 63%; five out of eight) (Fig. 1B). In those partial embryos, it seemed that every constituent cell of every partial embryo developed into muscle cells (Fig. 2B). By contrast, in dissociation of A4.1 descendants, muscle formation was never observed. In individual dissociation of B4.1 blastomeres, all seven partial embryos expressed myosin in 14 sets of experiments, six expressed myosin in three sets and four expressed myosin in one set. These results indicate that most partial embryos derived from B4.1 blastomeres assumed muscle fate, even though the B4.1 blastomere also has endoderm and mesenchyme fates. We further confirmed that an absence of Ca^{2+} in sea water during cell dissociation does not account for our results. We manually dissociated cleaved blastomeres in normal sea water with a fine glass needle instead of Ca^{2+}-free sea water, and we obtained essentially same results – namely, loss of endoderm and excessive muscle formation (data not shown).

Table 2. Periods when endoderm differentiation is sensitive to cell dissociation

Isolated blastomeres	Stage of dissociation	*n*	Endoderm (ALP)	Muscle (myosin)
B4.1	16 and 32 cell	60	3% (25%)	100% (75%)
B4.1	16 cell	58	24% (50%)	98% (100%)
B5.1	32 cell	31	38% (50%)	68% (50%)
A4.1	16 and 32 cell	76	53% (50%)	0% (0%)

Blastomeres were isolated at the eight- or 16-cell stage and descendant cells were dissociated once or twice. ALP and myosin were double stained. Expectations from the cell lineage tree (Fig. 1) are indicated in parentheses.

Cell interactions take place at the 16- to 32-cell stages

We simply isolated endoderm lineage cells at various stages, B4.1 at the eight-cell stage, B5.1 at the 16-cell stage and B6.1 at the 32-cell stage (Fig. 1B). In most cases (100% of 68 cases, 85% of 66 cases and 95% of 42 cases, respectively), the partial embryos expressed ALP (see Fig. 4A for B4.1 partial embryo).

Next we examined the period during which endoderm formation is sensitive to cell dissociation (Table 2). When isolated B4.1 blastomeres were dissociated during two successive cleavages at the 16- and 32-cell stage, most partial embryos developed into muscle, and endoderm formation was suppressed. By contrast, single dissociation of isolated B4.1 blastomeres at the 16-cell stage and single dissociation of isolated B5.1 blastomeres at the 32-cell stage resulted in partial reduction of endoderm formation. Again, dissociation of the A4.1 blastomeres had no effect. Therefore, it is likely that cell interactions take place at both the 16- and 32- cell stages, and continuous dissociation at both stages is required for complete suppression of endoderm formation and promotion of ectopic muscle formation.

FGF-MAPK signaling is involved in the cell interaction

In ascidian embryos, FGF signaling is involved in notochord

and mesenchyme induction (Nakatani et al., 1996; Kim et al., 2000). Especially in mesenchyme induction, FGF suppresses muscle fate and promotes mesenchyme formation in B-line blastomeres. The inductive signal is emanated from presumptive endoderm blastomeres at the 32-cell stage. Thus, one can reasonably infer that FGF signaling may also be involved in suppression of muscle fate in presumptive endoderm blastomeres, and endoderm precursors may send the signal to each other.

We examined this possibility by treating dissociated blastomeres with basic FGF (Table 3; Fig. 3A,B). B4.1 isolates were dissociated twice at the 16- and 32-cell stages in sea water containing 0.1% BSA and 4 ng/ml bFGF protein, then washed thoroughly with sea water and cultured as partial embryos. The concentration of bFGF is effective enough to induce notochord and mesenchyme formation in *Halocynthia* (Nakatani et al., 1996; Kim et al., 2000). In control partial embryos treated only with BSA, 99% of them expressed muscle myosin and only 2% expressed ALP. When partial embryos were treated with FGF, 22% of them developed ALP activity. The proportion was fairly close to expectation from the lineage (25%, one out of four). Muscle formation was greatly reduced to 21% (expectation is 75%, three out of four). Most ALP-negative partial embryos consisted of very small cells that resembled mesenchyme cells (Fig. 3A). Thus, FGF was efficient at suppressing muscle fate and inducing endoderm fate in the B-line blastomeres.

Table 3. Treatments of dissociated blastomeres with bFGF and BMP4 proteins

	n	Endoderm (ALP)	*n*	Muscle (myosin)
Expectation from lineage	–	25%	–	75%
Control (BSA)	169	2%	161	99%
bFGF	238	22%	214	21%
BMP4	99	16%	111	84%

B4.1 blastomeres were isolated at the eight-cell stage and descendant cells were dissociated twice at the 16- and 32-cell stages.

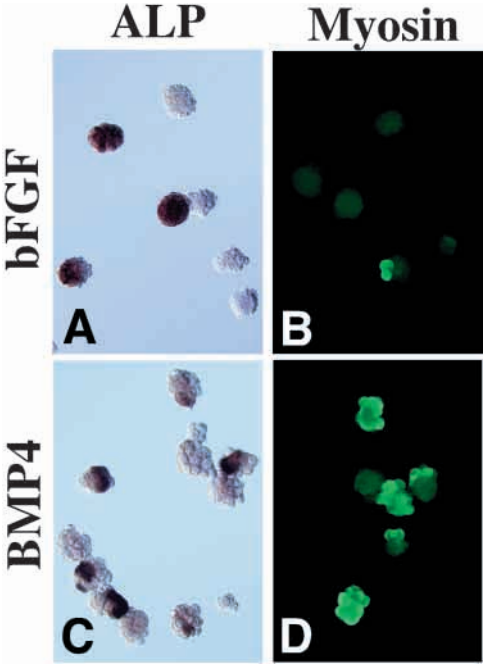

Fig. 3. Treatment of dissociated cells with bFGF and BMP4. Descendant cells of the isolated B4.1 blastomeres were dissociated at the 16- and 32-cell stages. Endoderm formation was detected by expression of ALP, and muscle formation was monitored by expression of myosin. (A,B) During dissociation, blastomeres were treated with bFGF protein in sea water. In A, ALP-negative partial embryos consist of very small cells that look like mesenchyme cells. (C,D) Blastomeres were treated with BMP4 protein.

Activation of the FGF receptor by the binding of ligands stimulates a multitude of downstream signaling cascades (Graves et al., 1994; Seger and Krebs, 1995; Triesman, 1996). A main pathway is a protein kinase transduction pathway, which includes FGFR, Ras, Raf, MEK and MAP kinase (MAPK). The signal transduction is also conserved in ascidian notochord and mesenchyme induction (Nakatani and Nishida, 1997; Kim and Nishida, 2001; Shimauchi, 2001). Among these molecules, the requirement of FGFR and MAPK activation in the formation of various cell types in ascidian larvae has been globally assessed by using inhibitors of FGFR and MEK, the kinase that activates MAPK (Kim and Nishida, 2001). As for endoderm formation, when isolated B4.1 blastomeres are treated with 2 μM MEK inhibitor, U0126, ALP expression is totally suppressed, and all constituent cells of the B4.1 partial embryos express muscle myosin without cell dissociation. The MEK inhibitor does not affect endoderm formation in A4.1 partial embryos. We reconfirmed these results (Fig. 4A,B,D,E,G) and further determined the period of sensitivity to the MEK inhibitor treatment (Fig. 4L). The results indicate that treatment initiated before and at the 32-cell stage was effective at suppressing ALP expression, but treatment initiated at the 64-cell stage was not effective. The period of sensitivity to the MEK inhibitor coincides well with the observation that the period of sensitivity to cell dissociation was the 16- and 32-cell stages. Thus, treatment with MEK inhibitor can substitute for cell dissociation in inhibiting cell interaction. Similar results was obtained when isolated B4.1 blastomeres are treated with 1.3-2.0 μM FGFR inhibitor, SU5402, from the eight-cell stage to fixation. Expression of ALP was observed in only 26% of the cases (n=23, Fig. 4H), and in 83% of cases (n=29), all constituent cells of the partial embryos express muscle myosin (Fig. 4I). The FGFR inhibitor also did not affect ALP expression in A4.1 partial embryos (Fig. 4K).

To verify that FGF-MEK signaling acts early on the specification of the posterior endoderm, fate conversion to muscle was examined by monitoring the expression of muscle actin (*HrMA4*) gene at the 64-cell stage. In *Halocynthia* embryos, muscle actin expression starts as early as 32-cell stage (Satou et al., 1995). At the 64-cell stage, two muscle precursor blastomere pairs (B7.4 and B7.8 pair) express actin. B4.1 partial embryos were fixed at the 64-cell stage, and actin expression was examined by in situ hybridization (Fig. 4C,F,J). In control partial embryos treated with DMSO, majority expressed actin in two blastomeres per partial embryo as expected (no blastomeres in 3% of cases, one in 3%, two in 64%, three in 23%, four in 7%; n=66). In 30% of cases, the number of positive blastomeres exceeded two, probably owing to inappropriate arrangement of blastomeres in the partial

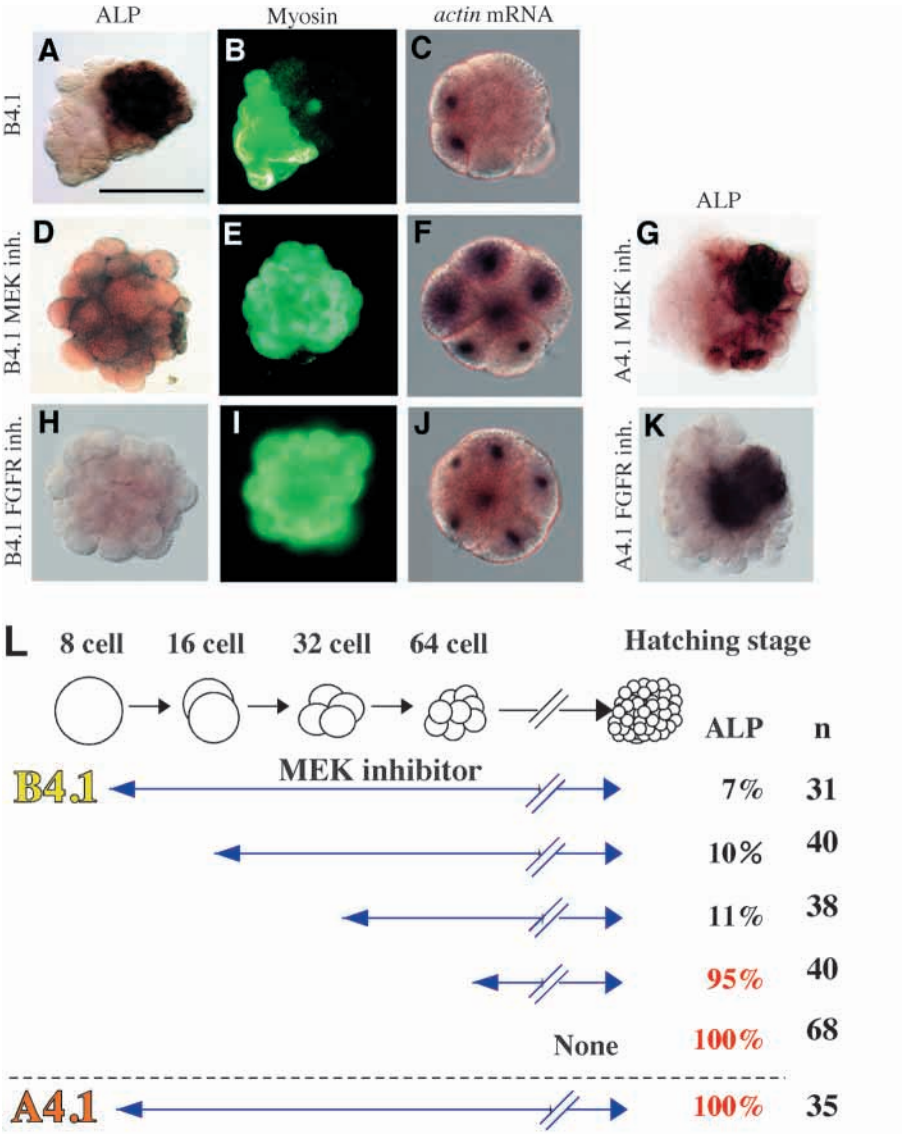

Fig. 4. Effects of MEK and FGFR inhibitor on formation of the posterior endoderm. (A,B) B4.1 blastomeres were isolated at the eight-cell stage and cultured as partial embryos until hatching stage. They expressed both ALP and myosin according to expectation from the cell lineage. Scale bar: 100 μm. (C) B4.1 partial embryos were fixed at the 64-cell stage. Muscle actin gene is expressed in the nuclei of two blastomeres. (D-F) Treatment with a MEK inhibitor, U0126, resulted in loss of endoderm differentiation. Every constituent cell of the partial embryos expressed myosin protein. Actin mRNA is detectable in six blastomeres. (G) MEK inhibitor did not affect endoderm formation in A4.1 isolates. (H-J) Treatment with FGFR inhibitor, SU5402, also resulted in loss of endoderm differentiation. Every constituent cell expressed myosin protein. Actin expression is detectable in six blastomeres. (K) FGFR inhibitor did not affect endoderm formation in A4.1 isolates. (L) Period of sensitivity to MEK inhibitor. B4.1 blastomeres were isolated at the eight-cell stage, then treatment with MEK inhibitor was initiated at various stages. The period of sensitivity ends at the 64-cell stage. A4.1 partial embryos were not sensitive to MEK inhibitor. *n*, number of partial embryos examined.

embryos, which would result in failure of correct cell interactions. By contrast, partial embryos treated with MEK inhibitor expressed actin gene in many blastomeres (two blastomeres in 4% of cases, three in 1%, four in 30%, five in 24%, six in 41%; n=91). Effect of the FGFR inhibitor was a bit weaker, but similar results was obtained (no blastomeres in 1% of cases, one in 1%, two in 16%, three in 15%, four in 31%, five in 22%, six in 13%; n=91). In treatment with either inhibitor, the maximum number of positive blastomeres per partial embryo was six, although the partial embryos should consists of eight blastomeres after three cell divisions. However, even in normal embryos, the most posterior muscle precursor blastomeres (B7.5 pair) do not initiate actin expression by the 64-cell stage. This is probably due to the general repression of zygotic gene expression in the two posterior blastomere pairs that are the putative germline lineage cells in ascidian embryos (Tomioka et al., 2002). Taking account of this, and even if the inhibitors caused two mesenchyme blastomeres to assumed muscle fate, the observed number of positive blastomeres indicates that some endoderm precursors initiated the expression of actin gene at the 64-cell stage in significant number of the partial embryos.

Suppression of macho-1 function is enough to allow posterior endoderm formation

Cell interaction mediated by FGF-MAPK signaling is suggested to play a role in suppression of muscle fate in posterior B-line endoderm precursors. Maternal *macho-1* mRNA has been identified as localized muscle determinants in ascidian eggs (Nishida and Sawada, 2001). The macho-1 product is necessary and sufficient for the formation of primary muscle cells of tadpole larvae. The next question we asked was whether suppression of the macho-1 function and consequent suppression of muscle fate is enough for specification of endoderm fate in the B-line. To do this, we injected morpholino antisense oligonucleotide (MO) complementary to *macho-1* mRNA into fertilized eggs. The efficiency of the *macho-1* MO has already been confirmed (K.K. and H.N., unpublished).

In control experiments, 300 pg of four-mismatch control oligonucleotide was injected into fertilized eggs, and B4.1

blastomeres were isolated at the eight-cell stage. Immediately after isolation, B4.1 blastomeres were treated with control DMSO or 2 μM MEK inhibitor until the larval stage (Table 4). Treatment with DMSO did not affect development of the B4.1 blastomeres, and both ALP and myosin were expressed in most of the partial embryos (Fig. 5A,B). Treatment with MEK inhibitor resulted in loss of endoderm, and every constituent cell of all partial embryos expressed myosin (Fig. 5E,F), confirming the previous results. Then 100-300 pg of MO was injected into eggs (Table 4). In DMSO-treated B4.1 partial embryos, ALP was expressed as in controls, but expression of myosin was completely inhibited, supporting the validity of *macho-1* MO (Fig. 5C,D). In partial embryos treated with MEK inhibitor, ALP expression was observed in 63% of cases and there was no myosin expression (Fig. 5G,H). Although the proportion of ALP-positive embryos was a little lower than in controls, suppression of macho-1 function restored endoderm formation in the presence of MEK inhibitor. Therefore, if there is no macho-1 activity, FGF-MEK signaling is not required to suppress muscle fate, and endoderm fate is autonomously executed in the B-line lineage.

Table 4. Effect of *macho-1* morpholino oligo on endoderm and muscle formation

	Control morpholino oligo		*macho-1* morpholino oligo	
	Endoderm (ALP)	Muscle (myosin)	Endoderm (ALP)	Muscle (myosin)
Control (DMSO)	89% (19)	100% (20)*	83% (30)	0% (30)
MEK inhibitor	0% (19)	100% (20)†	63% (30)	3% (29)

Morpholino oligos were injected into fertilized eggs. B4.1 blastomeres were isolated at the eight-cell stage and treated with DMSO or MEK inhibitor. Number of specimens is indicated in parentheses.

*Approximately half of constituent cells of each partial embryo were positive in every case.

†All constituent cells of each partial embryo were positive in every case.

Redundant signaling independent of MAPK activity is emanated from the anterior blastomeres

During our study of posterior endoderm formation, we encountered somewhat strange results that seem to contradict the above results. Even when the cleavage of ascidian embryos is permanently arrested at a cleavage stage, cleavage-arrested blastomeres continue some differentiation processes and eventually express endoderm and muscle differentiation characteristics, including ALP and myosin (Whittaker, 1977; Nishikata et al., 1987). This observation was reconfirmed by treating whole embryos with 2 μg/ml cytochalasin B at the 110-cell stage in order to inhibit subsequent cleavages. In DMSO-treated cleavage-arrested embryos, ALP expression was essentially observed in ten endoderm-lineage blastomeres, including anterior A-line and posterior B-line endoderm (A7.1, A7.2, A7.5, B7.1, B7.2 pairs), which are located in the vegetal pole region (Fig. 6A). In addition to these endoderm blastomeres, TLC precursors (A7.6) also expressed ALP, as observed by Whittaker (Whittaker, 1990). Myosin expression was also detected in all ten primary-muscle-lineage blastomeres, which are located at the posterior edge of the vegetal hemisphere (Fig. 6C). We treated the cleavage-arrested embryos with MEK inhibitor from the eight-cell stage to fixation. Unexpectedly, nothing was changed in ALP expression, and ALP was still expressed in B-line endoderm blastomeres (Fig. 6B, arrow). As for muscle formation, ectopic myosin expression was observed in four mesenchyme blastomeres (Fig. 6D, arrowheads). This is consistent with previous results that FGF-MEK signaling is required to suppress muscle fate in mesenchyme blastomeres (Kim et al., 2000; Kim and Nishida, 2001), confirming that MEK inhibitor worked in the present experiments. However, myosin expression was never observed in endoderm blastomeres. These results were confirmed by observing 20-40 cleavage-arrested embryos in each experiment.

The results suggest that suppression of muscle fate and

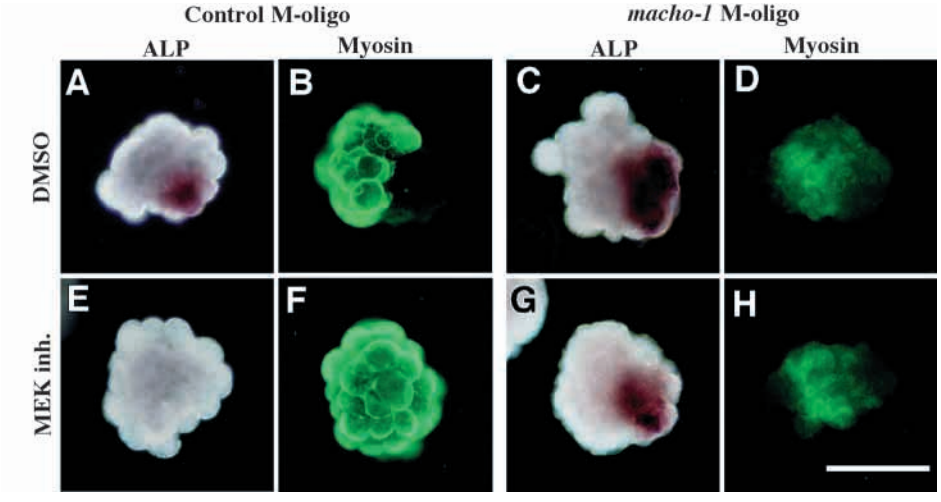

Fig. 5. Inhibition of macho-1 function is enough to allow endoderm formation in the absence of cell interaction. (A-D) B4.1 blastomeres were isolated from eight-cell embryos and cultured as partial embryos. Embryos were treated with DMSO as controls. Expression of ALP and myosin was detected to monitor the formation of endoderm and muscle cells. (E-H) The partial embryos were treated with a MEK inhibitor, U0126, to inhibit MEK-MAPK signaling. In F, every constituent cell expressed myosin. (A,B,E,F) Control morpholino antisense oligonucleotide (MO) was injected into fertilized eggs. (C,D,G,H) Antisense MO complementary to *macho-1* mRNA was injected to inhibit macho-1 translation. Slight staining in D and H is background caused by mitochondria. Muscle formation was totally suppressed. In G, ALP expression is visible even when embryos were treated with MEK inhibitor. Scale bar: 100 μm.

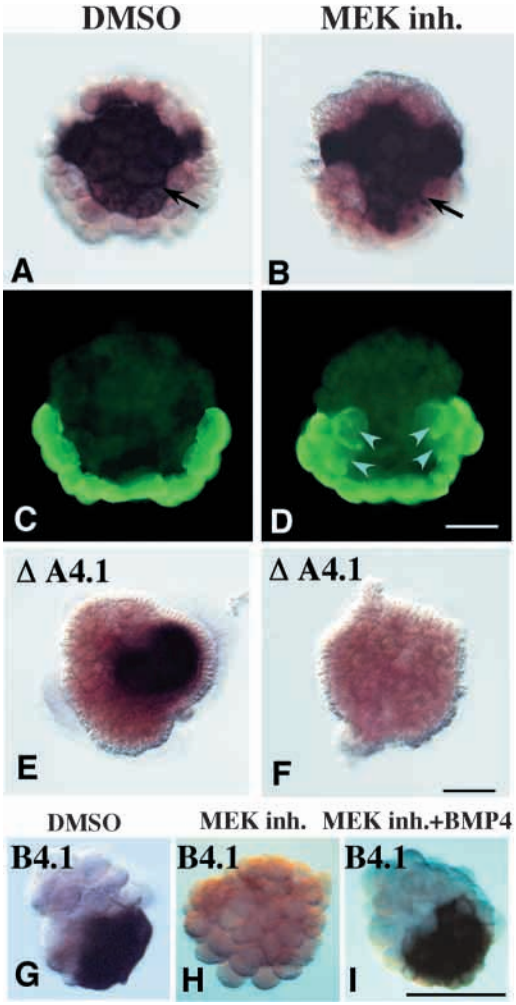

Fig. 6. Redundant BMP signaling induces posterior endoderm formation. (A-D) Cell divisions were permanently arrested at the 110-cell stage by cytochalasin B, and whole embryos were cultured until hatching stage. Then expression of ALP (A,B) and myosin (C,D) was detected. Vegetal views. Arrows indicate the posterior endoderm blastomeres. Treatment with MEK inhibitor did not affect ALP expression in the posterior endoderm blastomeres. Arrowheads indicate presumptive mesenchyme blastomeres that ectopically express muscle myosin. (E,F) The anterior-vegetal A4.1 blastomeres were removed from embryos (Δ A4.1) with a fine glass needle at the eight-cell stage. The embryos were cultured without cleavage-arrest until hatching stage, and ALP expression was detected. Without A4.1 blastomeres, embryos became sensitive to MEK inhibitor (F). (G-I) B4.1 partial embryos treated with DMSO (G), MEK inhibitor (H), and both MEK inhibitor and BMP4 protein (I). In I, BMP4 restored ALP expression. Scale bars: 100 μm.

formation of endoderm in the posterior-endoderm precursors may be operated by redundant mechanisms. If so, another signal must be emanated from blastomeres other than B-line cells, because the redundant signal did not act in B4.1 partial embryos that were sensitive to MEK inhibitor. Therefore, we ablated another vegetal blastomere pair, A4.1, at the eight-cell stage (Fig. 1A). Embryos were reared without cleavage arrest. In controls treated with DMSO, endoderm formation was observed in all cases (n=54) (Fig. 6E). By contrast, treatment with MEK inhibitor suppressed endoderm formation in 72% of cases (n=54) (Fig. 6F). In the other 28% of cases, we observed ALP staining only in a small region. The result suggests that a redundant signal emanates from the A-line anterior-vegetal blastomeres.

BMP is a candidate for the redundant signal

A plausible candidate for the signaling molecule is BMP. In *Halocynthia*, the *HrBMPb* gene (the ascidian *BMP2/4* homolog) is expressed at and after the 44-cell stage in anterovegetal blastomeres (anterior endoderm precursors: A7.1, A7.2 and A7.5 blastomeres; trunk lateral cell precursor: A7.6 blastomeres; Fig. 1A) (Darras and Nishida, 2001). In addition, BMP is a potent signaling molecule in notochord induction as well as FGF (Darras and Nishida, 2001). Therefore, we examined whether BMP is also able to induce posterior endoderm formation.

Similar to the above experiments used to test FGF, B4.1 isolates were dissociated twice at the 16- and 32-cell stages in sea water containing 0.1% BSA and 50 ng/ml BMP4 protein, then washed thoroughly with sea water and cultured as partial embryos (Table 3; Fig. 2C,D). The concentration of BMP4 is effective at inducing notochord in *Halocynthia*. When partial embryos were treated with BMP, 16% of them developed ALP activity. The proportion was close to expectation from the lineage (25%, one out of four). There is no statistical difference in ALP expression between FGF and BMP treatments ($0.2<P<0.3$, Table 3). Thus, BMP was also efficient at inducing endoderm fate in the B-line blastomeres. However, in contrast to FGF treatment, muscle formation was not significantly reduced, being observed in 84% (expectation is 75%, three out of four).

In the second set of experiments, B4.1 partial embryos were treated with BMP protein without cell dissociation. First, isolated B4.1 blastomeres were treated with BSA and DMSO. ALP expression was observed in all cases (n=5) (Fig. 6G). Second, B4.1 isolates was treated with BSA and MEK inhibitor (from eight-cell to larval stage). ALP expression was greatly reduced to 6% of cases (n=34), as observed in the previous experiments (Fig. 6H). Third, B4.1 isolates was treated with both BMP (from eight- to 64-cell stage) and MEK inhibitor. BMP restored ALP expression in 56% of cases (n=66) in the presence of MEK inhibitor (Fig. 6I). These results indicate that BMP is a possible candidate of the redundant signals, and MEK is not required for the signal transduction to induce endoderm by BMP.

DISCUSSION

Our results suggest that cell interactions are required for posterior endoderm formation in ascidian embryos, which has hitherto been thought to be solely regulated by localized egg cytoplasmic factors. Cell dissociation experiments revealed that cell interactions are required for suppression of muscle fate, and thereby promote endoderm differentiation in the B-line endoderm precursors. The cell interactions take place at the 16- and 32-cell stages. Suppression of cell signaling in B4.1 partial embryos by inhibitors of FGFR and MEK also supported this idea. Consistently, FGF was potent to induce

posterior endoderm. FGF signaling seems to acts early on the specification of the endoderm because some endoderm blastomeres in inhibitor-treated embryos already expressed muscle actin gene at the 64-cell stage. By contrast, such cell interactions are not required for formation of the anterior A-line endoderm. Another redundant signaling mechanism is present in the embryos and is likely to be mediated by BMP secreted from the anterior-vegetal blastomeres. The BMP signaling does not require MEK activity. Suppression of macho-1 function was enough to allow endoderm differentiation in the B-line. Therefore, the cell interactions induce endoderm formation by suppressing the function of macho-1, which is to promote muscle fate.

Maternal endoderm determinants and cell interactions

In our previous study, the expression of ALP was observed in continuously dissociated whole embryos (Nishida, 1992). The observation led us to conclude that endoderm formation is a cell-autonomous process. However, at that time we did not discriminate the anterior and posterior endoderm. In this study, we isolated B4.1 and A4.1 blastomeres at the eight-cell stage and dissociated their descendants to evaluate the role of cell interactions separately in each lineage. This uncovered a remarkable difference in endoderm specification between the two lineages. Only in the B-line are cell interactions required. The difference is caused by the presence of macho-1 products in the posterior region of the embryos, as discussed in the next section.

Experiments involving transfer of cytoplasm have been carried out to demonstrate the presence and localization of endoderm determinants, by fusing isolated blastomeres with cytoplasmic fragments (Nishida, 1993; Yamada and Nishida, 1996). The results suggested that endoderm determinants reside in the unfertilized egg and are partitioned into both A-line and B-line endoderm-lineage blastomeres during cleavages. When cytoplasm of B4.1 blastomeres was transferred into presumptive epidermis blastomeres, it promoted ectopic endoderm formation. The transferred cytoplasm probably also promoted ectopic cell interactions within the partial embryos to which it was introduced.

The requirement for cell interactions does not exclude the possibility that endoderm fate is basically specified by maternal localized endoderm determinants. In the absence of macho-1 function, embryos could form the posterior endoderm even when they were treated with MEK inhibitor. Imai et al. (Imai et al., 2000) report a role for β-catenin in vegetal fate specification in *Ciona*. Preferential β-catenin nuclear localization is observed in cells of the vegetal hemisphere, including B-line endoderm precursors. These observations support the view that endoderm specification is essentially mediated by a maternal mechanism even in the B-line, and that cell interactions overlie it as a parallel process to suppress inappropriate muscle fate.

Suppression of inappropriately distributed macho-1 activity is required for proper specification of B-line cell fates

Maternal *macho-1* mRNA has been identified as localized maternal muscle determinant within ascidian egg cytoplasm (Nishida and Sawada, 2001). *macho-1* encodes a putative transcription factor that has a zinc-finger domain. In this study, we demonstrated that muscle fate directed by macho-1 should be suppressed by cell interactions in the posterior endoderm. In the absence of macho-1 activity, endoderm specification was autonomously executed. Without suppression of macho-1 function, all descendants of the B4.1 blastomeres developed into muscle, and ALP expression was totally suppressed. Probably, macho-1 intensively directs muscle fate and overcomes the endoderm fate that is directed by β-catenin signaling. Cells may have mechanisms that prohibit an intermediate state between different cell types and force one or another cell fate. This will be an interesting issue for future study.

In mesenchyme precursor blastomeres, macho-1 products are inferred to be also present, and macho-1-directed muscle fate must be suppressed by FGF signaling for proper formation of mesenchyme cells (Kim and Nishida, 1999; Kim et al., 2000). The following observations further highlight the importance of suppression of muscle fate in the posterior vegetal region. In the posterior region of the vegetal hemisphere, precursor cells of trunk ventral cells (TVCs) are present (Fig. 7). When the precursors are isolated from embryos, they also differentiate into larval muscle cells (Nishida, 1992). Therefore, suppression of muscle fate by cell interactions is also required for the formation of the trunk ventral cells. This coincides well with our observation that every descendant cell of the B4.1 blastomeres assumed muscle fate when they were dissociated or treated with MEK inhibitor. Therefore, suppression of muscle fate would be necessary in all of the non-muscle lineages within the region derived from the B4.1 blastomere (Fig. 7, pink area).

macho-1 mRNA translocates to the posterovegetal region of eggs by ooplasmic segregation (Nishida and Sawada, 2001; Satou et al., 2002). During early cleavages, it is concentrated in the very restricted posterior region of the B4.1 blastomere pair in eight-cell embryos. Interestingly, the RNA is

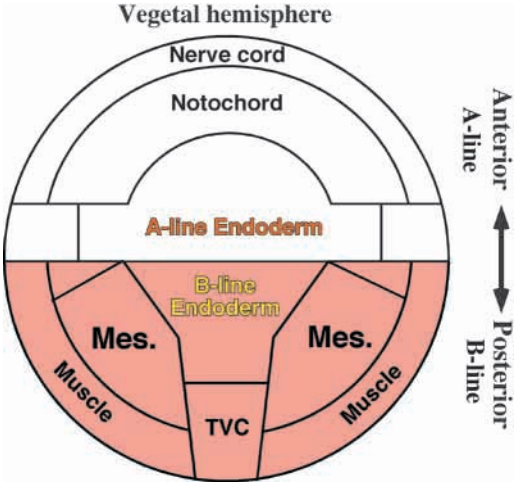

Fig. 7. Suppression of muscle fate in non-muscle lineages by cell interactions is required for proper patterning of the posterovegetal quarter of early ascidian embryos. When cell interactions were inhibited by cell dissociation or treatment with MEK inhibitor, every B-line cell assumed muscle fate as directed by macho-1. macho-1 protein would distribute into all B-line cells (pink area). Mes, mesenchyme; TVC, trunk ventral cell.

exclusively localized to a specific subcellular structure, the centrosome-attracting body (CAB) during cleavage stages (Hibino et al., 1998; Nishikata et al., 1999; Sasakura et al., 2000) (reviewed by Nishida, 2002). During three rounds of successive unequal cleavages after the eight-cell stage that occur only in the posterior region, *macho-1* mRNA always segregates into the smaller daughter cells located at the posterior pole (the B5.2 cell pair of the 16-cell embryo, the B6.3 cell pair of the 32-cell embryo, then the B7.6 cell pair of the 64-cell embryo). Thus, maternal mRNA of *macho-1* is not partitioned into all of the B4.1 descendants. Therefore, our prediction is that translation of maternal *macho-1* mRNA is initiated in the B4.1 blastomeres at the eight-cell stage, and the protein distributes into every descendant of the B4.1 cells. There would be a localization mechanism for the mRNA, but the protein may diffuse throughout the cytoplasm of the B4.1 cells upon translation. We have tried hard to produce antibody against macho-1 protein, but have so far been unsuccessful. The amount of the protein in the early embryo may be too low for immunohistochemical detection. In conclusion, our observations suggest the presence of novel mechanisms that suppress the functions of inappropriately distributed maternal determinants via cell interactions after embryogenesis starts. Such cell interactions would restrict the regions where maternal determinants work, and play a role in marking a precise boundary between precursor cells of different tissue types. It would be hard for embryos to precisely partition muscle determinants exclusively into multiple muscle precursor blastomeres.

Our recent results with macho-1 MO indicate that macho-1 is also required for mesenchyme formation (K.K., K. Sawada and H.N., unpublished). Therefore, macho-1 directs muscle fate in muscle cells; its function is probably modified by FGF signaling to promote mesenchyme fate in mesenchyme cells; and in endoderm cells the function is totally suppressed (Fig. 7). FGF is involved in cell signaling in both mesenchyme and endoderm formation. There will probably be intrinsic differences in responsiveness to FGF between mesenchyme and endoderm blastomeres, and the localization of endoderm determinants could account for the differences.

Redundant signaling is involved in posterior endoderm induction

When the B4.1 blastomeres were isolated and then dissociated or treated with inhibitors of FGF signaling, presumptive endoderm blastomeres assumed muscle fate. Consistently, FGF was potent to induce posterior endoderm in cell dissociation experiment. Recently, we have investigated the spatiotemporal pattern of activation of MAPK during embryogenesis of *Halocynthia*, using an antibody specific to the activated form of MAPK (Nishida, 2003). Inconsistent with the present results, phosphorylated and activated MAPK becomes detectable in the nuclei of every endoderm blastomere including both of the B- and A-line at the 44-cell stage when endoderm induction was completed. It has been also shown that the activation of MAPK in endoderm blastomeres is suppressed by MEK inhibitor treatment. In our previous study (Kim and Nishida, 2001), we reported that treatment with the FGFR inhibitor (SU5402) did not suppress the endoderm formation in isolated B-line blastomeres. In the experiments, embryos was treated form the 2- to early 32-cell stage. The results were confirmed again. It turns out that treatment up to the early 32-cell stage is not enough to suppress endoderm formation because effects of FGF inhibitor is reversible as shown by Kim and Nishida (Kim and Nishida, 2001), and that treatment should be continued at least up to the 64-cell stage (data not shown). This is reasonable because the endoderm induction take place during the 16- to 32-cell stage as revealed in the present study.

However, the cleavage-arrest experiment indicated the presence of redundant mechanisms in the induction of posterior endoderm. Both of FGF and BMP are candidates for signaling molecules because both proteins were potent to induce posterior endoderm in cell dissociation experiment. The FGF and BMP genes are expressed at the right time and in the right place for posterior endoderm induction during cleavage stages in ascidian (Darras and Nishida, 2001; Imai et al., 2002). FGF is expressed in both the anterior and posterior endoderm blastomeres, and BMP is expressed in the anterior endoderm blastomeres. FGF signaling required MEK activity, but BMP signaling did not, in accordance with the finding that BMP signaling is transduced mainly by Smad proteins in various animals (Whittman, 1998; Massague and Wotton, 2000). We tried to inhibit BMP signaling in cleavage-arrested and MEK-inhibitor-treated embryos by injection of MO complementary to *HrBMPb*. But it did not affect the endoderm and muscle formation. In our experience, some MOs worked well but others did not, depending on genes and targeted sequences in 5′ UTR. Although BMP is a promising candidate of signaling molecule secreted by anterior blastomeres, it is important to further elucidate the role of endogenous *HrBMPb* gene by loss-of-function type experiments in future study.

It is not clear how these distinct signaling mechanisms similarly promote endoderm formation. Treatment of dissociated blastomeres with FGF and BMP showed a slight difference. FGF treatment greatly reduced muscle formation, and most ALP-negative partial embryos seemed to develop into mesenchyme. In BMP treatment, however, ALP-negative partial embryos developed into muscle. This coincides with the observation that BMP is not effective in mesenchyme induction (H.N., unpublished). Therefore, FGF suppressed muscle fate in most blastomeres, but BMP is likely to suppress muscle fate only in presumptive endoderm blastomeres. Probably, the mechanism of suppression of muscle fate is different between FGF and BMP signaling.

There are similarities and differences between endoderm induction and notochord induction in ascidians. FGF and BMP are both potent at inducing notochord formation (Nakatani et al., 1996; Darras and Nishida, 2001). However, MEK is required for BMP to act in notochord induction. Therefore, events during both kinds of induction would be similar but not identical, although the details are unknown.

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