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A bHLH transcription factor gene, *Twist-like1*, is essential for the formation of mesodermal tissues of *Ciona* juveniles

Miki Tokuoka [a,*,1], Nori Satoh [a,b], Yutaka Satou [a]

[a] *Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*
[b] *CREST, Japan Science and Technology Agency, Kawaguchi, Saitama 330-0012, Japan*

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Abstract

Ascidian larval mesenchyme cells, comprising about 900 cells, are derived from the A7.6, B8.5 and B7.7 blastomere pairs in the 110-cell embryo. Previous studies showed that the properties of mesenchyme cells are not uniform among the three lines in embryos of *Ciona savignyi* and *Ciona intestinalis*. After metamorphosis, the larval mesenchyme cells form the mesodermal tissues or organs of the adult body. In the present study, the developmental fates of A7.6-, B8.5- and B7.7-line mesenchyme cells were traced using DiI to determine the origins of juvenile mesodermal tissues of *C. savignyi*. It was demonstrated that each of the A7.6-, B8.5- and B7.7-line mesenchyme cells is distributed in different positions of the larval trunk, and then give rise to the different mesodermal tissues of juveniles. *Twist-like1* is a transcription factor gene essential for the specification of larval mesenchyme cells. Knockdown of this gene with specific morpholino antisense oligonucleotides affected not only the specification of larval mesenchyme cells, but also the formation of most of the mesodermal tissues of juveniles. The juvenile mesodermal tissues in the *Twist-like1*-knockdown specimen were never compensated by the surrounding tissues. The present results therefore indicate that *Twist-like1* is required for the differentiation of most mesodermal precursors of adults.

Keywords: Cell lineages; Mesenchyme cells; *Ciona savignyi*; *Twist-like1*; Juvenile; Mesoderm development

Introduction

Ascidians belong to the subphylum Urochordata, which is one of three chordate groups. Their fertilized eggs develop into tadpole-type larvae, which consist of about 2600 cells that form several distinct types of tissues (Satoh, 1994). The tail of tadpole-type larvae contains the notochord, flanked dorsally by the nerve cord, ventrally by the endodermal strand and bilaterally by three rows of muscle cells. These features represent the basic body plan of all chordates (Kowalevsky, 1871). Then, swimming larvae metamorphose into sessile adults. Metamorphosis begins with the resorption of the tail into the trunk region with the subsequent outgrowth of ampullae (Cloney, 1978; Numakunai et al., 1964). In *Ciona intestinalis*, juveniles develop most of the adult organs, including the gonads, and become mature after approximately 2–3 months (Chiba et al., 2004). Although the overall appearance of an ascidian adult looks different from that of vertebrates, the gill openings and endostyle show a close phylogenetic relationship between ascidians and vertebrates. The gill openings are characteristic features of all chordates, and the endostyle is thought to be homologous to the vertebrate thyroid gland (Goodbody, 1974). Furthermore, the adult ascidian has blood cells, digestive organs, a heart and a gonad, all of which are not formed in the larva. Therefore, understanding the developmental mechanisms of ascidian adult tissues is as important as understanding those of larval tissues.

The ascidian larval mesenchyme is believed to be essential for making the adult body. The larval mesenchyme cells are derived from A7.6 (trunk lateral cells, TLCs), B8.5 and B7.7 blastomeres in the 110-cell embryo. These cells are specified by FGF9/16/20, which represents an ancestral form of vertebrate FGF9, FGF16 and FGF20 (Imai et al., 2002; Satou et al., 2002). In *Ciona* embryos, the FGF9/16/20 signal

* Corresponding author. Fax: +81 6 6850 5472.
E-mail address: miki@bio.sci.osaka-u.ac.jp (M. Tokuoka).
[1] Present address: Department of Biology, Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka 560-0043, Japan.

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activates a bHLH transcription factor gene, *Twist-like1*. *Twist-like1* is the most likely candidate for ortholog of *Twist* and triggers the genetic cascade for the differentiation of A7.6/B8.5/B7.7-line mesenchyme (Satou et al., 2003; Imai et al., 2003; Tokuoka et al., 2004). However, the genetic cascades downstream of *Twist-like1* are different in all the three lines (Tokuoka et al., 2004). Different properties of A7.6, B8.5 and B7.7-line mesenchyme cells suggest that these three lines contribute to different tissues in adults after metamorphosis.

In addition to these three lines of mesenchyme cells, ascidian larvae contain another mesenchymal cells, namely, trunk ventral cells (TVCs), which are derived from B7.5 blastomeres (Whittaker, 1990). In *Ciona savignyi*, a key gene for TVC differentiation is *Cs-Mesp*, a sole ortholog of vertebrate bHLH transcription factor genes, *Mesp* (Satou et al., 2004). The molecular mechanism of differentiation of TVCs is different from the other three lines of mesenchyme cells (Satou et al., 2004). Therefore, in the present study, the term "larval mesenchyme cells" is used to include A7.6, B8.5 and B7.7-line cells and does not include B7.5-line cells.

Hirano and Nishida traced the cell lineage after metamorphosis with horseradish peroxidase (HRP) in another ascidian, *Halocynthia roretzi*, and showed that most mesodermal tissues of juveniles are derived from larval mesenchyme cells (Hirano and Nishida, 1997). In *Halocynthia*, A7.6-line mesenchyme cells give rise to body-wall muscle (oral siphon and longitudinal mantle muscles), epithelium of 1st/2nd gill-slits and blood cells of the juvenile, while both the B8.5- and B7.7-line mesenchyme cells give rise to tunic cells. Tunic cells derived from B8.5-line are scattered throughout the entire tunic, and these, derived from the B7.7-line, tended to be concentrated near the holdfast. In other words, larval mesenchyme cells are "stem cells" of mesodermal tissues of juveniles and adults.

As mentioned above, a bHLH transcription factor gene, *Twist-like1*, is essential for the differentiation of larval mesenchyme cells. *Cs-Twist-like1* mRNA was detected in the larval mesenchyme precursors only between 64-cell stage and neurula stage (Imai et al., 2003) and never detected in tailbud embryos, larvae and mature juveniles when quantification of the amount of *Cs-Twist-like1* mRNA was performed with quantitative RT-PCR (data not shown). It is possible that *Twist-like1* is essential for the formation of "stem cells" of mesodermal tissues of juveniles. However, it is not yet clear whether the effect of *Twist-like1* on the differentiation of larval mesenchyme cells extends to the formation of juvenile mesodermal tissues derived from larval mesenchyme cells. Although the previous study showed the effect of *Twist-like1* on the differentiation of larval mesenchyme cells, the effects of *Twist-like1* on the morphology of metamorphosing larvae and juveniles were not examined (Imai et al., 2003; Tokuoka et al., 2004). It is also unclear whether the developmental fates of *Ciona* larval mesenchyme cells are the same as those of *Halocynthia* because the two species, *Halocynthia* and *Ciona*, are evolutionarily distant. *Halocynthia* is an Enterogona ascidian, and *Ciona* is a Pleurogona ascidian, these two being major and distinct orders of ascidians. Therefore, it should be determined whether the result of the lineage trace experiments leading to that conclusion is also valid for *Ciona*.

In the present study, we first examined the developmental fates of three types of mesenchyme, A7.6, B8.5 and B7.7 lines after metamorphosis in *C. savignyi*. Then, we examined whether *Twist-like1*, which is expressed in larval mesenchyme cells, is essential for the formation of juvenile mesodermal tissues.

Material and methods

Ascidian eggs, embryos and juveniles

C. savignyi adults were obtained from the Maizuru Fisheries Research Station of Kyoto University and the International Coastal Research Centre of the Ocean Research Institute, the University of Tokyo. They were maintained in aquaria in our laboratory at Kyoto University under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoducts. After insemination, eggs were reared at 18°C in millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate. We used the developmental staging of *Ciona* juveniles by Chiba et al. (2004).

Microinjection of morpholino oligo

In the present study, we used a 25-mer morpholino oligo (hereafter referred to as 'morpholino'; Gene Tools, LLC) for *Cs-Twist-like1* (5′-CTTGATTG-TACTCTAGTGATGTCAT-3′). The specificity of this morpholino was confirmed in the previous study (Imai et al., 2003). As a control, morpholino against *lacZ* was used as described in previous report (Satou et al., 2001). Morpholinos (15 pmol) were injected into intact eggs before fertilization or dechorionated eggs after fertilization, as described previously (Imai et al., 2003). Injected eggs were reared at about 18°C in MFSW containing 50 μg/ml streptomycin sulfate.

DiI labeling of blastomeres

DiI labeling of blastomeres was performed basically as described previously (Satou et al., 2004). DiI (CellTracker CM-DiI, Molecular Probes) was dissolved in soybean oil at a concentration of 10 mg/ml. We labeled A7.6, B8.5 and B7.7 blastomeres of the 64- and 110-cell stage embryo with or without intact chorions to trace the cell lineage. DiI-labeled embryos, larvae and juveniles were observed using fluorescent microscopy.

Iontophoretic injection and histochemical detection of HRP

Iontophoretic injection and histochemical detection of HRP were performed basically as described previously in Hirano and Nishida (1997), except that follicle cells were removed by pipetting after fertilization and that HRP and dextran tetramethylrhodamine were dissolved at concentrations of 4% and 0.5%, respectively.

Histochemical staining for acetylcholinesterase

Differentiation of juvenile muscle was examined by the histochemical reaction of acetylcholinesterase (AChE). Juveniles were relaxed with L-menthol and fixed with 5% formaldehyde in sea water for 10 min at room temperature. The specimens were washed with PBT twice and were replaced with AChE staining buffer (0.2 mg/ml acetylthiocholine iodide, 65 mM sodium acetate, 3 mM copper sulfate, 0.5 mM pottasium ferricyanide, 5 mM sodium citrate, pH 5.5). The reaction was performed for 2–3 h at room temperature.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed by using digoxigenin (DIG)-labeled antisense probes, as described previously (Satou, 1999). RNA

probes were prepared using a DIG RNA-labeling Kit (Roche). The differentiation of larval mesenchyme cells with probe for a mesenchyme-specific gene, *Cs-mech1* (Imai et al., 2002). Control embryos hybridized with a sense probe did not show any signal above background levels.

Results

Developmental fates of A7.6-, B8.5- and B7.7-line mesenchyme cells in Ciona

To determine whether A7.6, B8.5 and B7.7 blastomeres of *Ciona* embryos give rise to different mesodermal tissues of juveniles, a cell lineage tracing experiment was performed by DiI labeling. The A6.3 and B6.4 of the 32-cell embryo divide into A7.5 and A7.6, and B7.7 and B7.8 of the 64-cell stage embryo, respectively (Fig. 1). The A7.6 and B7.7 are retained in the 110-cell embryo (Fig. 1). On the other hand, B7.3 in the 64-cell embryo divides into B8.5 and B8.6 at the 110-cell stage (Fig. 1). The developmental fates of A7.6, B8.5 and B7.7 are restricted to give rise to mesenchyme cells, and these three blastomere pairs are the only source of larval mesenchyme cells. A7.6, B8.5 and B7.7 blastomeres of dechorionated embryos were each labeled with DiI between the 64- and 110-cell stages (Fig. 2). All embryos were confirmed to have the tracer in the correct cells at the 110-cell stage (data not shown). The labeled cells were observed in a region of the tailbud embryo expected from their lineages (Nishida, 1987; Figs. 2A, C, E). At the swimming larva stage, the labeled cells were observed to be dispersed in the trunk between the endoderm and epidermis. However, the mode of dispersion was different among the three lines. When A7.6 was traced, a cluster of cells located at the central region of the trunk, as well as cells dispersed in the trunk region, were labeled (in 25 of 25 cases; Fig. 2B). Judging from the position, this cluster is likely the primordium of the 1st gill-slit (Chiba et al., 2004). When B8.5 was labeled, the labeled cells seemed to migrate away from posterior region in the trunk and form a cluster at the anterior trunk region (in 20 of 20 cases; Fig. 2D). When B7.7 was labeled, the labeled cells seemed to migrate anteriorly, but the number of these cells was not large enough to form a cluster at the anterior trunk region (in 19 of 19 cases, Fig. 2F).

Dechorionation of ascidian eggs often inhibits the metamorphosis of larvae (Sato and Morisawa, 1999). Therefore, embryos with intact chorions were labeled to observe the developmental fates of mesenchyme cells in normally metamorphosed juveniles. We investigated juveniles at Stage-4, which was defined by Chiba et al. (2004). When A7.6 was labeled with DiI, blood cells, oral siphon muscle and epithelium of the 1st/2nd gill-slits were labeled (Fig. 3A; Table 1). In addition, a part of the stomach, small granules within the tunic (hereafter referred to as 'tunic granules') and very few tunic cells (1–5 cells/juvenile) were labeled (Figs. 3A, B; Table 1). Longitudinal mantle muscle was not labeled (Fig. S1B; Table 1). Because longitudinal mantle muscle was derived from A7.6 in *Halocynthia* (Hirano and Nishida, 1997), we further confirmed this result by injection of HRP into A7.6 at the 64-cell stage, which was a method adopted in *Halocynthia* (Hirano and Nishida, 1997). Longitudinal mantle muscle was not stained by histochemical detection of HRP (in 9 of 9 cases; Figs. S1C, D). Therefore, the embryonic origin of

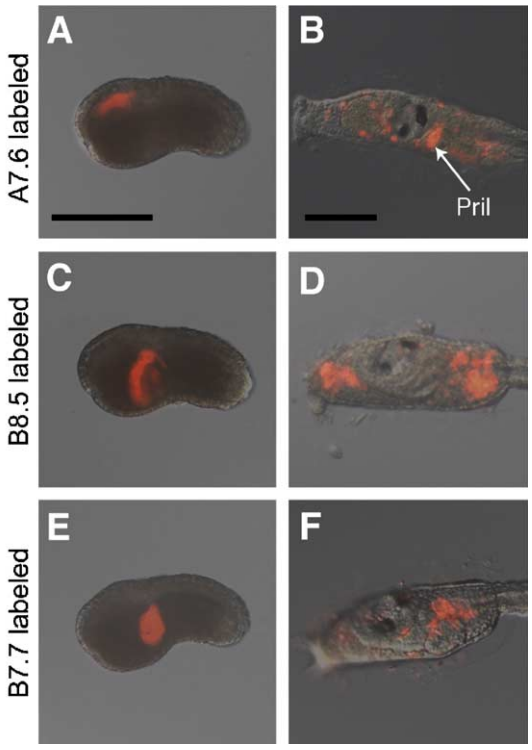

Fig. 2. Lineage tracing by DiI labeling of A7.6 (A, B), B8.5 (C, D) and B7.7 (E, F) blastomeres at the tailbud stage (A, C, E) and the swimming larva stage (B, D, F). Anterior is to the left. PriI: primordium of 1st gill-slit. Scale bars, 100 μm.

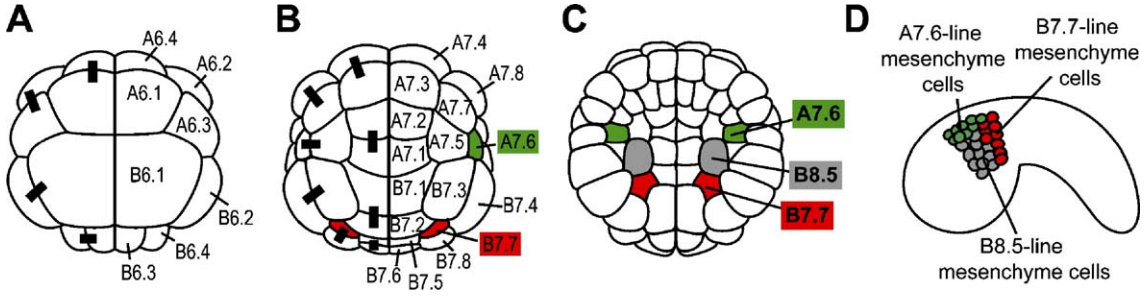

Fig. 1. A schematic illustration of fate map of the ascidian embryo with respect to A7.6, B8.5 and B7.7-line mesenchyme cells. (A–C) Vegetal view of the (A) 32-, (B) 64- and (C) 110-cell stage embryos. Anterior is to the up. (D) Lateral view of the tailbud embryo. Anterior is to the left. A7.6 and its descendants are colored in green, B8.5 and its descendants are colored in gray, and B7.7 and its descendants are colored in red. Black bars indicate relationships of sister blastomeres.

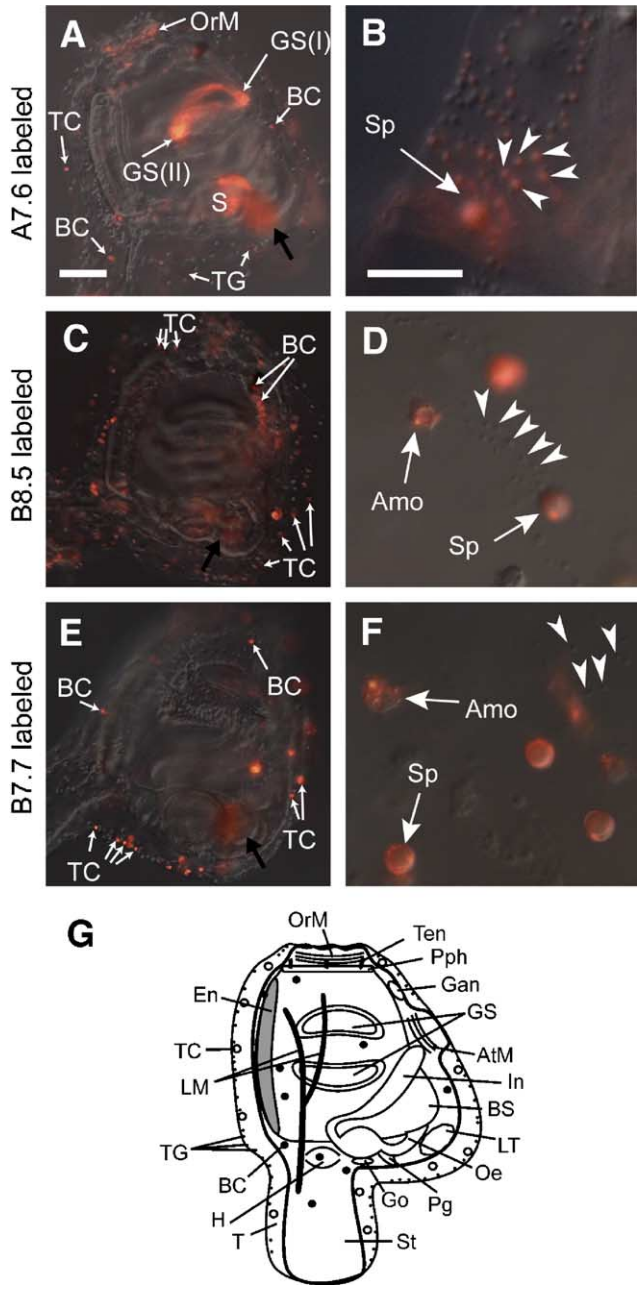

Fig. 3. Lineage tracing by DiI labeling of A7.6 (A, B), B8.5 (C, D) and B7.7 (E, F) blastomeres after metamorphosis. (A, C, E) Overviews of experimental juveniles. Dorsal is to the right. Black arrows indicate degenerated larval tissues, which were observed as weak autonomous fluorescences. (B, D, F) A higher magnification to show the morphology of tunic cells. (G) Diagram illustrating the inner structure of a *Ciona* juvenile at Stage-4. Dorsal is to the right. AtM, atrial siphon muscle; BC, blood cells; BS, branchial sac; En, endostyle; Gan, ganglion; Go, gonad; GS, gill-slits; GS(I), 1st gill-slit; GS(II), 2nd gill-slit; H, heart; In, intestine; LM, longitudinal mantle muscle; LT, larval tissues; Oe, oesophagus; OrM, oral siphon muscle; Pg, pyloric gland; Pph, peripharyngeal band; S, stomach; St, stalk; T, tunic; TC, tunic cells; TG, tunic granules; Ten, tentacle; Amo, amoebocyte-like tunic cells; Sp, sphere-shaped tunic cells. Scale bars represent (A) 100 μm and (B) 25 μm, which are applicable to panels (C, E) and (D, F), respectively.

longitudinal mantle muscle of *Ciona* is highly likely different from that of *Halocynthia*. On the other hand, when B8.5 and B7.7 were labeled with DiI, blood cells, as well as tunic cells, were labeled, although tunic granules were not labeled (Figs. 3C, E; Table 1).

We found that there are two populations of tunic cells classified by their morphology. Tunic cells of one group were sphere-shaped, while tunic cells of the other group were amoebocyte-like with pseudopodia. Both types of tunic cells were labeled when either B8.5 or B7.7 was labeled (Figs. 3D, F). On the other hand, when A7.6 was labeled, only the sphere-shaped cells were labeled (Fig. 3B). The tunic cells derived from B8.5 and B7.7 were dispersed throughout the entire tunic (Figs. 3C, E). The tunic cells derived from A7.6 did not seem to be concentrated in any region.

In the present study, 'blood cells' are defined as free cells within the fluid residing in the connective tissues. Many blood cells were observed to move within the connective tissues, although some blood cells were observed to move infrequently (Fig. S2). Both types of blood cells were differentiated from each of A7.6, B8.5 and B7.7 larval mesenchyme cells. We could not determine how many types of blood cells the ascidian juvenile at Stage-4 had and whether the types of blood cells derived from A7.6, B8.5 and B7.7 were different or not (see Discussion).

Effects of functional suppression of Cs-Twist-like1 on the larval and juvenile morphogenesis

In both *C. savignyi* and *C. intestinalis*, a bHLH transcription factor gene, *Twist-like1*, is essential for the specification of A7.6, B8.5 and B7.7 mesenchyme cells (Imai et al., 2003; Tokuoka et al., 2004). The larval mesenchyme cells give rise to several types of mesodermal tissues after metamorphosis as described above, suggesting important roles of the larval mesenchyme as primordia of the adult tissues. To address this issue, we examined the effect of the functional suppression of *Twist-like1* on the metamorphosis and subsequent development.

Larval morphology

When a morpholino against *Cs-Twist-like1* was injected into eggs with intact chorions, all larvae examined were unable to hatch. In *C. savignyi*, normal larvae hatch and start swimming at about 20 h post-fertilization (hpf), and they usually initiate tail resorption until 48 hpf at 18°C (Sato and Morisawa, 1999). However, the larvae injected with *Cs-Twist-like1* morpholino

Table 1
Developmental fates of larval mesenchyme cells after metamorphosis

	Labeled blastomeres			Unlabeled control
	A7.6	B8.5	B7.7	
Specimen	9[a]	4	6	5
Tunic cells	4[b]	4	6	0
Blood cells	9	4	6	0
Longitudinal mantle muscle	0	0	0	0
Oral siphon muscle	9	0	0	0
Epithelium of 1st/2nd gill-slits	9	0	0	0
Stomach	7	0	0	0
Larval tissues	9[c]	3[c]	6[c]	5[c]

[a] Tunic granules were also labeled (in 9 of 9 cases).
[b] Very few labeled cells were observed.
[c] Weak autonomous fluorescence was observed.

never hatched (in 30 of 30 cases; Fig. 4B). As a control, a morpholino against *lacZ* was injected, and this injection showed no effects on hatching (in 29 of 30 cases; Fig. 4A). Most of the *lacZ* morpholino-injected larvae initiated tail resorption until 48 hpf (in 29 of 30 cases; Fig. 4C). *Twist-like1*-knockdown larvae also initiated tail resorption at 48 hpf within the chorion (in 30 of 30 cases; Fig. 4D). All of the *Twist-like1*-knockdown larvae died

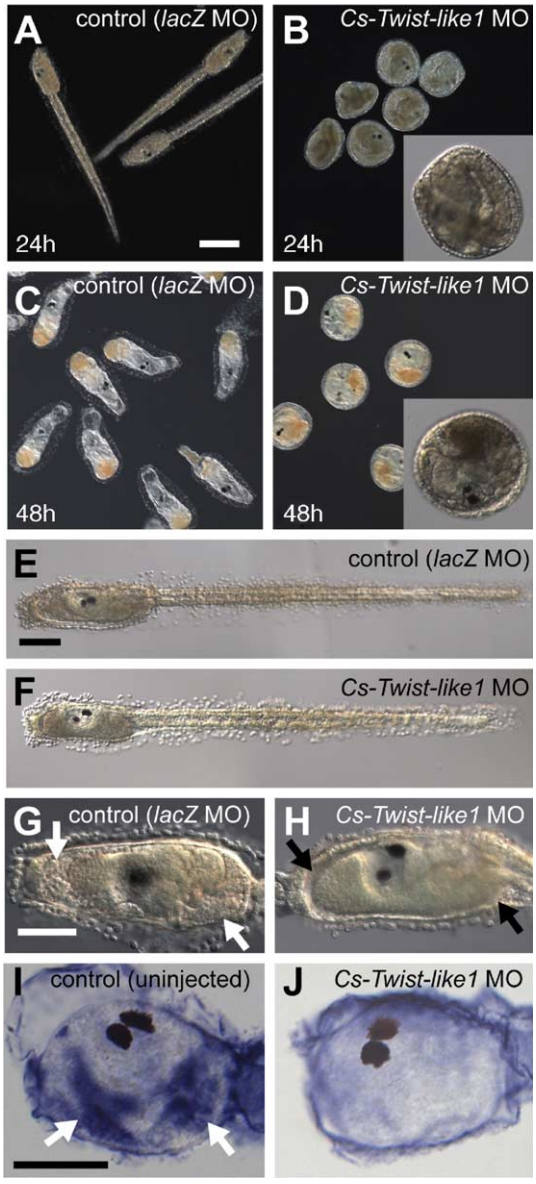

Fig. 4. Effects of functional suppression of *Cs-Twist-like1* on hatching and metamorphosis (A–D) and larval morphology (E–J). (A, C, E, G) Control larvae in which *lacZ* morpholino (MO) were injected. (I) Uninjected control larvae. Insets in panels (B) and (D) are enlargements. (B, D, F, H, J) Larvae in which *Cs-Twist-like1* MO were injected. (A, B, E, F, G, H) 24, (C, D) 48 and (I, J) 22 h after fertilization (hpf). (E–H) Larvae dechorionated manually at 20 hpf with sharpened tungsten needles. (I, J) Whole-mount in situ hybridization to examine the expression of *Cs-mech1*. The tunic gives rise to non-specific background signals. (E–J) Lateral view. Anterior is to the left. Control larva has mesenchyme (E, G, I, white arrows), but *Cs-Twist-like1*-MO-injected larva does not have mesenchyme (F, H, J, black arrows). Scale bars represent (A) 200 μm, (E) 100 μm, (G) 50 μm and (I) 50 μm, which are applicable to panels (B–D), (F), (H) and (J), respectively.

1 week after fertilization without hatch (data not shown). These phenomena have not been well observed in the previous study (Imai et al., 2003) because *Cs-Twist-like1* morpholino was injected into dechorionated eggs.

To help the experimental larvae to metamorphose successfully, we manually dechorionated at 20 hpf with sharpened tungsten needles. These larvae appeared morphologically normal and started swimming normally (Figs. 4E, F). However, no mesenchyme cells were observed under the light microscope (Figs. 4G, H), which was confirmed by the expression of a mesenchyme specific gene, *Cs-mech1*, in *Twist-like1*-knockdown larva (at 22 hpf; Figs. 4I, J). These results coincide well with the previous report, in which the expression of *Cs-mech1* was lost in *Twist-like1*-knockdown tailbud embryos (at 10 hpf; Imai et al., 2003).

Juvenile morphology

Twist-like1-knockdown larvae could swim, and they metamorphosed into juveniles. However, knockdown of *Cs-Twist-like1* caused morphological abnormalities in the Stage-4 juveniles and death about 1 week after fertilization (5–6 days after the initiation of tail resorption; data not shown). Because normal stage-4 juveniles differentiated most of the adult tissues and organs (Fig. 5A; Table 2; Chiba et al., 2004), we examined whether all of these adult tissues developed in *Twist-like1*-knockdown juveniles at Stage-4. As described above, oral siphon muscle, gill slits (epithelium of 1st/2nd gill-slits), tunic cells (and tunic granules), blood cells and a part of stomach are derived from the larval mesenchyme cells. Of these tissues, oral siphon muscle and gill-slits were not observed in *Twist-like1*-knockdown juveniles (Fig. 5B; Table 2). In addition, atrial siphon muscle and longitudinal mantle muscle were not formed in the experimental juveniles (Table 2), although these two muscle tissues were not labeled when the larval mesenchyme cells were labeled with DiI in the normal development (Fig. 3; Table 1). To further confirm that the three types of body-wall muscle (oral siphon, atrial siphon and longitudinal mantle muscles) were not formed in *Twist-like1*-knockdown juveniles, we examined the enzyme activity of acetylcholinesterase (AChE). AChE is present in ascidian muscle cells, and, in normal juveniles, body-wall muscle and heart show AChE activity (Fig. 5E; T. Matsuoka and Y. Sasakura, personal communication). Histochemical detection of AChE showed that the three types of body-wall muscle were lost in *Twist-like1*-knockdown juveniles, although the heart was formed normally (Fig. 5F). AChE activity was also detected in some granules that were disrupted within the larval tissues in experimental juveniles (Fig. 5F).

The stomach, a part of which was derived from A7.6, was observed in *Twist-like1*-knockdown juveniles (Table 2; Fig. 5B). Tunic cells and blood cells were mostly lost, but a very few cells were observed within the tunic and body cavity of *Twist-like1*-knockdown juveniles (Figs. 5B, C, D), even when a high dose (45 pmol) of *Cs-Twist-like1* morpholino was injected (in 6 of 9 cases; data not shown). Because the sizes of several tissues, including tentacle, pyloric gland and gonad, are very small even in normal juveniles at stage-4, these tissues were not distinguishable in *Twist-like1*-knockdown juveniles in

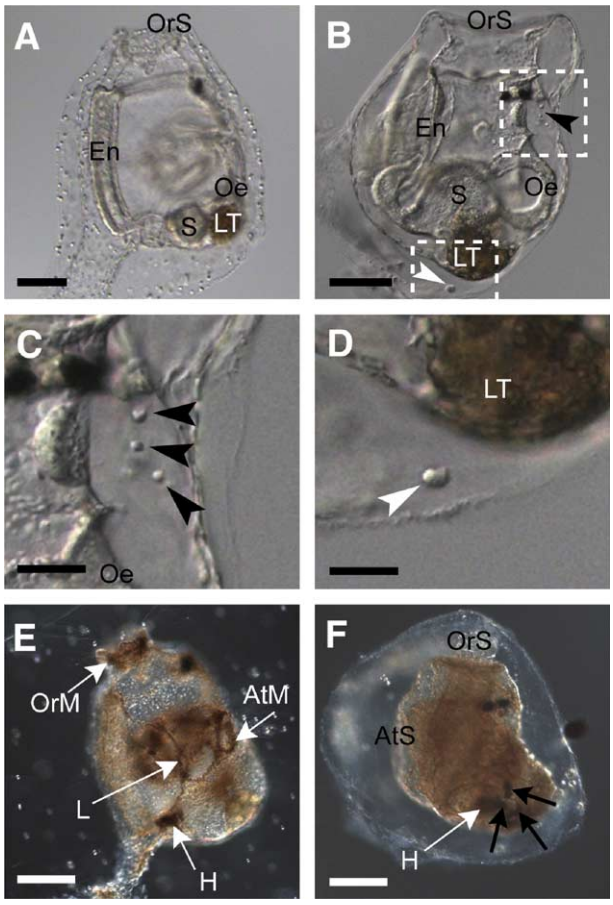

Fig. 5. Effects of the functional suppression of *Cs-Twist-like1* on juvenile morphology. (A, E) Uninjected control juveniles and (B, C, D, F) juveniles developed from egg injected with *Cs-Twist-like1* MO. The juveniles injected with *Cs-Twist-like1* MO have fewer cells within the body cavity (black arrowheads) and tunic (white arrowheads). (C, D) Close up of white boxes in panel (B). (E, F) Histochemical staining of juveniles for AChE activity. The longitudinal mantle muscle was not observed in *Cs-Twist-like1*-MO-injected juveniles, although AChE activity was seen in some granules (black arrows) and heart. Dorsal is to the right. AtM, atrial siphon muscle; AtS, atrial siphon; OrM, oral siphon muscle; OrS, oral siphon; L, longitudinal muscle; H, heart; En, endostyle; LT, larval tissues; Oe, oesophagus; S, stomach. Scale bars, (A, B, E, F) 100 μm; (C, D) 25 μm.

which the morphology of the whole body of juvenile was disrupted. Because *Twist-like1*-knockdown juveniles died within about 1 week after fertilization, fates of these tissues could not be followed further into later stages.

Developmental fate of A7.6, B8.5 and B7.7 cells in Twist-like1-knockdown juveniles

Because knockdown of *Cs-Twist-like1* is shown to suppress the formation of mesodermal tissues of juveniles, we then examined what types of tissues A7.6, B8.5 or B7.7 give rise to in the *Twist-like1*-knockdown embryos and juveniles. The developmental fates of each of the A7.6, B8.5 and B7.7 lineages in the morpholino-injected embryos were traced with DiI labeling between the 64- and 110-cell stages. In experimental tailbud embryos, no labeled cells were formed in the region where A7.6, B8.5 and B7.7-line mesenchyme cells should have been positioned (Fig. 6). Instead, when A7.6 of the *Twist-like1*-knockdown embryos was labeled with DiI, the labeled cells were located at the dorsal portion of the endoderm in the tailbud embryo and in the larva (in 7 of 8 cases; Figs. 6B, D). This suggests that A7.6 descendants with a mesenchymal fate converted their fate to that of larval endodermal cells. When B8.5 was labeled, cells located at the posterior region of the trunk and ventral region of the tail were labeled during development into larvae (in 5 of 5 cases; Figs. 6F, H), suggesting that B8.5 descendants with a mesenchymal fate converted their fate to that of larval endodermal cells and endodermal strand cells. When B7.7 was injected, labeled cells were located at the posterior region of the trunk and anterior region of the tail (in 5 of 5 cases; Figs. 6J, L). This suggests that B7.7 descendants with a mesenchymal fate converted their fate to that of larval muscle cells. The latter two observations coincide with the previous observations using cleavage-arrested embryos and molecular markers (Imai et al., 2003).

The developmental fates of A7.6, B8.5 and B7.7 after metamorphosis in *Twist-like1*-knockdown embryos are shown in Fig. 7. The A7.6-line cells of the uninjected control embryos gave rise to oral siphon muscle, the epithelium of 1st/2nd gill-slits, tunic cells (and tunic granules), blood cells and stomach (Figs. 3A, 7A). However, in *Twist-like1*-knockdown juveniles, no A7.6-derived cells were observed in these tissues. Instead, a part of branchial sac was labeled (in 5 of 5 cases; Fig. 7B). The

Table 2
Effects of functional suppression of *Cs-Twist-like1* on morphogenesis of juvenile tissues and organs

Tissues and organs observed in normal juveniles (Stage-4)	Formation of tissues and organs in juvenile (Stage-4) developed from *Cs-Twist-like1* MO-injected embryos (%)
Oral siphon	28/28 (100%)
Oral siphon muscle	0/28 (0%)
Tentacle	ND
Atrial siphon	26/28 (93%)
Atrial siphon muscle	0/28 (0%)
Ganglion	25/28 (89%)
Longitudinal mantle muscle	1/28 (4%)
Gill-slits	0/28 (0%)
Branchial sac	27/28 (96%)
Heart	28/28 (100%)
Tunic cells	0/28[a,b] (89%)
Blood cells	0/28[a] (89%)
Intestine	28/28 (100%)
Oesophagus	28/28 (100%)
Stomach	27/28 (96%)
Pyloric gland	ND
Gonad	ND
Endostyle	28/28 (100%)
Peripharingeal band	26/28 (93%)
Stalk	25/28 (89%)
Atrial cavity	27/28 (96%)

ND: because these tissues are small at Stage 4, it was not distinguished whether these organs were existed in *Twist-like1*-knockdown juveniles.

[a] A very few cells were observed within the tunic and body cavity in 25 of 28 cases, although it was not confirmed whether these cells were indeed tunic cells/blood cells.

[b] Tunic granules were not observed in experimental juveniles (in 0 of 28 cases; 100%).

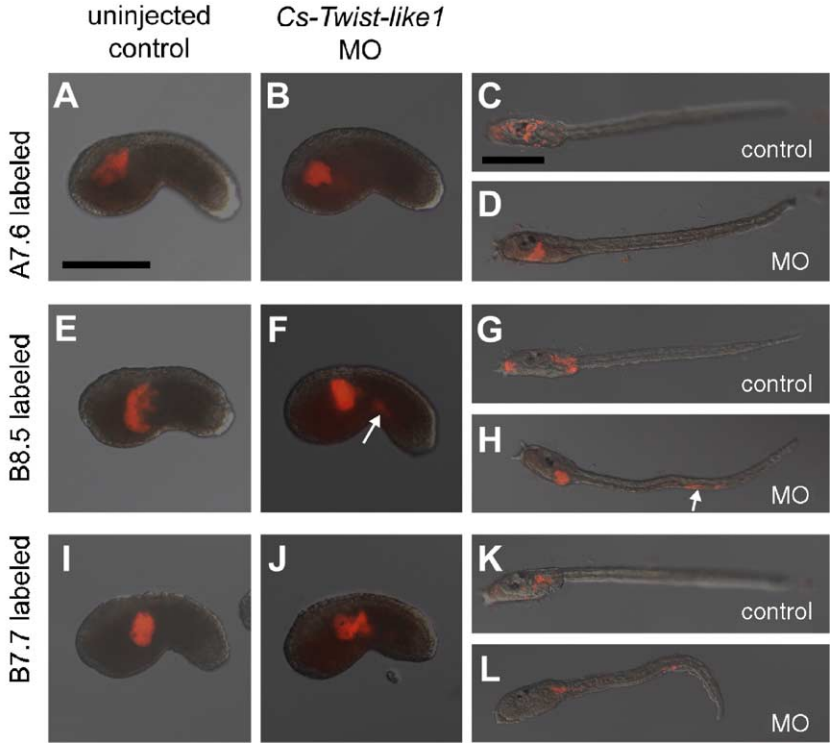

Fig. 6. The developmental fate of A7.6, B8.5 and B7.7 blastomeres in *Cs-Twist-like1*-knockdown embryos (B, F, J) and larvae (D, H, L). (A, E, I) Uninjected control embryos and (C, G, K) uninjected control larvae. Anterior is to the left. White arrows indicate the endodermal strand. Scale bars in panels (A) and (C) represent 100 μm, which are applicable to (B, E, F, I, J) and (D, G, H, K, L), respectively.

B8.5- and B7.7-line cells of the control embryos gave rise to tunic cells and blood cells (Figs. 3C, E; Figs. 7D, G), while, in *Twist-like1* knockdown juveniles, the B8.5 and B7.7 descendants were found in a part of the intestine (in 4 of 5 cases; Fig. 7E) and disintegrating larval tissues (in 5 of 5 cases; Fig. 7H), respectively. Although a few cells were observed within the tunic and body cavity of *Twist-like1*-knockdown juveniles (Figs. 5C, D), A7.6, B8.5 and B7.7 descendants were not found in the tunic and body cavity (Figs. 7C, F, I). This suggests that a few cells found in the tunic and body cavity of *Twist-like1*-knockdown juveniles are derived from other cells than larval mesenchyme cells.

Discussion

The developmental fates of larval mesenchyme cells in the formation of adult organs in C. savignyi

The present study showed similarities and dissimilarities in the mesenchyme cell lineage between *Ciona* and *Halocynthia*. A previous study of lineage tracing with HRP in *Halocynthia* showed that A7.6-line cells give rise to oral siphon muscle, longitudinal mantle muscle, epithelium of the 1st/2nd gill-slits and blood cells and that B8.5- and B7.7-line cells only give rise to tunic cells (Hirano and Nishida, 1997). In the present study, we traced the developmental fates of the larval mesenchyme cells in *C. savignyi* with DiI. In *Ciona*, the A7.6-line larval mesenchyme cells gave rise to oral siphon muscle, epithelium of the 1st/2nd gill-slits and blood cells, as in *Halocynthia*, but did not contribute to longitudinal mantle muscle. In *Ciona*, tunic cells, tunic granules and stomach were additionally derived from the A7.6-line mesenchyme. Tunic granules were first described in the present study, and its function has not yet been clarified. The B8.5- and B7.7-line cells gave rise not only to tunic cells but also to blood cells in *Ciona*. Therefore, the developmental fates of larval mesenchyme are largely similar between these two divergent species, but there are also significant differences between the two species.

Furthermore, A7.6-line mesenchyme cells gave rise to a part of the stomach in *Ciona* juveniles. In vertebrates, digestive organs are derived from the endoderm and visceral mesoderm (Grapin-Botton and Melton, 2000). Therefore, there may be a conserved mechanism of forming the stomach within chordates. However, there is another possibility in which A7.6-derived mesenchyme cells have intrinsic endoderm cell properties as well as mesodermal cell properties because A7.6-line cells exhibit alkaline phosphatase activity, an enzyme expressed in endoderm cells (Whittaker, 1990).

In *Halocynthia*, the tunic cells derived from B7.7-line cells tend to be concentrated near the holdfast, while the tunic cells derived from B8.5-line cells are scattered throughout the entire tunic, although the morphological differences between B8.5- and B7.7-derived tunic cells were not reported (Hirano and Nishida, 1997). In *Ciona*, both of the larval B8.5 and B7.7 mesenchyme cells gave rise to blood cells and tunic cells of juveniles, and no differences in the distribution and the morphology of the tunic cells were observed between B8.5- and B7.7-derived cells.

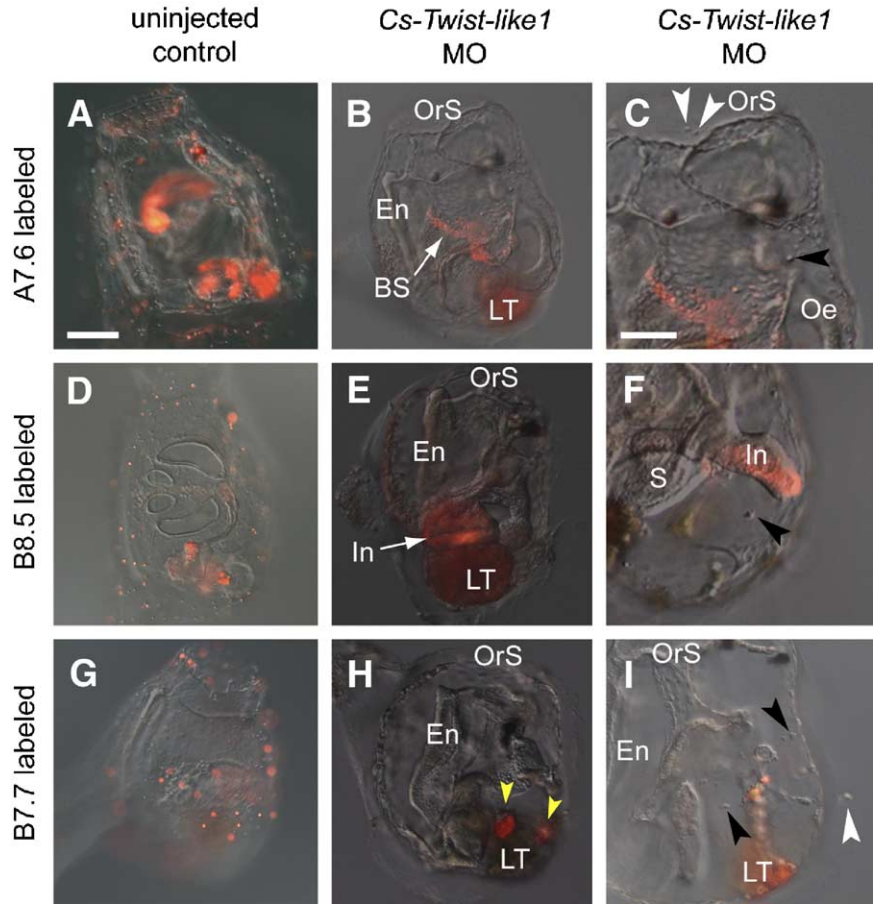

Fig. 7. Control juveniles (A, D, G) and juveniles developed from eggs injected with *Cs-Twist-like1* MO (B, C, E, F, H, I). A7.6 (A, B, C), B8.5 (D, E, F) or B7.7 (G, H, I) is DiI labeled at the 64- and 110-cell stage. Dorsal is to the right. In juveniles developed from eggs injected with *Cs-Twist-like1* MO, A7.6, B8.5 and B7.7 give rise to branchial sac (B, white arrow), intestine (E, white arrow) and larval tissues (H, yellow arrowheads), respectively. Black arrowheads and white arrowheads indicate cells in the body cavity and tunic, respectively. BS, brachial sac; En, endostyle; In, intestine; LT, larval tissues; Oe, esophagus; OrS, oral siphon; S, stomach. Scale bars in panels (A) and (C) represent 100 μm and 50 μm, which are applicable to panels (B, D, E, G, H) and (F, I), respectively.

The ultrastructures of tunic cells and blood cells of *C. intestinalis* adults (about 10 cm in length) have already been reported (De Leo et al., 1981; Rowley, 1981). The tunic cells of *Ciona* adults comprise at least four types, while blood cells comprise nine types. Although ultrastructural analysis has not yet been performed in developing juveniles, we found that two morphologically different tunic cells, namely, amoebocyte-like cells and sphere-shaped cells have three different but overlapping developmental origins. It is possible that these two groups of tunic cells further differentiate into four types and that each cell type has a different developmental origin, which should be resolved in future studies. Although the present study cannot discriminate between types of blood cells, such an ultrastructural analysis is also applicable to blood cells.

Twist-like1 is required for the formation of mesodermal tissues of Ciona juveniles

The larvae developed from eggs injected with *Cs-Twist-like1* morpholino lack mesenchyme cells. This result coincides with our previous results (Imai et al., 2003) that *Twist-like1* is essential for the differentiation of the larval mesenchyme fate. In the present study, it was also shown that *Twist-like1*-knockdown larvae cannot hatch, suggesting that *Twist-like1* is required for the synthesis or secretion of hatching enzyme.

We also demonstrated that the knockdown of *Twist-like1* causes the failure of development of most juvenile mesodermal tissues. All types of body-wall muscle (oral siphon, longitudinal mantle and atrial siphon muscles) were not formed in *Twist-like1*-knockdown juveniles. Consistently, external stimuli could not cause any twitch in *Twist-like1*-knockdown juveniles (data not shown). The only muscle derived from the larval mesenchyme cells is oral siphon muscle (Table 1). There are two possibilities to explain why longitudinal mantle muscle and atrial siphon muscle were not differentiated in the experimental embryos. The first possibility is that these two muscles were lost due to an indirect effect of *Twist-like1* knockdown. That is, tissues lost in the *Twist-like1*-knockdown embryos would be required for formation of these muscles. The second is that *Twist-like1* would be expressed in these two muscles or their precursor in the later stage and thus would directly control their development. However, *Twist-like1* was transiently expressed in the larval mesenchyme precursors only between 64-cell stage and neurula stage during embryogenesis (Imai et al., 2003), and the expression of this gene was not detected thereafter by the quantitative RT-PCR method

(Tokuoka, unpublished). Therefore, the second possibility is unlikely, and it is likely that the failure of development of atrial siphon muscle and longitudinal mantle muscle is due to an indirect effect of *Twist-like1*-knockdown.

In *Twist-like1*-knockdown juveniles, a few cells were observed within the tunic and body cavity. Even when a high dose (45 pmol) of *Cs-Twist-like1* morpholino was injected, these cells were considerably reduced but were not completely lost, suggesting that these cells are not derived from A7.6, B8.5 and B7.7 blastomeres. We could not determine whether these cells are tunic cells and/or blood cells. But if so, it is possible that the origins of these cells are not only the larval mesenchyme.

Knockdown of Twist-like1 converted the developmental fates of A7.6, B8.5 and B7.7

In *Twist-like1*-knockdown juveniles, A7.6-line cells gave rise to a part of the branchial sac and B8.5-line cells gave rise to intestine, although B7.7-line cells failed to give rise to juvenile tissues. These results may be explained by conversions of the developmental fates of A7.6- and B8.5-line cells when *Cs-Twist-like1* was suppressed. The present and previous studies showed that A7.6 and B8.5 descendants converted their fate morphologically and molecularly to larval endoderm cells in *Twist-like1*-knockdown embryos, while B7.7 descendants converted their fate to larval muscle cells. However, as discussed above, A7.6 has been restricted to mesenchyme but still has endoderm potential, and the loss of *Twist-like1* function may only suppress the mesenchyme potential in A7.6-line cells.

In *Halocynthia*, branchial sac and intestine are derived from larval endoderm (Hirano and Nishida, 2000). Therefore, if this is also applicable to *Ciona*, it is reasonable that A7.6- and B8.5-line cells, which converted their developmental fates to larval endoderm, gave rise to juvenile endodermal tissues in the *Twist-like1*-knockdown juvenile. It is also expected that B7.7-line cells, which converted their developmental fates to larval muscle cells, did not contribute to any juvenile tissues in the *Twist-like1*-knockdown juvenile because larval muscle cells are degenerated during metamorphosis and do not contribute to juvenile muscle (Hirano and Nishida, 1997; Satou et al., 2004).

The previous studies showed that *Twist-like1* is essential for the specification of larval mesenchyme cells in *Ciona* embryos (Imai et al., 2003; Tokuoka et al., 2004). The present study showed that the function of *Twist-like1* in the differentiation of larval mesenchyme cells reflects on the formation of juvenile mesodermal tissues derived from larval mesenchyme cells. That is, *Twist-like1* is required for the differentiation of most mesodermal precursors of *Ciona* adults.

The ascidian tadpole larva is regarded as a prototype of the ancestral chordate, and the development of the ascidian tadpole larva may provide clues concerning the origin and evolution of chordates (Satoh, 2003). As mentioned above, understanding the developmental mechanisms of ascidian adult tissues is also important. Further studies using ascidians may help us understand the mechanism of specification of embryonic/larval stem cells, which are likely conserved not only in chordates, but also in invertebrate animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.09.018.

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