Maternal *macho-1* is an intrinsic factor that makes cell response to the same FGF signal differ between mesenchyme and notochord induction in ascidian embryos

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Summary

An extracellular signaling molecule acts on several types of cells, evoking characteristic and different responses depending on intrinsic factors in the signal-receiving cells. In ascidian embryos, notochord and mesenchyme are induced in the anterior and posterior margins, respectively, of the vegetal hemisphere by the same FGF signal emanating from endoderm precursors. The difference in the responsiveness depends on the inheritance of the posterior-vegetal egg cytoplasm. We show that *macho-1*, first identified as a localized muscle determinant, is also required for mesenchyme induction, and that it plays a role in making the cell response differ between notochord and mesenchyme induction. A zygotic event involving *snail* expression downstream of maternal *macho-1* mediates the suppression of notochord induction in mesenchyme precursors.

Key words: Mesenchyme, Notochord, Induction, Responsiveness, Intrinsic factor, *macho-1*, FGF, *snail*, Ascidian

Introduction

In animal development, various organisms use limited kinds of signaling molecules for intercellular communication. The same signals are used over and over again in different cells at different stages, with different biological outcomes depending on the spatial and temporal context. This reflects the 'responsiveness' or 'competence' of the signal-receiving cells. In inductive interactions, extracellular signaling molecules and intrinsic factors are thought to combine in signal-receiving cells, conferring particular cell fates. Intrinsic factors of responding cells play roles in defining the response. To understand animal development, it is important to clarify how embryonic cells can respond differently to the same signal. Ascidian embryos provide a good system for elucidating this issue.

The structure of the ascidian tadpole larva is relatively simple. As shown in Fig. 1A, larval muscle cells lie laterally on both sides of the notochord, which is aligned in the center of the tail. The posterior nerve cord is located on the dorsal side of the notochord in the tail. On each side of the trunk region, there is a cluster of mesenchyme cells. Fig. 1B,C show fate maps of the vegetal hemisphere at the blastula stage (32- and 64-cell stage in ascidians) (Nishida, 1987; Kim et al., 2000; Minokawa et al., 2001). Nerve cord, notochord, mesenchyme and muscle cells are derived from the anterior and posterior margins of the vegetal hemisphere. The endoderm originates from the central zone. These five tissue-forming areas are aligned along the anteroposterior axis. From the anterior, nerve cord, notochord, endoderm, mesenchyme and muscle precursors are present in this order.

Mesenchyme and notochord fates are determined by inductive interactions: Endoderm precursors induce mesenchyme in the posterior region of embryos (lower side in Fig. 1B,C), whereas they induce notochord in the anterior region (upper side) (reviewed by Nishida, 2002). Induction of mesenchyme and notochord shares several common features (Fig. 1D).

(1) In the posterior region, the precursor of both mesenchyme and muscle of the 32-cell embryo divides into mesenchyme and muscle precursors at the sixth division. In the anterior region, the precursor of both notochord and nerve cord of the 32-cell embryo divides into notochord and nerve cord precursors (Nishida, 1987).

(2) Inductive interactions take place during the 32- and 44-cell stage. Then mesenchyme and notochord precursors acquire developmental autonomy (Kim and Nishida, 1999; Nakatani and Nishida, 1994).

(3) Directed signaling and asymmetric division play a crucial role in fate specification in both anterior and posterior vegetal marginal zones. Induced cells of 32-cell stage embryos respond to the signal by asymmetric divisions that produce daughter cells with distinct fates. The daughter cell that faces the inducing endoderm blastomere assumes a mesenchyme or

notochord fate (induced fate). However, another daughter cell follows a muscle or nerve cord fate (default fate) (Kim and Nishida, 1999; Minokawa et al., 2001).

(4) Endoderm blastomeres are the inducers, and FGF signaling mediates the induction. Mesenchyme and notochord are induced by the same FGF signal. The FGF signal is transduced by FGF receptor, Ras, MEK and MAPK (ERK1/2) in both kinds of precursor (Nakatani et al., 1996; Kim et al., 2000; Shimauchi et al., 2001; Kim and Nishida, 2001; Imai et al., 2002; Nishida, 2003).

(5) When the inductive influence is inhibited by isolating blastomeres or by inhibitors of FGF signaling, both daughter cells adopt the muscle or nerve chord fate (default fate) (Fig. 1D, part c). By contrast, when the mother cell is isolated from the embryos at the 32-cell stage and it receives the signal over its entire surface by treatment with FGF, both daughter cells adopt the mesenchyme or notochord fate (induced fate) (Fig. 1D, part d) (Kim et al., 2000; Minokawa et al., 2001).

Thus, there are striking similarities at the cellular and molecular levels between mesenchyme and notochord induction, and a similar mechanism symmetrically functions in both the anterior and posterior marginal zones. In particular, the signaling cascade from FGF to MAPK (ERK1/2) is remarkably conserved among mesenchyme and notochord induction in ascidian embryos, as well as in other organisms that have been studied. But mesenchyme and notochord blastomeres show distinct responses to the same FGF signal. Removal and transplantation of egg cytoplasm by microsurgery revealed that the difference in their responsiveness is caused by the cytoplasmic factor of the responding blastomeres, which is inherited from the egg (Kim et al., 2000) (Fig. 1E,F). The posterior-vegetal cytoplasm (PVC) of eggs confers the muscle and mesenchyme fate on the posterior blastomeres. Removal of the PVC resulted in anteriorization of the embryos. Blastomeres positioned where mesenchyme blastomeres are normally located were converted to notochord, so that central endoderm blastomeres were encircled by notochord blastomeres. Thus, removal of the PVC causes ectopic formation of notochord and loss of mesenchyme in the posterior region (Fig. 1E). However, transplantation of the PVC to the anterior region of another intact egg suppressed notochord formation and promoted ectopic formation of mesenchyme in the anterior blastomeres (Fig. 1F). Therefore, the factors that are localized in the PVC are involved in differentiating cell response to the FGF signal. In the presence of the PVC factors, blastomeres respond by forming mesenchyme; and, in their absence, blastomeres respond by developing into notochord.

The molecular nature of the PVC factors that determine cellular responsiveness is unknown. The PVC is the region corresponding to Conklin's myoplasm at completion of ooplasmic segregation (Conklin, 1905). Recently, it was shown that maternal mRNA of *macho-1*, which is localized in the PVC region, is an ascidian muscle determinant (Nishida and Sawada, 2001). *macho-1* encodes a putative transcription factor with zinc-finger domains. *macho-1* would be a good candidate for the PVC factor that regulates cellular responsiveness. Other evidence to support this idea is that without induction, mesenchyme blastomeres assume muscle fate directed by *macho-1* (Kim and Nishida, 1999). Therefore, *macho-1* products are supposed to be present also in mesenchyme blastomeres, and can play a role as the PVC factor. To examine this hypothesis, we investigated the formation of mesenchyme, notochord, muscle and nerve cord in *macho-1*-deficient and *macho-1*-overexpressing embryos. Our results showed that *macho-1* not only is a muscle determinant but also plays a pivotal role as an intrinsic factor that controls the responsiveness of mesenchyme blastomeres.

Downstream of the maternal PVC factor, zygotic events would be involved in suppression of the notochord fate in mesenchyme blastomeres. *snail* is a possible candidate, because it is expressed in muscle and mesenchyme precursors at the 32-cell stage or the 44-cell stage (Erives et al., 1998; Wada and Saiga, 1999). Furthermore, Snail is a zinc-finger protein known to be a transcription repressor. *Brachyury* is a key transcription factor that is involved in notochord formation in ascidians (Yasuo and Satoh, 1998; Takahashi et al., 1999a). Misexpression of *snail* in notochord-lineage cells driven by a heterologous promoter suppresses at least the expression of the reporter gene driven by the *Brachyury* minimal promoter through Snail-binding sites within it, although the formation of notochord was not suppressed in experiments (Fujiwara et al., 1998). We showed that *snail* is a downstream target of maternal *macho-1*. To examine the function of *snail* in mesenchyme and notochord induction, we also injected *snail* mRNA into eggs, where it suppressed endogenous *Brachyury* expression and formation of notochord.

Materials and methods

Animals and embryos

Halocynthia roretzi were collected near the Asamushi Marine Biological Station, Aomori, Japan, and the Otsuchi Marine Research Center, Iwate, Japan. Naturally spawned eggs were fertilized with a suspension of non-self sperm. Embryos were cultured in Millipore-filtered seawater containing 50 μg/ml streptomycin and 50 μg/ml kanamycin at 9-13°C. Tadpole larvae hatched after 35 hours of development at 13°C.

Injection of MO and synthetic mRNAs

To suppress translation of *macho-1*, we used antisense morpholino oligonucleotides (MO; Gene Tools) complementary to the 5′ UTR of *macho-1* (GenBank Accession Number: AB045124) (5′-AATTGCAAAACACAAAAATCACACG-3′, antisense to a 25-nucleotide sequence spanning nucleotides 13-37 of *macho-1* cDNA). In control experiments, we used 4-mismatch control MO (5′-AATTCCAAATCACAATAATCTCACG-3′, mismatch underlined). Capped mRNAs of *macho-1* and *Hrsna* were synthesized as described previously (Nishida and Sawada, 2001), except for the use of the mMessage mMachine kit (Ambion). Mutant *macho-1* mRNA lacking a zinc-finger domain (Nishida and Sawada, 2001) and *lacZ* mRNA were used as controls. MO and/or synthetic mRNA was suspended in sterile distilled water and injected into intact eggs after fertilization. For microinjection, we followed the method described previously (Miya et al., 1997).

Isolation of blastomeres and inhibition of cell division

Embryos were manually devitellinated with tungsten needles and reared in 1.2% agar-coated plastic dishes filled with seawater. Blastomeres were identified and isolated from embryos with a fine glass needle under a stereomicroscope (SZX-12; Olympus). Isolated blastomeres were cultured separately as partial embryos in agar-coated plastic dishes, then the partial embryos were fixed for immunohistochemistry or in situ hybridization. To inhibit cell division, cleavage was permanently arrested with 2.5 μg/ml cytochalasin B (Sigma) at the 110-cell stage.

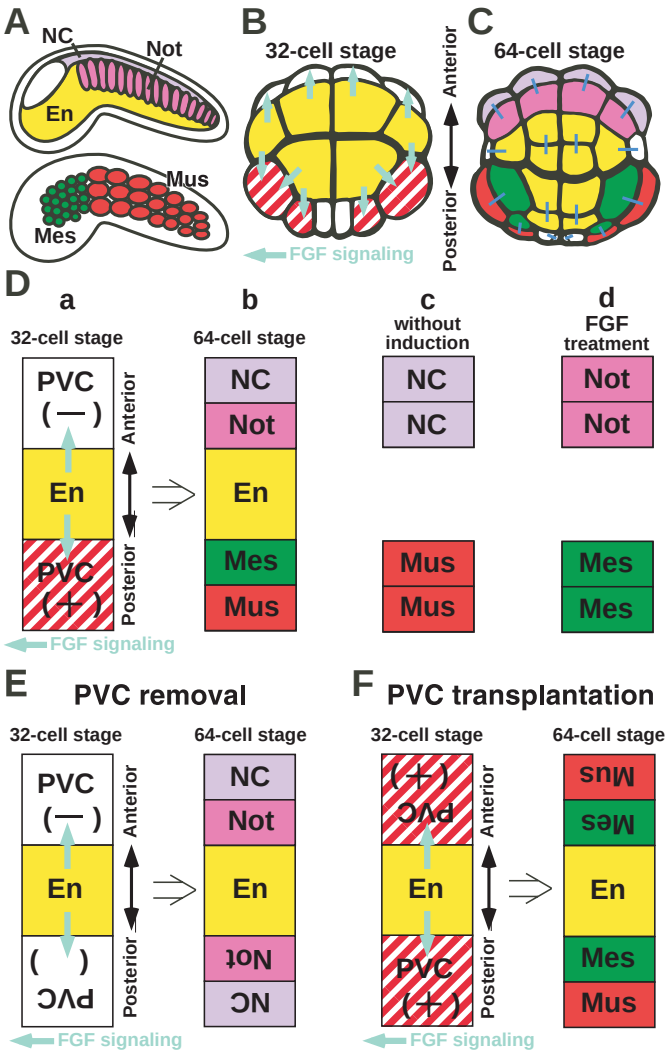

Fig. 1. Fate specification in the vegetal hemisphere of ascidian embryos. (A-C) Endoderm (En)-lineage cells are yellow. Mesenchyme (Mes)-lineage cells are shown in green and muscle (Mus)-lineage cells in red. Notochord (Not)- and nerve cord (NC)-lineage cells are pink and purple, respectively. (A) Tailbud embryos. Lateral view. Anterior is towards the left. Upper and lower diagrams illustrate midsagittal and parasagittal sections, respectively. (B) 32-cell stage embryo. Vegetal view. Light-blue arrows indicate direction of induction of FGF signaling. Red hatching indicates the location of the posterior-vegetal cytoplasm (PVC). (C) 64-cell stage embryo. Blastomeres connected with blue bars are sister blastomeres. (D) A directed signaling and asymmetric division model of the tissue specification mechanism in the vegetal hemisphere of the ascidian embryo. The model is applicable to both the anterior and posterior margins of the vegetal hemisphere. Light-blue arrows indicate direction of induction of FGF signaling. (a) Schematic drawing representing embryo at the 32-cell stage. Endoderm precursors (En) emanate the inductive FGF signal (light-blue arrows) to neighboring anterior and posterior blastomeres and polarize them. The PVC (red hatching) brings about different responsiveness of posterior marginal cells. (b) Asymmetric divisions occur at the 64-cell stage in both the anterior and posterior marginal zones. For precise positions of blastomeres, see (C). (c) Without inductive signal, both daughter blastomeres in the anterior region assume the default nerve cord fate (NC), and those in the posterior region assume the default muscle fate (Mus). (d) When isolated blastomeres receive inducing FGF signal all over the surface, both daughter cells develop into notochord (Not) or mesenchyme (Mes), depending on absence or presence of PVC, respectively. (E,F) The results of PVC removal and PVC transplantation, respectively. Light-blue arrows indicate direction of induction of FGF signaling. (E) When the PVC is removed, ectopic notochord is induced in the position of presumptive mesenchyme in the posterior region. Mesenchyme formation is suppressed. (F) PVC transplantation to the anterior region of intact eggs results in ectopic mesenchyme and muscle formation in the anterior region. On the other hand, notochord formation is suppressed. NC, nerve cord; Not, notochord; En, endoderm; Mes, mesenchyme; Mus, muscle.

Removal and transplantation of egg cytoplasm

Removal and transplantation of PVC were carried out as described previously (Nishida, 1994). After completion of ooplasmic segregation, fertilized eggs were oriented by using the position of the polar bodies and the posterior transparent myoplasm. Egg fragments containing the PVC, which was 8%-15% of the total egg volume, were removed from the eggs by severing the eggs with a fine glass needle. The eggs were cultured as PVC-deficient embryos. For transplantation of the PVC, an egg fragment containing PVC that had been severed from an egg was transplanted into the anterior-vegetal region of another intact egg by using polyethylene glycol and electric field-mediated fusion.

Treatment with FGF

Isolated blastomeres were transferred into seawater that contained 0.1% bovine serum albumin (BSA; Sigma) and 2 ng/ml recombinant human bFGF protein (Amersham). This concentration of FGF is effective enough to induce notochord and mesenchyme formation in *Halocynthia* (Nakatani et al., 1996; Kim et al., 2000). In controls, blastomeres were treated with BSA in seawater.

Immunohistochemistry

Formation of mesenchyme cells was monitored by staining with the Mch-3 monoclonal antibody (Kim and Nishida, 1998). The monoclonal antibody Mu-2 was used for monitoring muscle formation (Nishikata et al., 1987). It recognizes the myosin heavy chain of *Halocynthia* (Makabe and Satoh, 1989). The specimens were fixed after the hatching stage for 10 minutes in methanol at –20°C. The monoclonal antibody Not1 recognized a component of the notochordal sheath that is secreted by notochord cells (Nishikata and Satoh, 1990). At the middle tailbud stage, this antibody is strictly specific to notochord cells (Nakatani and Nishida, 1994). Specimens were fixed at the middle tailbud stage for Not1 staining. Indirect immunofluorescence detection was carried out by standard methods using a TSA fluorescein system (PerkinElmer Life Sciences) according to the manufacturer's protocol.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Wada et al., 1995). Specimens were hybridized by using digoxigenin-labeled *HrBra*, *HrETR-1* and *Hrsna* antisense probes. *HrBra*, encoding *Halocynthia Brachyury* gene, was used to assess notochord specification (Yasuo and Satoh, 1993). The expression of *HrBra* was monitored at the 110-cell stage. *HrETR-1*, encoding an RNA-binding protein of the Elav family, was used as a molecular marker for nerve cord specification (Yagi and Makabe, 2001). The expression of *HrETR-1* was monitored at the 118-cell stage or the neural-plate stage in cleavage-arrested 110-cell embryos. *Hrsna* encodes a Snail homolog in *Halocynthia* (Wada and Saiga, 1999). The expression was examined at the 64-cell stage.

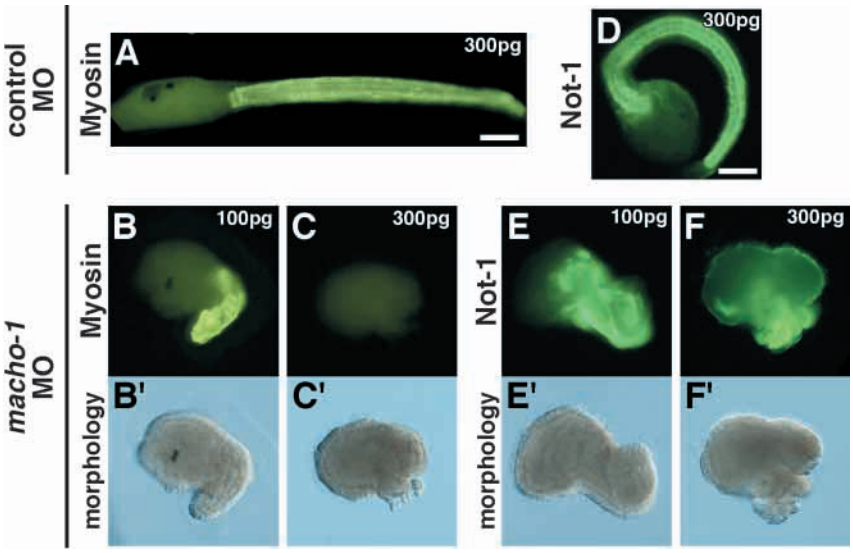

Fig. 2. Effect of suppression of *macho-1* function by MO on formation of muscle and notochord. (A-C) Expression of the muscle myosin protein. (A) Control larva in which 300 pg of control MO was injected into fertilized egg. Anterior is towards the left. (B) 100 pg and (C) 300 pg of *macho-1* MO was injected. (D-F) Expression of notochord-specific Not1 antigen. (D) Control tailbud embryo that was injected with 300 pg of control MO. (E) 100 pg and (F) 300 pg of *macho-1* MO was injected. (B′,C′,E′,F′) Morphologies of embryos are shown in B,C,E,F, respectively. Scale bars: 100 μm.

Results

Inhibition of *macho-1* function disrupts the anterior-posterior axis

In previous studies to characterize *macho-1* as a muscle determinant, we used antisense phosphorothioate DNA oligonucleotides (S-DNA) to deplete *macho-1* maternal messages (Nishida and Sawada, 2001). Recently antisense morpholino oligonucleotides (MO) have been introduced to study maternal and zygotic gene functions in ascidian embryos (Satou et al., 2001). In this study, we also used MO to prevent the function of *macho-1* by inhibiting its translation, and MO was more effective than S-DNA. In control larvae derived from fertilized eggs injected with 300 pg of 4-mismatch control MO, their morphology looked normal and muscle cells were normally detected by immunostaining of muscle myosin protein (Fig. 2A). By contrast, when a low dose (100 pg) of *macho-1* MO was injected into eggs, the tail was shortened and primary muscle cells were lost (Fig. 2B,B′). This phenotype is similar to that observed in the previous study using S-DNA (Nishida and Sawada, 2001). However, at a high dose (300 pg), resultant malformed larvae did not have a distinct head and tail, and they seem to be almost radially symmetrical along the animal-vegetal axis without an obvious anterior-posterior axis, which is normally perpendicular to the animal-vegetal axis in ascidian embryos (Fig. 2C′). This morphology was very similar to that of the larvae from which the posterior-vegetal egg cytoplasm (PVC) containing maternal *macho-1* mRNA was removed (Nishida, 1994). There were no muscle cells in these embryos (Fig. 2C). This phenotype was unlikely to be the result of nonspecific toxic effects, because notochord cells that express notochord-specific Not1 antigen were formed in every *macho-1*-deficient embryo (Fig. 2E,F). And the muscle formation was restored in isolated primary-muscle-lineage (B4.1) blastomeres by injection of *macho-1* MO (300 pg) together with *macho-1* mRNA (100 pg) that had no complementary sequence to the MO in the 5′ UTR (n=24) (data not shown). Furthermore, it has been shown that the effects of MO are dose dependent (Heasman, 2002). Thus, the phenotype of the high dose seems to be result of complete inhibition of the *macho-1* function. Therefore, we used *macho-1* MO to inhibit the *macho-1* function in the following experiments.

macho-1 confers on blastomeres the responsiveness to be induced to form mesenchyme

Removal of the PVC caused loss of mesenchyme in the posterior region (Fig. 1E). However, transplantation of the PVC to the anterior region promoted ectopic formation of mesenchyme in the anterior blastomeres (Fig. 1F). To examine the possibility that *macho-1* plays a role as the PVC factor that

Table 1. Expression of mesenchyme-specific Mch3 antigen in *macho-1* mRNA-injected partial embryos derived from isolated blastomeres

	Partial embryos with Mch3 expression/embryos examined (%)					
	Control (mutant)		*macho-1*			
Isolated blastomeres	100 pg		50 pg		100 pg	
	BSA	FGF	BSA	FGF	BSA	FGF
A4.1	0/19 (0%)	0/11 (0%)	17/22 (**77%**)	16/19 (**84%**)	5/17 (29%)	10/16 (**63%**)
B4.1	11/11 (**100%**)	16/16 (**100%**)	8/10 (**80%**)	7/9 (**78%**)	4/13 (31%)	8/13 (**62%**)
a4.2	0/10 (0%)	0/10 (0%)	0/10 (0%)	7/10 (**70%**)	0/12 (0%)	0/10 (0%)
b4.2	0/10 (0%)	0/10 (0%)	0/10 (0%)	6/10 (**60%**)	0/10 (0%)	2/12 (17%)

Synthetic mRNAs were injected into eggs, then each blastomere was isolated at the eight-cell stage. Isolated blastomeres were treated only with BSA-seawater or with FGF in BSA-seawater. Control mRNA encodes mutant Macho-1 that lacks the zinc-finger domain (Nishida and Sawada, 2001).

Proportions over 50% are indicated in bold.

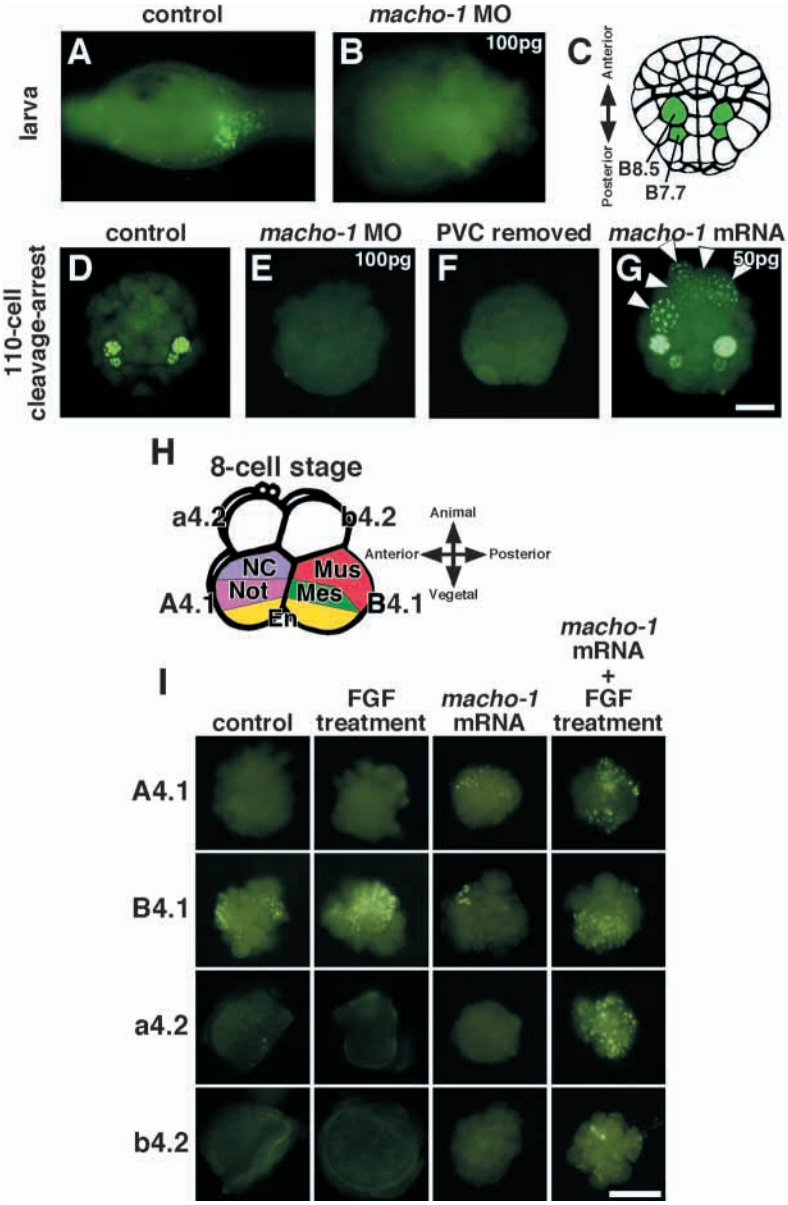

Fig. 3. Formation of mesenchyme detected with Mch-3 antibody in *macho-1*-deficient and -overexpressing embryos. (A,B) Expression of mesenchyme-specific Mch-3 antigen. (A) Control larva in which control MO was injected. Anterior is towards the left. (B) 100 pg of *macho-1* MO was injected. (C) Diagram illustrating the vegetal hemisphere of the 110-cell stage embryo. Presumptive mesenchyme blastomeres are indicated in green. (D-G) Expression of mesenchyme-specific Mch-3 antigen. Embryos whose cleavages were arrested at the 110-cell stage. (D) Control embryo injected with control MO. Anterior is upwards. (E) 100 pg of *macho-1* MO was injected. (F) PVC-removed embryo. (G) 50 pg of *macho-1* mRNA was injected. White arrowheads indicate ectopic expression of Mch-3 antigen in the anterior half. Anterior is upwards. (H) Lateral view of the eight-cell stage embryo and fate map. Nerve cord (NC), notochord (Not), endoderm (En), mesenchyme (Mes) and muscle (Mus)-forming areas are indicated. Color of each area is the same as in Fig. 1. (I) Expression of mesenchyme-specific Mch-3 antigen in the partial embryos derived from isolated blastomere of the eight-cell embryos. Embryos were injected with 50 pg of control *macho-1* mRNA that lacks the zinc-finger domain or 50 pg of *macho-1* mRNA. After isolation, blastomeres were treated with FGF or BSA only. Scale bars: 100 μm.

controls the responsiveness of mesenchyme blastomeres to endodermal inducing signal, we investigated mesenchyme formation in *macho-1*-deficient embryos in order to ask whether the phenotypes of *macho-1*-deficient embryos reproduce those of the PVC-removed embryos.

In control larvae derived from fertilized eggs injected with 4-mismatch control MO, mesenchyme cell clusters were normally detected by immunostaining of mesenchyme-specific Mch3 antigen in 20 cases (Fig. 3A). By contrast, injection of 100 pg of *macho-1* MO abolished the expression of the antigen in all 13 cases (Fig. 3B). This was also confirmed in another way. In ascidian embryos, even when cleavages were permanently arrested with cytochalasin B at a cleavage stage, cleavage-arrested blastomeres continued some differentiation processes and eventually expressed different features according to their developmental fates (Whittaker, 1973; Nishikata and Satoh, 1990). In embryos injected with control MO, four mesenchyme precursors (B8.5 and B7.7 blastomere pairs) eventually expressed the mesenchyme marker, as expected from the cell lineage when cleavage and morphogenesis were arrested at the 110-cell stage (Fig. 3C,D) (four cells were stained in 74% of 15 cases). Expression of the mesenchyme marker was also suppressed by *macho-1* MO injection in cleavage-arrested 110-cell embryos (Fig. 3E) (four cells were stained in 8% of 13 cases). This phenotype coincides well with the PVC-deficient embryos (Fig. 3F) (Kim et al., 2000). As will be described later, mesenchyme precursors in *macho-1*-deficient embryos assumed a notochord fate.

Next, to investigate in an opposite way whether *macho-1* plays a role as the PVC factor, we over- and/or mis-expressed *macho-1* by injecting 50 pg of synthetic *macho-1* mRNA into fertilized eggs. This resulted in ectopic formation of mesenchyme in the anterior region of the cleavage-arrested 110-cell embryos (ectopic staining: 71% of 17 cases; Fig. 3G, arrowheads). This phenotype coincides with the PVC-transplanted embryos (Kim et al., 2000). Mch-3 antibody recognizes particles in mesenchyme cells. For an unknown reason, the particles were somewhat dispersed in the anterior marginal zone. But the presence of these particles indicates that mesenchyme differentiates ectopically in the anterior blastomeres, which is never observed in the controls. This experiment was somewhat tricky, because when too much *macho-1* mRNA (100 pg) was injected into fertilized eggs, mesenchyme formation tended to be suppressed in the entire embryos (no staining: 43% of 14 cases). Every cell is likely to assume muscle fate in such condition, as we observed previously (Nishida and Sawada, 2001) (also see Fig. 5D).

Therefore, ectopic mesenchyme formation in the anterior region was confirmed in another way by isolating blastomeres of the eight-cell embryos (Table 1, Fig. 3H,I). Fig. 3H shows

the developmental fate of each blastomere of the eight-cell embryo. In normal embryos, mesenchyme cells originate from the posterior-vegetal blastomeres (B4.1 pairs), but not from the anterior-vegetal (A4.1 pairs) or animal blastomeres (a4.2 pairs and b4.2 pairs). When these blastomeres were isolated from control embryos injected with mRNA that encodes a mutant form of *macho-1* lacking the putative DNA-binding zinc-finger domain (Nishida and Sawada, 2001) and treated with BSA, only B4.1 partial embryos developed mesenchyme features, in all cases. FGF treatment causes muscle precursors to develop into mesenchyme cells (Kim et al., 2000). Therefore, the amount of mesenchyme cells increased in each B4.1 partial embryo treated with 2 ng/ml FGF protein, but still mesenchyme formation was restricted only to the B4.1 partial embryos. In embryos injected with wild type *macho-1* mRNA (50 pg), A4.1 blastomeres ectopically developed mesenchyme cells without FGF treatment. Further treatment with FGF increased the amount of formed mesenchyme cells in each A4.1 partial embryo (Fig. 3I). Most interestingly in embryos injected with *macho-1* mRNA, treatment with FGF led to ectopic formation of mesenchyme cells even in animal blastomeres (a4.2 pairs and b4.2 pairs). This is strong evidence that *macho-1* confers on blastomeres the responsiveness to be induced to mesenchyme when the cells receive FGF. Too much *macho-1* mRNA (100 pg) resulted in a decrease in the frequency of positive partial embryos forming mesenchyme cells (Table 1) as well as the amount of mesenchyme cells in each positive partial embryo again. The reduction of mesenchyme formation was restored to a certain extent by FGF treatment (Table 1).

Knockdown of *macho-1* results in ectopic notochord formation, and the overexpression suppresses notochord induction

Next, we investigated notochord formation in *macho-1*-deficient and -overexpressing embryos. Notochord formation was monitored by expression of two distinct markers. One was *HrBra*, a *Brachyury* homolog of *Halocynthia roretzi*, which has been shown to be a key transcription factor involved in notochord formation (Yasuo and Satoh, 1998; Takahashi et al., 1999a). The expression of *HrBra* is induced by an endodermal FGF signal at the 32-cell stage and is initiated at the 64-cell stage. The expression is strictly restricted to notochord precursors in ascidian embryos (Fig. 4A) (Yasuo and Satoh, 1993; Nakatani et al., 1996). Another marker was the notochord-specific Not1 antigen. As shown in Fig. 2D, Not1 antigen is expressed in differentiated notochord cells of the middle tailbud embryos. Fig. 4I shows the fate map of 110-cell embryos. Ten presumptive notochord blastomeres are colored pink. In cleavage-arrested 110-cell embryos, the antigen is expressed in notochord precursor blastomeres, and the maximum number of Not1-positive blastomeres never exceeded 10 [i.e. the number of notochord precursor blastomeres at the 110-cell stage (Fig. 4E)]. It has been shown that removal of the PVC results in mirror image duplication of the anterior half in the posterior region of the early embryos, and in ectopic expression of Not1 in the posterior region of cleavage-arrested 110-cell embryos (Nishida, 1994). We reconfirmed the phenotype by monitoring expression of *HrBra* in the 110-cell embryos and Not1 in the cleavage-arrested 110-cell embryos (Fig. 4B,F). In PVC-removed embryos, ectopic

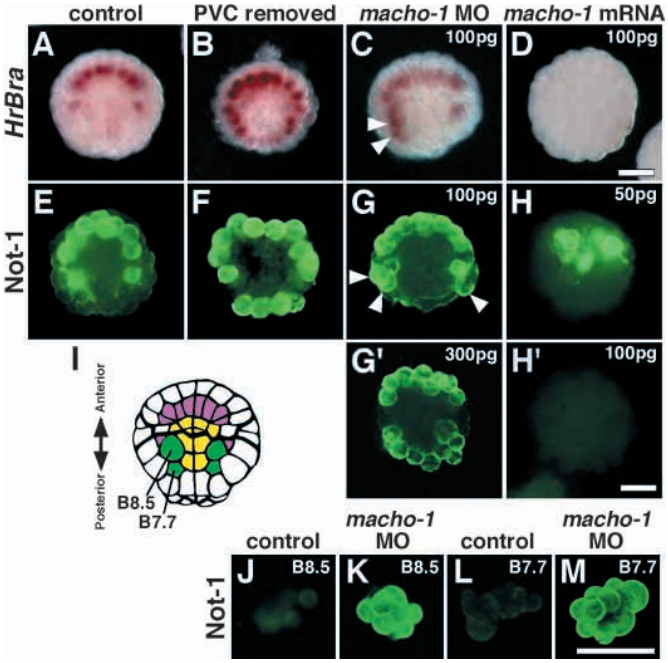

Fig. 4. Expression of notochord markers in *macho-1*-deficient and -overexpressing embryos. (A-D) Expression of *HrBra* gene at the 110-cell stage. (E-H,G′,H′) Expression of notochord-specific Not1 antigen in embryos whose cleavage was arrested at the 110-cell stage. (A,E) Embryos injected with control MO. Anterior is upwards. (B,F) PVC-removed embryos. (C,G,G′) Embryos injected with (C,G) 100 pg and (G′) 300 pg of *macho-1* MO. White arrowheads indicate ectopic expression in mesenchyme precursors. Anterior is upwards. (D,H,H′) Embryos injected with (D,H′) 100 pg and (H) 50 pg of *macho-1* mRNA. (I) The vegetal hemisphere of the 110-cell stage embryo. Color of blastomeres is the same as in Fig. 1. (J-M) Expression of Not1 antigen in the partial embryos derived from isolated mesenchyme precursor blastomeres. (J,K) B8.5 partial embryos. (L,M) B7.7 partial embryos. (J,L) Isolates from control embryos. (K,L) Isolates from embryos injected with 100 pg of *macho-1* MO. Scale bars: 100 μm.

notochord blastomeres were formed in the posterior region, and notochord blastomeres encircled the central endodermal area. The maximum number of Not1-positive blastomeres was 16 (Table 2).

We examined notochord formation in embryos injected with *macho-1* MO. Not1 antigen was expressed in every larva injected with *macho-1* MO (Fig. 2E,F). However, it was hard to tell the amount of notochord cells in such larvae. So cleavage was arrested at the 110-cell stage. In control embryos injected with control MO (300 pg), presumptive notochord cells expressed both markers, *HrBra* and Not1 (Fig. 4A,E, Table 2). In embryos injected with *macho-1* MO, ectopic expression of *HrBra* and Not1 in the posterior region was observed. The number of ectopically formed notochord cells was increased dose dependently. When a low dose (100 pg) of MO was injected, the maximum number of Not1-positive blastomeres was 14 (Fig. 4G, arrowheads). In these specimens, blastomeres in the posterior region never expressed Not1 antigen. We obtained the same results for the expression of *HrBra* (Fig. 4C, arrowheads). At a high dose (300 pg), the maximum number of Not1-positive blastomeres reached 17 (Table 2). They

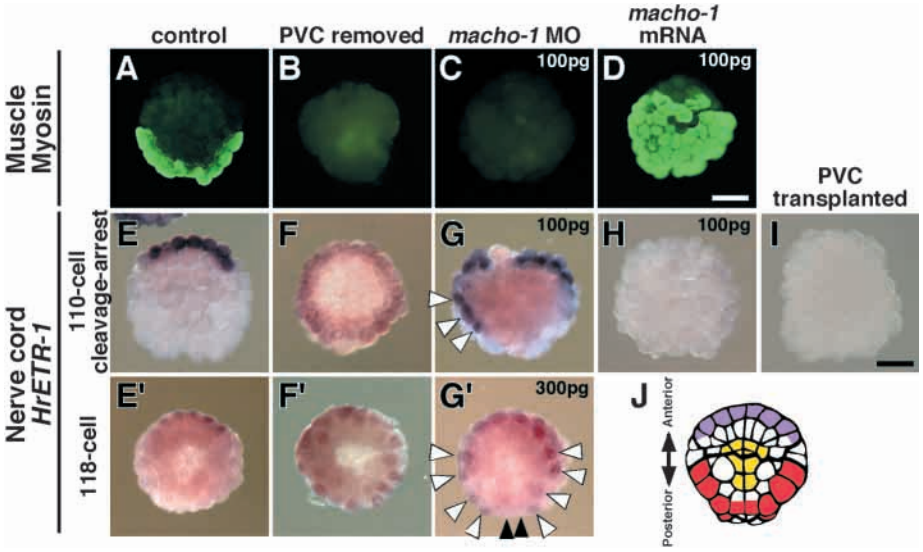

Fig. 5. Muscle and nerve cord formation in *macho-1*-deficient and -overexpressing embryos. (A-D) Expression of muscle myosin protein in embryos whose cleavage was arrested at the 110-cell stage. (E-I,E′-G′) Expression of *HrETR-1* in (E-I) cleavage-arrested 110-cell embryos and (E′-G′) 118-cell stage embryos without cleavage arrest. (A,E,E′) Embryos injected with control MO. Anterior is upwards. (B,F,F′) PVC-removed embryos. (C,G,G′) Embryos injected with (C,G) 100 pg and (G′) 300 pg of *macho-1* MO. White arrowheads indicate ectopic expression of *HrETR-1*. Black arrowheads indicate the posterior blastomeres that show no signal. Anterior is upwards. (D,H) 100 pg of *macho-1* mRNA was injected. (I) PVC-transplanted embryo. (J) The vegetal hemisphere of the 110-cell stage embryo. Color of blastomeres is the same as in Fig. 1. Scale bars: 100 μm.

encircled the central endodermal area (Fig. 4G′), as observed in the PVC-removed embryos (Fig. 4F).

To directly confirm the fate conversion of mesenchyme to notochord, presumptive mesenchyme blastomeres (B8.5 and B7.7 in Fig. 4I) were identified and isolated at the 110-cell stage and cultured as partial embryos without cleavage-arrest. Expression of Not1 was never observed in B8.5 (n=21) or B7.7 (n=18) partial embryos injected with control MO (Fig. 4J,L). By contrast, B8.5 and B7.7 partial embryos isolated from embryos injected with *macho-1* MO (100 pg) expressed Not1 in 63% (n=40) and 39% (n=53) of cases, respectively (Fig. 4K,M).

Transplantation of the PVC to the anterior region suppressed notochord formation (Fig. 1F). To examine whether *macho-1* overexpression reproduces the phenotype of PVC-transplanted embryos, we examined notochord formation in *macho-1*-overexpressing embryos. Injection of *macho-1* mRNA resulted in the decrease (50 pg, n=32) or loss (100 pg, n=29) of Not1 expression in cleavage-arrested 110-cell embryos and *HrBra* expression at the 110-cell stage (Fig. 4D,H,H′). In these cases, as mentioned in the previous section, presumptive notochord blastomeres presumably failed to develop into notochord because they assumed mesenchyme or muscle fates.

Muscle precursor blastomeres assume nerve cord fate without *macho-1*

macho-1-deficient embryos lose primary muscle cells (Fig. 2B,C) (Nishida and Sawada, 2001). However, it is not known what kind of tissue cell the muscle precursor cells are converted to in those embryos, although we expected it to be nerve cord (Fig. 1E). Formation of nerve cord has not yet been examined in PVC-removed and -transplanted embryos or in *macho-1*-deficient and -overexpressing embryos. Therefore, we examined muscle and nerve cord formation in these embryos to fully understand fate specification in the marginal zone of early ascidian embryos, although this issue is not directly relevant to the mechanisms that control cellular responsiveness.

Fig. 5J shows a fate map of 110-cell stage embryos; 10 presumptive primary-muscle blastomeres are colored red. First, we examined muscle formation in *macho-1*-deficient and -overexpressing embryos. In control embryos, when cleavage was arrested at the 110-cell stage, 10 muscle precursors eventually expressed muscle myosin, as expected from the fate map (Fig. 5A). In embryos injected with *macho-1* MO, expression of muscle myosin was suppressed (Fig. 5C), as in the PVC-deficient embryos (Fig. 5B). By contrast,

Table 2. Number of Not1-positive blastomeres in cleavage-arrested 110-cell embryos

			Number of blastomeres (%)							
	Dose	n	≤10	11	12	13	14	15	16	17
PVC removal	–	16	**44**	**6**	**13**	**6**	**13**	**13**	**6**	0
Control MO	300 pg	15	**100**	0	0	0	0	0	0	0
macho-1 MO	100 pg	23	**43**	**13**	**22**	0	**22**	0	0	0
macho-1 MO	300 pg	13	**38**	**23**	0	0	**8**	0	**23**	**8**

			Number of blastomeres (%)							
			3	4	5	6	7	8	9	10
lacZ mRNA	100 pg	15	0	0	0	0	0	0	**7**	**93**
Hrsna mRNA	100 pg	31	**6**	**6**	**19**	**26**	**10**	**13**	**6**	**13**

Cleavages of embryos were permanently arrested at the 110-cell stage.
Positive proportions are indicated in bold.

overexpression of the *macho-1* mRNA resulted in ectopic formation of muscle cells (Fig. 5D), as observed previously in PVC-transplanted embryos (Nishida, 1994). All of these results reconfirm the previous results, showing the validity of *macho-1* MO and mRNA, as well as cytoplasmic removal and transplantation in the present study.

Eight nerve cord precursor blastomeres are colored purple in the fate map (Fig. 5J). We investigated nerve cord formation by monitoring the expression of a neural plate marker gene, *HrETR-1*, in PVC-removed embryos and *macho-1*-deficient embryos. The expression of *HrETR-1* is restricted in neural plate precursors at the 110- or 118-cell stage in ascidian embryos (Fig. 5E,E′) (Yagi and Makabe, 2001). *HrETR-1* gene expression was monitored in cleavage-arrested 110-cell embryos and 118-cell embryos without cleavage-arrest. In cleavage-arrested 110-cell embryos (*n*=30) and 118-cell embryos (*n*=33) injected with control MO, nerve cord precursors expressed *HrETR-1*, as expected from fate map (Fig. 5E,E′). The number of *HrETR-1*-positive blastomeres in the vegetal hemisphere ranged form six to eight.

Removal of the PVC (total number examined=60) resulted in ectopic expression of *HrETR-1* in the entire marginal zone (Fig. 5F,F′). Thus, presumptive muscle blastomeres assumed nerve cord fate in these embryos. In embryos injected with *macho-1* MO (total number examined=87), ectopic expression of *HrETR-1* was also observed in the lateral and posterior region, which corresponds to muscle blastomeres (Fig. 5G,G′, white arrowhead). Ectopic nerve cord formation in the posterior-vegetal region in *macho-1*-deficient embryos was also confirmed by isolation of blastomeres at the eight-cell stage (data not shown). The nerve cord phenotype was similar in both *macho-1*-deficient and PVC-deficient embryos. However, in *macho-1*-deficient embryos, the posterior (B7.5) cells never expressed *HrETR-1* (Fig. 5G′, black arrowheads), while in PVC removed embryos, the posterior cells were ectopically expressed *HrETR-1*. We noticed that the posterior (B7.5) blastomeres in *macho-1*-deficient embryos assumed an endoderm fate but not a nerve cord fate (data not shown). We then examined nerve cord formation in *macho-1*-overexpressing embryos and PVC-transplanted embryos. In embryos injected with *macho-1* mRNA (100 pg), *HrETR-1* expression was completely suppressed (*n*=34) (Fig. 5H), as well as in the PVC-transplanted embryos (*n*=8) (Fig. 5I). These results indicate that muscle precursors assume a nerve cord fate without *macho-1*.

Zygotic expression of *snail* is downstream of *macho-1* and inhibits notochord formation

snail seems a good candidate for mediating suppression of a notochord fate in presumptive mesenchyme precursors. We first examined whether zygotic expression of a *snail* homolog, *Hrsna*, occurs downstream of maternal *macho-1* (Fig. 6A). Injection of control mutant form of *macho-1* mRNA (100 pg) had no effect on *Hrsna* expression at the 64-cell stage (*n*=32). When *macho-1* mRNA (100 pg) was injected, *Hrsna* expression was ectopically activated (*n*=39). However, injection of *macho-1* MO (100 pg) suppressed *Hrsna* expression (*n*=22). Therefore, *macho-1* is necessary and sufficient for *Hrsna* expression.

Then we injected synthetic *Hrsna* mRNA to investigate the effects of *Hrsna* on notochord formation. Injection of *Hrsna* mRNA (100 pg) resulted in short-tailed embryos (Fig. 6B). To evaluate notochord formation, we carried out a cleavage-arrest experiment. As mentioned previously, 10 notochord precursor blastomeres eventually expressed Not1 antigen when cleavage was permanently arrested at the 110-cell stage. Injection of control *lacZ* mRNA (100 pg) had no effect on Not1 expression. By contrast, the number of Not1-positive blastomeres was significantly reduced in embryos injected with *Hrsna* mRNA (100 pg) (Fig. 6C, Table 2). Muscle and mesenchyme formation detected with the Mu-2 (*n*=37) and Mch-3 (*n*=33) antibodies was not affected in the cleavage-arrested 110-cell embryos injected with *Hrsna* mRNA (data not shown). We also examined *HrBra* expression in notochord blastomeres at the 110-cell stage (Fig. 6D). The expression became weak and punctate, and the number of *HrBra*-positive blastomeres decreased in embryos injected with *Hrsna* mRNA (*n*=13), but not in embryos injected with control *lacZ* mRNA.

Discussion

The same signal is used to elicit different outcomes in different cells in animal embryogenesis. The response to inductive signals depends on the internal state of the signal-receiving cells. Intrinsic factors thus determine the way a cell responds. We have focused on this issue using ascidian embryos. In ascidian embryos, mesenchyme and notochord fates are induced by the same FGF signaling molecule originating from endoderm precursors. The PVC of eggs causes the difference in the responsiveness of mesenchyme and notochord precursor blastomeres. We have demonstrated that *macho-1*, first identified as a muscle determinant, also plays a role as an intrinsic factor that controls the responsiveness of mesenchyme blastomeres to an inducing signal.

The effect of *macho-1* MO

We used antisense morpholino oligonucleotides (MO) to prevent the function of *macho-1* by inhibiting its translation. The phenotype caused by a low dose injection (100 pg) is almost the same as that seen in our previous study using phosphorothioate DNA oligonucleotides (S-DNA) to deplete *macho-1* mRNA (Nishida and Sawada, 2001). However, a high dose injection (300 pg) resulted in a phenotype that is similar to that of PVC-removed larvae. This phenotype is unlikely to have been the result of non-specific toxic effects, because notochord cells were formed in every *macho-1*-deficient embryo, and embryonic cells transfated rather than failed to differentiate. Thus, it is plausible that MO inhibited the *macho-1* functions more efficiently than did S-DNA. This conclusion is supported by the observation that injection of another MO that covers a different region of *macho-1* mRNA produced similar phenotypes as the main MO we used in this studies (data not shown).

No muscle cells formed in high-dose-injected embryos (Fig. 2C). This phenotype could be an indirect effect of the prevention of *macho-1* functions. Formation of the primary lineage (B-line) of muscle cells depends on maternal *macho-1* (Nishida and Sawada, 2001). However, the fate of the secondary lineage (A-line and b-line) of muscle cells is specified by cell interactions, probably during gastrulation (Nishida, 1990). Although the inducer cells and the inducing signal involved in secondary muscle formation are as yet

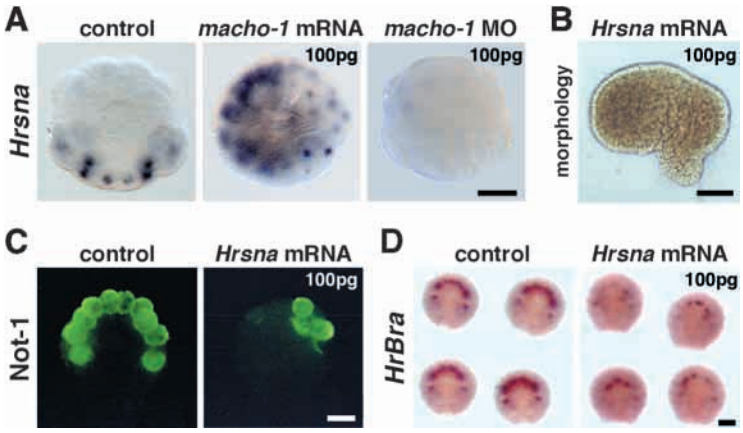

Fig. 6. *Hrsna* is downstream of *macho-1* and inhibits notochord formation. (A) Expression of *Hrsna* at the 64-cell stage in embryos injected with mutant *macho-1* mRNA (100 pg, control), *macho-1* mRNA (100 pg) and *macho-1* MO (100 pg). Anterior is upwards. (B) Morphology of the embryo injected with 100 pg of *Hrsna* mRNA. (C) Expression of notochord-specific Not1 antigen in cleavage-arrested 110-cell embryos injected with *lacZ* mRNA (100 pg, control) or *Hrsna* mRNA (100 pg). Anterior is upwards. (D) Expression of *HrBra* in the 110-cell stage embryos injected with *lacZ* mRNA (100 pg, control) or *Hrsna* mRNA (100 pg). Anterior is upwards. Scale bars: 100 μm.

unknown, it is possible that inhibition of the *macho-1* function would perturb secondary muscle induction.

macho-1 is not only a muscle determinant but also a main component of the PVC factor

We investigated the formation of mesenchyme and notochord in *macho-1*-deficient and -overexpressing embryos. In *macho-1*-deficient embryos, mesenchyme formation was completely suppressed, and instead ectopic notochord formation was promoted in the presumptive mesenchyme precursors. Conversely, in *macho-1*-overexpressing embryos, notochord formation was suppressed, and ectopic mesenchyme formation was observed in the anterior-vegetal region. These phenotypes were the same as those of PVC-removed embryos and PVC-transplanted embryos, respectively. These results support the idea that maternal mRNA of *macho-1*, first identified as a muscle determinant, also plays a role as an intrinsic factor that controls the responsiveness of mesenchyme blastomeres. Most importantly, in embryos injected with *macho-1* mRNA, treatment with FGF led to ectopic mesenchyme formation even in animal blastomeres. This result provides strong evidence that *macho-1* plays a key role in determining that cells are induced to develop into mesenchyme when they receive the FGF signal.

macho-1-deficient embryos reproduced only some of the phenotypes of the PVC-removed embryos. In the PVC-removed embryos, in addition to loss of muscle and mesenchyme, the cleavage pattern of the posterior-vegetal region was converted to that of the anterior-vegetal region (Nishida, 1994). The cleavage pattern of *macho-1*-deficient embryos was normal at least up to the gastrula stage. Therefore, PVC is likely to have two distinct functions. One function is muscle and mesenchyme formation, and is accounted for by maternal mRNA of *macho-1*. Another function of PVC is the generation of the posterior cleavage pattern. It has been reported that a unique subcellular structure designated the centrosome-attracting body (CAB), which exists in the posterior pole cortex of cleaving embryos, plays essential roles in generating the posterior cleavage pattern and the unequal cleavages within it (Hibino et al., 1998; Nishikata et al., 1999; Iseto and Nishida, 1999). Removal of the PVC results in loss of the CAB. Transplantation of the PVC into the anterior region causes ectopic formation of the CAB in the anterior region, and the cleavage pattern of the anterior region converts to the posterior type (Nishida, 1994; Nishikata et al., 1999). Therefore, another molecule involved in the formation of the CAB would also be present in the PVC. Several kinds of maternal mRNA show a similar localization pattern to that of *macho-1*, namely localization to PVC in fertilized eggs (Yoshida et al., 1996; Satou and Satoh, 1997; Sasakura et al., 1998a; Sasakura et al., 1998b; Sasakura et al., 2000; Satou, 1999; Caracciolo et al., 2000; Makabe et al., 2001; Nishida and Sawada, 2001; Nishikata et al., 2001; Nakamura et al., 2003).

Snail is downstream of *macho-1* and mediates suppression of notochord fate in mesenchyme precursors

Expression of ascidian *snail* preferentially starts in the mesenchyme-muscle precursor blastomeres at the 32-cell stage or the 44-cell stage (Erives et al., 1998; Wada and Saiga, 1999). Our preliminary results show that the expression of *snail* depends on the presence of the PVC (A. Yamada, H. Yamamoto and H. Nishida, unpublished). Similarly, the phenotype of *macho-1*-deficient and -overexpressing embryos indicates that *macho-1* is necessary and sufficient for zygotic *Hrsna* expression in these blastomeres. The overexpression of *Hrsna* reduced *HrBra* and Not1 antigen expression. These results suggest that zygotic *Hrsna* expression mediates the suppression of notochord fate by maternal *macho-1* in the posterior region. This idea is supported by the following observations. Snail is a zinc-finger protein known to be a transcription repressor in *Drosophila* (Ip et al., 1992). In *Ciona intestinalis*, misexpression of *snail* in notochord-lineage cells driven by a heterologous promoter suppresses at least the expression of the reporter gene driven by the *Brachyury* minimal promoter through Snail-binding sites within it. However, neither endogenous *Brachyury* expression nor the formation of notochord was suppressed in experiments (Fujiwara et al., 1998), contrary to our results.

The difference between these previous results and ours is the mode of misexpression. In *Ciona*, misexpression of *snail* was driven by the *Brachyury* promoter, which promotes misexpression after the 64-cell stage, whereas *snail* expression starts at the 32-cell stage or the 44-cell stage in normal embryos. That stage could be too late for misexpressed *snail* to suppress initiation of endogenous *Brachyury* expression. In the present study, we injected synthetic *snail* mRNA into eggs. Therefore, enough protein could accumulate before the initiation of *Brachyury* expression. Of course, the difference may be attributed to species difference. But this explanation is

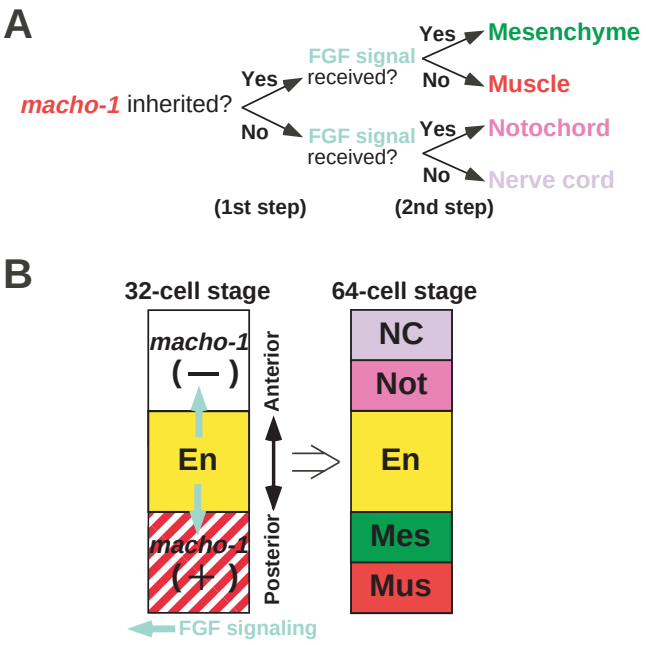

Fig. 7. Two-step model of binary fate specification in the marginal cells of the vegetal hemisphere in ascidian embryos. (A) There are two distinct steps to specify the four cell types. The first step is inheritance (or not) of *macho-1*. The second step is receipt (or not) of the FGF signal. (B) Presence or absence of *macho-1* is responsible for making cell responses different in mesenchyme and notochord induction. The molecular identity of the PVC factor shown in Fig. 1D is the *macho-1* product.

unlikely, because the promoters of *Ciona* and *Halocynthia Brachyury* are interchangeable and are able to drive notochord expression in either species (Takahashi et al., 1999b). It is not known whether there is a Snail-binding site in the *Halocynthia Brachyury* promoter, but above-mentioned observations suggests that a similar mechanism operates in *Ciona* and *Halocynthia*. Thus, our results confirm that *snail* is indeed involved in suppression of notochord fate in the posterior blastomeres. To confirm the role of *Hrsna* in suppression of notochord fate, we injected MO complementary to *Hrsna*. However, there was no ectopic notochord formation. At the moment, it is not clear whether the *Hrsna* MO was not sufficiently effective or there is a redundant mechanism to suppress notochord fate other than that involving *Hrsna*. Furthermore, overexpression of *Hrsna* was not enough to promote ectopic mesenchyme formation, suggesting that *Hrsna* is involved only in suppression of a notochord fate but not in promotion of a mesenchyme fate.

Model for fate specification in the vegetal-marginal cells of ascidian embryos: two-step model

The default fates of mesenchyme precursors and notochord precursors are muscle and nerve cord, respectively (Fig. 1D). In this study, we also investigated formation of muscle and nerve cord in *macho-1*-deficient and -overexpressing embryos. In *macho-1*-deficient embryos, the formation of muscle was suppressed, and *HrETR-1*, a neural plate marker gene, was ectopically expressed in the presumptive muscle blastomeres. On the other hand, in *macho-1*-overexpressing embryos, *HrETR-1* expression was suppressed and ectopic formation of muscle was observed. These results together led us to propose a simple model for fate specification in ascidian embryos (Fig. 7).

Two steps of binary specification of cell fates operate in the marginal zone of the vegetal hemisphere. Depending on presence or absence of *macho-1* (the first step), and depending on reception of the FGF signal (the second step), four types of cell are generated in the marginal zone of the vegetal hemisphere (Fig. 7A). The marginal cells receive an endodermal FGF signal from the vegetal pole at the 32-cell stage (Fig. 7B), and only one of the daughter cells facing the endoderm assumes an induced cell fate, namely mesenchyme or notochord. A directed signal that emanates from endoderm blastomeres polarizes the responding blastomeres at the 32-cell stage and promotes asymmetric divisions that operate in both the anterior and posterior regions (Kim et al., 2000; Minokawa et al., 2001; Nishida, 2002). The posterior marginal cells inheriting Macho-1 protein divide into two daughters that assume default muscle and induced mesenchyme fates. The anterior marginal cells without Macho-1 protein divide into two daughters that assume default nerve cord and induced notochord fates. Thus, although *macho-1* was first identified as a muscle determinant, it is also required in mesenchyme formation. Indeed, *macho-1* promotes muscle fate as a default fate, but it directs the mesenchyme pathway when cells receive the FGF signal. It is noteworthy that if too much Macho-1 protein was present, the cells were assigned to default muscle fate irrespective of reception of the FGF signal (Fig. 5D; Table. 1).

The future question is how cells integrate the intrinsic activity of *macho-1* with information from extrinsic cues that are delivered into the cell by the signal-transduction machinery. The Macho-1 protein has five CCHH-type zinc-finger repeats that show similarity with Zic, GLI and odd-paired proteins (Nishida and Sawada, 2001). All of these proteins are transcription factors. Because Macho-1 protein synthesized from FLAG-tagged mRNAs accumulates in the nuclei during the cleavage stage, it was suggested that Macho-1 functions as a transcription factor (Nishida and Sawada, 2001). Our recent results using VP16 and En^R fusion protein further support the possibility that Macho-1 indeed functions as a transcription activator (K. Sawada and H. Nishida, unpublished). Recently, we also found that an Ets transcription factor is the target activated by FGF-MAPK (ERK1/2) signaling and is involved in notochord and mesenchyme induction in ascidians (Miya and Nishida, 2003). Thus, it is important to elucidate how these two transcription factors cooperate to promote mesenchyme fate. There are two possibilities. The Ets transcription factor is known to interact with other transcription factors to direct signals for the transcription of specific target genes (Sharrocks, 2001). For example, it has been shown that mammalian Ets1 interacts with Pit-1, a pituitary-specific POU-homeodomain protein, and activates the transcription of pituitary-specific genes (Bradford et al., 1997). Another possibility is that inputs from the signal resulting in Ets activation and Macho-1 activity could be combined at the level of regulatory regions of the target genes without direct interaction of either Ets or Macho-1 protein. Both factors might independently bind to *cis*-regulatory elements and cooperate to activate or silence the target gene transcription.

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References

Bradford, A. P., Wasylyk, C., Wasylyk, B. and Gutierrez-Hartmann, A. (1997). Interaction of Ets-1 and the POU-homeodomain protein GHF-1/Pit-1 reconstitutes pituitary-specific gene expression. *Mol. Cell. Biol.* **17**, 1065-1074.

Caracciolo, A., Di Gregorio, A., Aniello, F., Di Lauro, R. and Branno, M. (2000). Identifcation and developmental expression of three *Distal-less* homeobox containing genes in the ascidian *Ciona intestinalis*. *Mech. Dev.* **99**, 173-176.

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

Erives, A., Corbo, J. C. and Levine, M. (1998). Lineage-specific regulation of the *Ciona snail* gene in the embryonic mesoderm and neuroectoderm. *Dev. Biol.* **194**, 213-225.

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

Heasman, J. (2002). Morpholino oligos: making sense of antisense? *Dev. Biol.* **243**, 209-214.

Hibino, T., Nishikata, T. and Nishida, H. (1998). Centrosome-attracting body: A novel structure closely related to unequal cleavages in the ascidian embryo. *Dev. Growth Differ.* **40**, 85-95.

Imai, K. S., Satoh, N. and Satou, Y. (2002). Early embryonic expression of *FGF4/6/9* gene and its role in the induction of mesenchyme and notochord in *Ciona savignyi* embryos. *Development* **129**, 1729-1738.

Ip, Y. T., Park, R. E., Kosman, D., Bier, E. and Levine, M. (1992). The dorsal gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. *Genes Dev.* **6**, 1728-1739.

Iseto, T. and Nishida, H. (1999). Ultrastructual studies on the centrosome-attracting body: Electron-dense matrix and its role in unequal cleavage in ascidian embryos. *Dev. Growth Differ.* **41**, 601-609.

Kim, G. J., and Nishida, H. (1998). Monoclonal antibodies against differentiating mesenchyme cells in larvae of the ascidian *Halocynthia roretzi*. *Zool. Sci.* **15**, 553-559.

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

Kim, G. J. and Nishida, H. (2001). Role of the FGF and MEK signaling pathway in the ascidian embryo. *Dev. Growth Differ.* **43**, 521-533.

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

Makabe, K. W., Kawashima, T., Kawashima, S., Minokawa, T., Adachi, A., Kawamura, H., Ishikawa, H., Yasuda, R., Yamamoto, H., Kondoh, K. et al. (2001). Large-scale cDNA analysis of the maternal genetic information in the egg of *Halocynthia roretzi* for a gene expression catalog of ascidian development. *Development* **128**, 2555-2567.

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

Minokawa, T., Yagi, K., Makabe, K. W. and Nishida, H. (2001). Binary specification of nerve cord and notochord cell fates in ascidian embryos. *Development* **128**, 2007-2017.

Miya, T., Morita, K., Suzuki, A., Ueno, N. and Satoh, N. (1997). Functional analysis of an ascidian homologue of vertebrate *Bmp-2/Bmp-4* suggests its role in the inhibition of neural fate specification. *Development* **124**, 5149-5159.

Miya, T. and Nishida, H. (2003). An Ets transcription factor, *HrEts*, is target of FGF signaling and involved in induction of notochord, mesenchyme, and brain in ascidian embryos. *Dev. Biol.* **261**, 25-38.

Nakamura, Y., Makabe, K. W. and Nishida, H. (2003). Localization and expression pattern of type I postplasmic mRNAs in embryos of the ascidian *Halocynthia roretzi*. *Gene Expr. Patterns* **3**, 71-75.

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

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

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

Nishida, H. (1990). Determinative mechanisms in secondary muscle lineages of ascidian embryos: development of muscle-specific features in isolated muscle progenitor cells. *Development* **108**, 559-568.

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

Nishida, H. (2002). Patterning the marginal zone of early ascidian embryos: localized maternal mRNA and inductive interactions. *BioEssays* **24**, 613-624.

Nishida, H. (2003). Spatio-temporal pattern of the activation of MAP kinase in embryos of the ascidian *Halocynthia roretzi*. *Dev. Growth Differ.* **45**, 27-37.

Nishida, H. and Sawada, K. (2001). macho-1 encodes a localized mRNA in ascidian egg that specifies muscle fate during embryogenesis. *Nature* **409**, 724-729.

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

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

Nishikata, T., Hibino, T. and Nishida, H. (1999). The centrosome-attracting body, microtubule system, and posterior egg cytoplasm are involved in positioning of cleavage planes in the ascidian embryo. *Dev. Biol.* **209**, 72-85.

Nishikata, T., Yamada, L., Mochizuki, Y., Satou, Y., Shin-i, T., Kohara, Y. and Satoh, N. (2001). Profiles of maternally expressed genes in fertilized eggs of *Ciona intestinalis*. *Dev. Biol.* **238**, 315-331.

Sasakura, Y. Ogasawara, M. and Makabe, K. W. (1998a). *HrWnt-5*: a maternally expressed ascidian *Wnt* gene with posterior localization in early embryos. *Int. J. Dev. Biol.* **42**, 573-580.

Sasakura, Y. Ogasawara, M. and Makabe, K. W. (1998b). Maternally localized RNA encoding a serine/threonine protein kinase in the ascidian, *Halocynthia roretzi*. *Mech. Dev.* **76**, 161-163.

Sasakura, Y. Ogasawara, M. and Makabe, K. W. (2000). Two pathways of maternal RNA localization at the posterior-vegetal cytoplasm in early ascidian embryos. *Dev. Biol.* **220**, 365-378.

Satou, Y. (1999). *posterior end mark 3* (*pem-3*), an ascidian maternally expressed gene with localized mRNA encodes a protein with *Caenorhabditis elegans* MEX-3-like KH domains. *Dev. Biol.* **212**, 337-350.

Satou, Y. and Satoh, N. (1997). *posterior end mark 2 (pem-2)*, *pem-4*, *pem-5*, and *pem-6*: maternal genes with localized mRNA in the ascidian embryo. *Dev. Biol.* **192**, 467-481.

Satou, Y., Imai, K. S. and Satoh, N. (2001). Action of morpholinos in *Ciona* embryos. *Genesis* **30**, 103-106.

Sharrocks, A. D. (2001). The ETS-domain transcription factor family. *Nature Rev. Mol. Cell Biol.* **2**, 827-837.

Shimauchi, Y., Murakami, S. D. and Satoh, N. (2001). FGF signals are involved in the differentiation of notochord cells and mesenchyme cells of the ascidian *Halocynthia roretzi*. *Development* **128**, 2711-2721.

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

Takahashi, H., Mitani, Y., Satoh, G. and Satoh, N. (1999b). Evolutionary alterations of the minimal promoter for notochord-specific *Brachyury* expression in ascidian embryos. *Development* **126**, 3725-3734.

Wada, S. and Saiga, H. (1999). Cloning and embryonic expression of *Hrsna*, a *snail* family gene of the ascidian *Halocynthia roretzi*: Implication in the

origins of mechanisms for mesoderm specification and body axis formation in chordates. *Dev. Growth Differ.* **41**, 9-18.

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

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

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

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

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

Yoshida, S., Marikawa, Y. and Satoh, N. (1996). *posterior end mark*, a novel maternal gene encoding a localized factor in the ascidian embryo. *Development* **122**, 2005-2012.