

A Twist-like bHLH gene is a downstream factor of an endogenous FGF and determines mesenchymal fate in the ascidian embryos

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SUMMARY

Ascidian larvae develop mesenchyme cells in their trunk. A fibroblast growth factor (FGF9/16/20) is essential and sufficient for induction of the mesenchyme in *Ciona savignyi*. We have identified two basic helix-loop-helix (bHLH) genes named *Twist-like1* and *Twist-like2* as downstream factors of this FGF. These two genes are phylogenetically closely related to each other, and were expressed specifically in the mesenchymal cells after the 110-cell stage. Gene-knockdown experiments using a specific morpholino oligonucleotide demonstrated that *Twist-like1* plays an essential role in determination of the mesenchyme and that *Twist-like2* is a downstream factor of *Twist-like1*. In addition, both overexpression and misexpression of *Twist-like1* converts non-mesenchymal cells to mesenchymal cells.

We also demonstrate that the upstream regulatory mechanisms of *Twist-like1* are different between B-line mesenchymal cells and the A-line mesenchymal cells called ‘trunk lateral cells’. FGF9/16/20 is required for the expression of *Twist-like1* in B-line mesenchymal precursor cells, whereas FGF, FoxD and another novel bHLH factor called NoTrlc are required for *Twist-like1* to be expressed in the A-line mesenchymal precursor cells. Therefore, two different but partially overlapping mechanisms are required for the expression of *Twist-like1* in the mesenchymal precursors, which triggers the differentiation of the mesenchyme in *Ciona* embryos.

Key words: Ascidian, *Ciona savignyi*, *Ciona intestinalis*, Mesenchyme, Basic helix-loop-helix Transcription factors

INTRODUCTION

Ascidians belong to the subphylum Urochordata, which is one of the 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 trunk of the larva contains a dorsal central nervous system (CNS) with two sensory organs (otolith and ocellus), endoderm, mesenchyme, trunk lateral cells and trunk ventral cells. The tail contains the notochord, which is flanked dorsally by the nerve cord, ventrally by the endodermal strand and bilaterally by three rows of muscle cells. The entire surface of the larva is covered by an epidermis. This configuration of the ascidian tadpole is thought to represent one of the most simplified and primitive chordate body plans (reviewed by Satoh and Jeffery, 1995; Di Gregorio and Levine, 1998; Satou and Satoh, 1999; Wada and Satoh, 2001; Nishino and Satoh, 2001; Satoh, 2003).

The ascidian larval mesenchyme cells consist of about 900 cells that form the adult body after metamorphosis (reviewed by Satoh, 1994). The mesenchymal cells are derived from the A7.6, B7.7 and B8.5 blastomere pairs of the 110-cell embryo (see Fig. 2A). The mesenchymal cells derived from A7.6 blastomeres are called trunk lateral cells (TLCs), and they mainly give rise to adult blood cells and body-wall muscle (Nishide et al., 1989; Hirano and Nishida, 1997). The other two lineages of mesenchymal cells (B7.7 and B8.5) are mesenchymal cells only in a narrow sense, and contribute mainly to tunic cells after metamorphosis (Hirano and Nishida,

1997). Although TLCs have been described as distinct from the other two lineages of mesenchyme cells (B-line mesenchyme), numerous genes are expressed specifically in both TLCs and B-line mesenchyme cells, suggesting that these cells share some features (Satou et al., 2001; Kusakabe et al., 2002). In the present study, we use the term ‘mesenchyme’ for all of these cells, except where indicated otherwise.

In addition to these three lineages of mesenchymal cells, ascidian larvae develop trunk ventral cells (TVCs), which are often regarded as a kind of mesenchymal cell because they give rise to adult body wall muscle and heart (Hirano and Nishida, 1997). In our previous comprehensive studies, we could not find any genes that are expressed specifically in both TVCs and mesenchyme at the tailbud stage, whereas many genes expressed in muscle cells were also found to be expressed in TVCs, suggesting that TVCs and muscle cells share some features (Satou et al., 2001; Kusakabe et al., 2002). This gene expression profile suggests that TVCs are quite different from the other three lineages of mesenchymal cell. In the present study, the term ‘mesenchyme’ does not include TVCs.

Ascidian larval development has been regarded as a ‘mosaic’ development, in which each tissue is specified by maternally supplied ‘determinants’. Actually, the endoderm of *Ciona* is autonomously specified by maternally supplied β -catenin (Imai et al., 2000). In another ascidian, *Halocynthia roretzi*, muscle cells are autonomously specified by a maternally supplied Zic-related transcription factor, macho1 (Nishida and Sawada, 2001). However, the specification of the remaining

mesodermal tissues, mesenchyme and notochord, requires inductive cell-to-cell interaction. The notochord and the mesenchyme (except for TLCs) have been demonstrated to be induced by presumptive endodermal cells around the 32-cell stage (Nakatani and Nishida, 1994; Kim et al., 2000). TLCs are induced by cells in the animal hemisphere at the 16-cell stage in *Halocynthia* (Kawaminani and Nishida, 1997). In a previous study, we demonstrated that a fibroblast growth factor, Cs-FGF9/16/20 [previously called Cs-FGF4/6/9 (Imai et al., 2002a); renamed by Satou et al. (Satou et al., 2002a)], which is expressed in precursors for endoderm, TLCs, notochord and nerve cord at the 16- and 32-cell stages, and in the nerve cord and the muscle precursors at the 64- and 110-cell stages, is essential for the induction of mesenchyme, including TLCs, in *Ciona* embryos (Imai et al., 2002a). However, it is not yet known either when this FGF induces TLCs or from where the FGF signal comes. In addition, the mechanism of the determination of mesenchymal cells after induction has not been clarified yet. In the present study, we identified genes for bHLH factors that work downstream of FGF9/16/20 and determine the mesenchymal cell fate.

MATERIALS AND METHODS

Ascidian eggs and embryos

Ciona savignyi adults were cultivated at the Maizuru Fisheries Research Station of Kyoto University, Maizuru city, facing the Sea of Japan. 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 gonads. After insemination, eggs were reared at about 18°C in Millipore-filtered seawater (MFSW) containing 50 µg/ml streptomycin sulfate.

Isolation of cDNAs and sequence determination

Ciona intestinalis cDNA clones were obtained from the *Ciona intestinalis* gene collection (Satou et al., 2002b). Their *Ciona savignyi* counterparts were first searched for in genome sequences that were produced by whole-genome shotgun sequencing and that are deposited in the Trace archive of NCBI. Based on the genomic sequences, we amplified cDNAs from gastrula and tailbud cDNA libraries by PCR.

Nucleotide sequences of both strands were determined using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM 377 DNA sequencer (Perkin Elmer, Norwalk, CT, USA).

Whole-mount in situ hybridization

To determine mRNA distribution in eggs and embryos, RNA probes were prepared using a DIG RNA-labeling Kit (Roche). Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense probes as described previously (Satou and Satoh, 1997).

Morpholino oligonucleotides and synthetic capped mRNAs

In the present study, we used 25-mer morpholino oligonucleotides (hereafter referred to as 'morpholinos'; Gene Tools, LLC) for *Cs-Twist-like1* (5'-CTTGATTGTA CTACTAGTGATGTCAT-3') and *Cs-NoTrlc* (5'-ATTTCTCATTACTTCTGTTGACATG-3'). Synthetic capped mRNAs for *Cs-Twist-like1* and *Cs-NoTrlc* were synthesized from their cDNAs cloned into the pBluescript RN3 vector (Lemaire et al., 1995) using a Megascript T3 kit (Ambion). To obtain capped mRNA, the concentration of GTP was lowered to 1.5 mM and the cap analog 7mGpppG was added at 6 mM. For rescue experiments, synthetic mRNAs were designed to lack the morpholino target

sequence and, therefore, the morpholinos did not recognize these synthetic mRNAs. We also used a morpholino against *Cs-Fgf9/16/20* (Imai et al., 2002a). After insemination, fertilized eggs were dechorionated, and microinjected with 15 pmol of morpholinos and/or synthetic capped mRNAs in 30 µl of solution using a micromanipulator (Narishige Scientific Instrument Laboratory, Tokyo), as described (Imai et al., 2000). Injected eggs were reared at ~18°C in MFSW containing 50 µg/ml streptomycin sulfate. Cleavage of some embryos was arrested at the 110-cell stage using cytochalasin B, and the embryos were then further cultured until the control embryos reached the desired stage.

Detection of differentiation markers

The following cell-specific markers were used to assess the differentiation of embryonic cells, and were detected by whole-mount in situ hybridization: *Cs-MAI*, a larval muscle-specific actin gene (Chiba et al., 1998); *Cs-Epi1*, an epidermis-specific gene (Chiba et al., 1998); *Cs-fibrinogen-like* (*Cs-fibrn*), a notochord-specific gene (Imai et al., 2002a); *Cs-Mech1*, a mesenchyme-specific gene (Imai et al., 2002a); and *Cs-ETR*, a pan-neural marker gene (Imai et al., 2002a). The differentiation of endoderm cells in experimental embryos was monitored by the histochemical detection of alkaline phosphatase as previously described (Whittaker and Meedel, 1989).

Quantitative RT-PCR

Twenty-five embryos at the 110-cell stage were lysed in 200 µl of GTC solution [4 M guanidinium thiocyanate, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 2% sarkosyl, 1% β-mercaptoethanol] and total RNA prepared. The RNA was used for cDNA synthesis with oligo(dT) primers as described previously (Imai et al., 2000). Real-time RT-PCR was performed using SYBR Green PCR Master Mix and an ABI prism 7000 (Applied Biosystem). A one-embryo-equivalent quantity of cDNA was used for each real-time RT-PCR. The cycling conditions were 15 seconds at 95°C and 1 minute at 60°C, according to the supplier's protocol. The experiment was repeated twice with different batches of embryos. Relative expression values were calculated by comparison with the level of expression in uninjected control embryos. Control samples lacking reverse transcriptase in the cDNA synthesis reaction failed to give specific products in all cases. Dissociation curves were used to confirm that single specific PCR products were amplified.

The primers used in the present study were as follows:

Cs-NoTrlc, 5'-CTCGCCACCAAACGATCAAT-3' and 5'-CGCGA-ATGTTGCCAAGTGT-3'; and

Elongation factor 2 (*EF2*), 5'-CGCAGTGAAACCGAATGATTC-3' and 5'-CCCGATCTTCAATGTCAATGC-3'.

RESULTS

Genes expressed in presumptive mesenchymal cells of early *Ciona* embryos

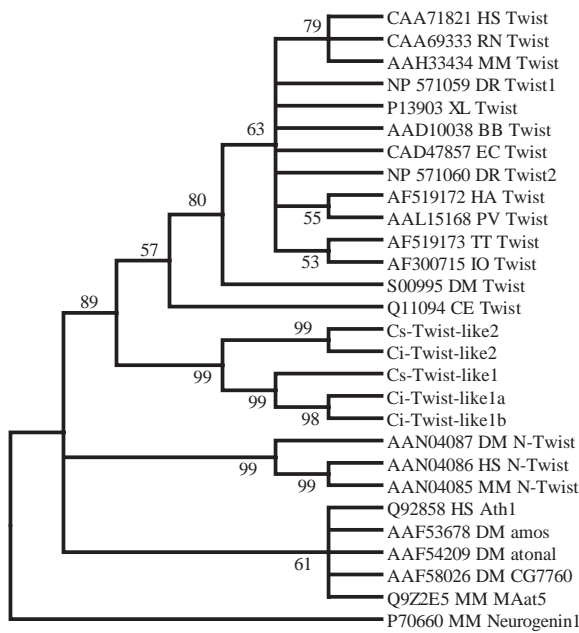
In previous studies (Satou et al., 2003; Wada et al., 2003), we identified whole gene sets for bHLH factors and homeobox transcription factors encoded in the *Ciona intestinalis* genome, the draft sequence of which we recently determined (Dehal et al., 2002). We comprehensively examined their expression patterns during embryogenesis, and thereby found that five bHLH genes and one homeobox gene were expressed predominantly in mesenchymal cells. Because embryos of a closely related species, *Ciona savignyi*, are more amenable to embryological manipulations, we isolated cDNA clones for *Ciona savignyi* orthologs of the *Ciona intestinalis* genes.

Three of the *Ciona intestinalis* bHLH genes were closely related to one another. A previous phylogenetic analysis failed

A



B



to predict their orthologous relationship with known bHLH proteins (Satou et al., 2003), although the best-hit proteins by BlastP searches were Twist and Atonal (E-value>3E-07). We identified two *Ciona savignyi* cDNA clones for their orthologs (AB105881 and AB105882). One was 1069 bp in length and encoded a polypeptide of 318 amino acid residues, and the other was 1211 bp in length and encoded a polypeptide of 255 amino acid residues. Alignment of the bHLH regions of these proteins and a phylogenetic analysis using the neighbor-joining method indicated that the last common ancestor of the two *Ciona* species had two Twist and Atonal-like genes, and that one of them was duplicated in the *Ciona intestinalis* lineage after the divergence of the two species (Fig. 1A,B). As described below, because these are most likely to be orthologous to Twist proteins from their function, these two genes were renamed as *Twist-like1* and *Twist-like2* [they were previously called *twist-and-atonal-like* genes (Satou et al., 2003)]. *Ciona savignyi* has only one *Twist-like1* gene (*Cs-Twist-like1*) and one *Twist-like2* gene (*Cs-Twist-like2*), whereas *Ciona intestinalis* has two *Twist-like1* genes (*Ci-Twist-like1a* and *Ci-Twist-like1b*) and one *Twist-like2* gene (*Ci-Twist-like2*).

The phylogenetic relationship of the fourth bHLH gene was not clearly predicted either [by using a gene model called

Fig. 1. (A) Alignment of amino acid residues of the bHLH domain of Twist-like1 and Twist-like2 of two *Ciona* species, and Twist proteins, atonal proteins and N-twist proteins of other animals. (B) A molecular phylogenetic tree generated by the neighbor-joining method based on the alignment of the bHLH domain. The number on each node indicates the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. The tree is shown as a bootstrap consensus tree (cutoff value=50%). Mouse neurogenin1 is used as an outgroup protein. Proteins of animals other than ascidians are shown by their accession number, species abbreviation and protein name. Species abbreviations are as follows: MM, mouse; HS,

human; RN, rat; XL, *Xenopus laevis*; DR, zebrafish; BB, *Branchiostoma belcheri*; DM, *Drosophila melanogaster*; CE, *Caenorhabditis elegans*; EC, *Enchytraeus coronatus*; HA, *Helix aspersa*; PV, *Patella vulgata*; TT, *Transennella tantilla*; IO, *Ilyanassa obsoleta*.

Ci0100140298/graill.146.25.1 of the *Ciona intestinalis* genome (Satou et al., 2003)]. A cDNA clone isolated for the *Ciona savignyi* ortholog was 1100 bp long and encoded a polypeptide of 295 amino acid residues (AB105884). A BlastP search showed that the best-hit protein in the non-redundant public protein database was *Xenopus* dHand although the E-value (>1e-11) was high. Because we have already found that another gene representing an ancestral form of *dHand* and *eHand* is encoded in the *Ciona intestinalis* genome, we do not believe that the fourth bHLH gene is an ortholog of *dHand* and *eHand* (Satou et al., 2003). The gene was designated as *NoTrlc* (No Trunk lateral cells; *Cs-NoTrlc* in *Ciona savignyi* and *Ci-NoTrlc* in *Ciona intestinalis*), because it is essential for the differentiation of TLCs, which give rise to blood cells and body-wall muscles after metamorphosis, as described below.

The fifth bHLH gene is an ortholog of vertebrate Mist genes [*Ci-Mist* (Satou et al., 2003)]. A cDNA clone for the *Ciona savignyi* ortholog was 1909 bp long and encoded a polypeptide of 537 amino acid residues (AB105883). This gene was designated *Cs-Mist*.

The homeobox gene predominantly expressed in mesenchymal cells is an ortholog of *Hex* [*Ci-Hex* (Wada et al., 2003)]. A cDNA clone for the *Ciona savignyi* ortholog was 2296 bp long and encoded a polypeptide of 524 amino acid residues (AB105885). This gene was designated *Cs-Hex*.

Expression patterns of mesenchyme-specific transcription factors

Expression profiles of these genes were re-examined in *Ciona savignyi* embryos. The expression pattern of *Cs-Twist-like1* is shown (Fig. 2B). No maternal expression of *Cs-Twist-like1* was detected by whole-mount in situ hybridization. An extensive EST analysis of developmentally relevant genes of *Ciona intestinalis* did not yield any ESTs for *Ci-Twist-like1a/b* in an egg cDNA library [http://ghost.zool.kyoto-u.ac.jp (Satou et al., 2002b)], supporting the notion that there was no maternal expression of this gene. The first zygotic expression was detected in one pair of the presumptive mesenchymal cells (B7.7) of the 64-cell embryo. Thereafter, *Cs-Twist-like1* was expressed in the remaining two lineages (B8.5 and A7.6 lines)

of mesenchymal cells by the early gastrula stage. This *Cs-Twist-like1* expression continued until the neurula stage and was then downregulated so that it was rarely detected in the tailbud embryo.

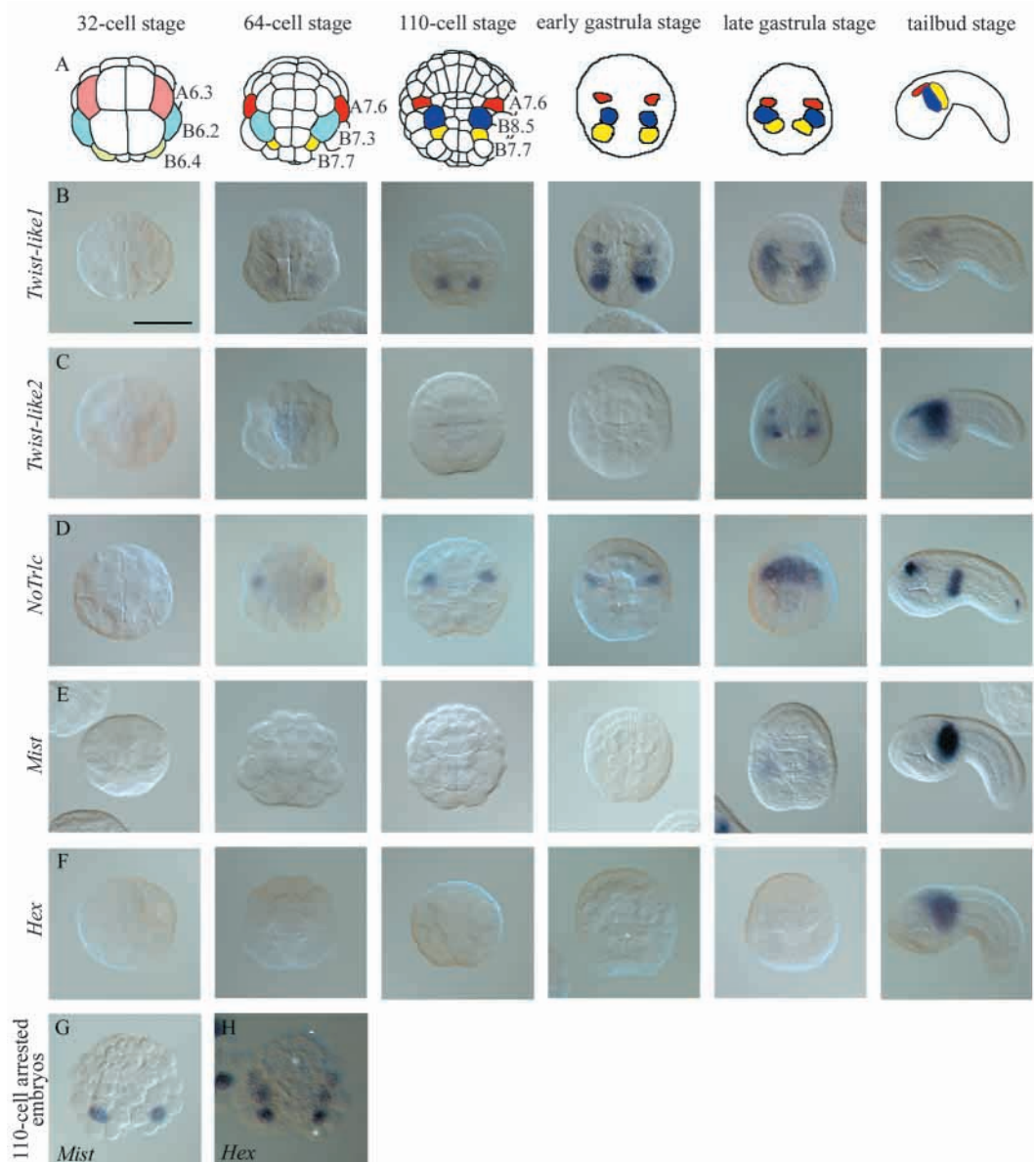
The expression of *Cs-Twist-like2* was not detected in early embryos up to the early gastrula stage (Fig. 2C); *Cs-Twist-like2* expression began in cells of every mesenchymal lineage at the late gastrula stage (refer to 110-cell stage arrested embryos in Fig. 7B and Fig. 9A) and continued strongly in mesenchymal cells of tailbud embryos. This temporal profile of *Cs-Twist-like2* expression is consistent with the EST data of the *Ciona intestinalis* counterpart [<http://ghost.zool.kyoto-u.ac.jp> (Satou et al., 2002b)].

Cs-NoTrlc was also zygotically expressed (Fig. 2D). Expression began in the presumptive TLCs (A7.6) of the 64-cell embryo. The expression was observed continuously up until the early gastrula stage and it was then downregulated. No mesenchymal cells other than TLCs expressed the gene, but non-mesenchymal cells also expressed it. For example, from

the middle gastrula stage, *Cs-NoTrlc* began to be expressed in the endodermal cells. TLC and endoderm expression disappeared by the tailbud stage, and novel expression was detected in the anterior trunk region, the TVCs and the posterior-most part of the dorsal nerve cord in the tailbud embryo.

The expression pattern of *Cs-Mist* is shown in Fig. 2E. No maternal *Cs-Mist* mRNA was detected. The zygotic expression of *Cs-Mist* began in cells of the B7.7 line mesenchyme between the late gastrula and early neurula stages, and the expression continued thereafter throughout embryogenesis. No mesenchymal cells other than the B7.7 line cells expressed *Cs-Mist*. This was confirmed in embryos arrested at the 110-cell stage and cultivated up to the stage corresponding to tailbud embryos. Treatment of 110-cell embryos with cytochalasin B disturbs cytokinesis but not the intrinsic differentiation program, which facilitates identification of the lineage of cells with *Cs-Mist* expression. The treated embryos showed *Cs-Mist* expression only in the B7.7 line cells (Fig. 2G).

Fig. 2. Expression profiles of genes characterized in the present study, as revealed by whole-mount in situ hybridization. (A) Schematic representation of ascidian embryos from the 32-cell stage to the tailbud stage. A7.6-line mesenchyme cells (TLCs) are shown in red. At the 32-cell stage, progenitor cells (A6.3) of A7.6 are shown in pink, because the developmental fate of A6.3 is not restricted to mesenchyme. B8.5-line and B7.7-line mesenchymal cells are shown in blue and yellow. Prior to the 110-cell stage and the 64-cell stage, the progenitors of B8.5 and B7.7 are not restricted to mesenchyme and, therefore, they are shown in light blue and light yellow, respectively. Up to the 110-cell stage, the colors identify individual blastomeres and the names for the colored blastomeres are shown. After the 110-cell stage, the colors identify cell populations sharing their origins. (B-F) Expression of *Cs-Twist-like1* (B), *Cs-Twist-like2* (C), *Cs-NoTrlc* (D), *Cs-Mist* (E) and *Cs-Hex* (F). (G,H) Expression of *Cs-Mist* (G) and *Cs-Hex* (H) in embryos arrested at the 110-cell stage. Embryos from the 32-cell stage to late gastrula stage are shown in a vegetal view and tailbud embryos are shown in a lateral view. Scale bar represents 100 μm .



The expression of *Cs-Hex* was also zygotic (Fig. 2F). Expression was not detected in embryos at the neurula stage or earlier, but zygotic expression was detected in all the mesenchymal cells of the tailbud embryo. This was confirmed in embryos arrested at the 110-cell stage and cultivated up to the stage corresponding to tailbud embryos (Fig. 2H).

FGF9/16/20 regulates the expression of the mesenchyme-specific transcription factors

A previous study demonstrated that *Cs-Fgf9/16/20* induces mesenchymal identification in ascidian embryos, because inhibition of the translation of *Cs-Fgf9/16/20* mRNA with a specific morpholino resulted in failure of the differentiation of mesenchymal cells, and overexpression of *Cs-Fgf9/16/20* mRNA resulted in ectopic differentiation of mesenchymal cells (Imai et al., 2002a). We examined here, whether or not the transcription of the five transcription factor genes identified above is under the control of *Cs-FGF9/16/20*. For this purpose, *Cs-Fgf9/16/20* morpholino was microinjected into fertilized eggs of *Ciona savignyi*. The specificity of this morpholino was demonstrated in a previous study (Imai et al., 2002a). The expression of *Cs-Twist-like1*, *Cs-Twist-like2*, *Cs-Mist* and *Cs-Hex* was completely suppressed in the morpholino-injected embryos (0/22 for *Cs-Twist-like1*; 0/16 for *Cs-Twist-like2*; 0/8

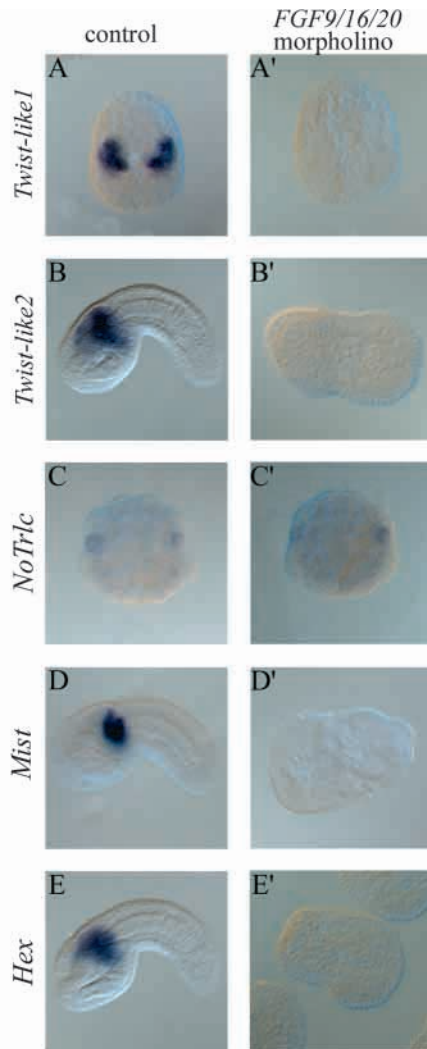
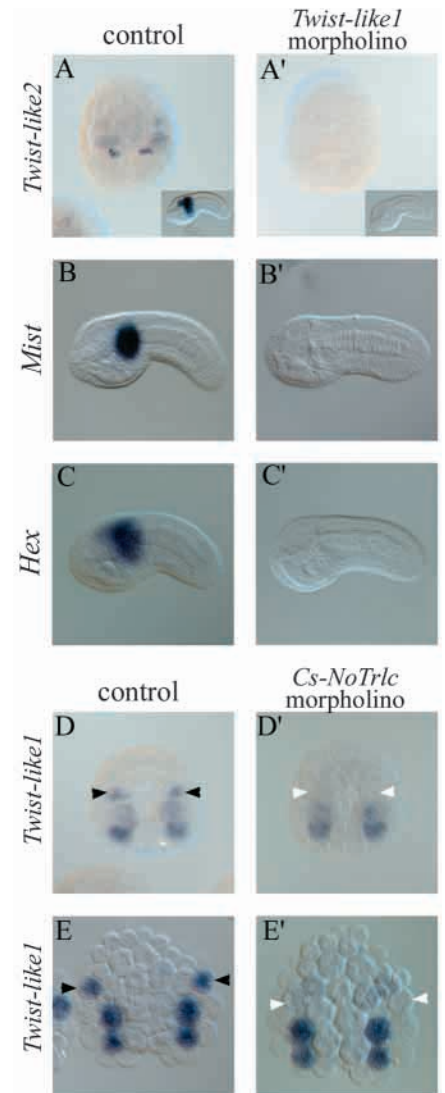


Fig. 3. Effects of functional suppression of *Cs-Fgf9/16/20* on the expression of *Cs-Twist-like1* in the late gastrula (A,A'), *Cs-Twist-like2* in the tailbud embryo (B,B'), *Cs-NoTrlc* in the 64-cell embryo (C,C'), *Cs-Mist* in the tailbud embryo (D,D') and *Cs-Hex* in the tailbud embryo (E,E'), as assessed by in situ hybridization. (A-E) Control embryos and (A'-E') embryos developed from eggs injected with the *Cs-Fgf9/16/20* morpholino.

for *Cs-Mist* and 0/12 for *Cs-Hex*), indicating that transcription of the genes for these four factors is regulated by *Cs-FGF9/16/20* (Fig. 3). However, *Cs-NoTrlc* expression was not downregulated in most experimental embryos (9/14), suggesting that the expression of this transcription factor gene is at least partly regulated by factor(s) other than *Cs-FGF9/16/20*.

Of these five transcription factor genes, *Cs-Twist-like1* is the first gene expressed in B-line mesenchymal cells, whereas *Cs-NoTrlc* is the first gene expressed in A-line mesenchymal cells (TLCs), as shown in Fig. 2. Therefore, it is possible that *Cs-Twist-like1* or *Cs-NoTrlc* regulates the expression of the remaining transcription factor genes, and we therefore examined this possibility. Microinjection of a morpholino against *Cs-Twist-like1* suppressed the expression of *Cs-Twist-like2* (0/51 at the late gastrula stage, and 0/16 at the tailbud stage), *Cs-Mist* (0/9 at the tailbud stage) and *Cs-Hex* (0/17 at the tailbud stage), indicating that *Cs-Twist-like2*, *Cs-Mist* and *Cs-Hex* are regulated downstream of *Cs-Twist-like1* (Fig. 4A'-C').

Fig. 4. Genetic cascade among the transcription factors identified in the present study. (A-C) Control embryos. (A'-C') Experimental embryos developed from eggs injected with the *Cs-Twist-like1* morpholino. Expression of *Cs-Twist-like2* in the late gastrula embryo (A,A') and in the tailbud embryo (insets in A and A'). (B,B') *Cs-Mist* expression in the tailbud embryo. (C,C') *Cs-Hex* expression in the tailbud embryo. (D,D',E,E') Expression of *Cs-Twist-like1*. (D,E) In control early gastrulae, *Cs-Twist-like1* was evident in A7.6 blastomeres (black arrowheads). (D',E') In early gastrulae developed from eggs injected with the *Cs-NoTrlc* morpholino, *Cs-Twist-like1* expression was lost in A7.6 blastomeres (white arrowheads). Embryos shown in E, E' were arrested at the 110-cell stage and cultivated until the control untreated embryos developed to the late gastrula stage.



Microinjection of a morpholino against *Cs-NoTrlc* into fertilized eggs resulted in loss of expression of *Cs-Twist-like1* in TLCs at the early gastrula stage (white arrowheads in Fig. 4D'; 0/15). To demonstrate this result more clearly, cleavage-arrested embryos were used. In embryos arrested at the 110-cell stage (Fig. 4E'), *Cs-Twist-like1* was expressed in B7.7 and B8.5 cells, but not in A7.6 (TLC) cells (0/33), whereas the control embryos (Fig. 4E) expressed *Cs-Twist-like1* in A7.6 (TLC) cells in addition to B7.7 and B8.5. Therefore, the expression of *Cs-Twist-like1* in the A7.6 (TLC) lineage is under the control of *Cs-NoTrlc*.

Cs-Twist-like2 and *Cs-Hex* are also thought to be under the control of *Cs-NoTrlc*, because *Cs-Twist-like2* and *Cs-Hex* are under the regulation of *Cs-Twist-like1*. Injection of the *Cs-NoTrlc* morpholino resulted in loss of expression of *Cs-Twist-like2* in TLCs (0/27; refer to Fig. 7B'). *Cs-Mist* was not examined in the present study, because it is not expressed in TLCs. These results strongly suggest that *Cs-Twist-like1* and *Cs-NoTrlc* play important roles in determination of the mesenchymal fate. Therefore, we further examined the functions of these two genes as described in the following sections.

***Twist-like1* is essential for determination of mesenchymal fate**

Microinjection of the *Cs-Twist-like1* morpholino into fertilized eggs resulted in larvae that appeared morphologically normal (Fig. 5A,A'). The differentiation of major tissues was examined with molecular markers at the tailbud stage. Marker genes for epidermis (*Cs-Epi1*), nervous system (*Cs-ETR*) and notochord (*Cs-fibrn*) were expressed in experimental embryos (Fig. 5B'-D') as they are in control embryos (Fig. 5B-D), suggesting that the differentiation of these tissues is not disturbed by injection of the *Cs-Twist-like1* morpholino. A marker gene for muscle (*Cs-MAI*) was expressed in the tail muscle cells of experimental embryos (Fig. 5E') as it was in control embryos (Fig. 5E). However, in all cases, the anterior boundary of *Cs-MAI*-positive cells was extended anteriorly (16/16), suggesting ectopic expression of the muscle marker gene (arrow in Fig. 5E'). Differentiation of endoderm appeared normal, as indicated by examination of the histochemical staining of endoderm-specific alkaline phosphatase (AP) in larvae (Fig. 5F,F'; 13/13). However, the differentiation marker for mesenchyme (*Cs-Mech1*) was completely lost in experimental embryos (Fig. 5G,G'; 0/17), suggesting an essential role for *Twist-like1* in the determination of mesenchymal fate.

To examine to which tissues the presumptive mesenchymal

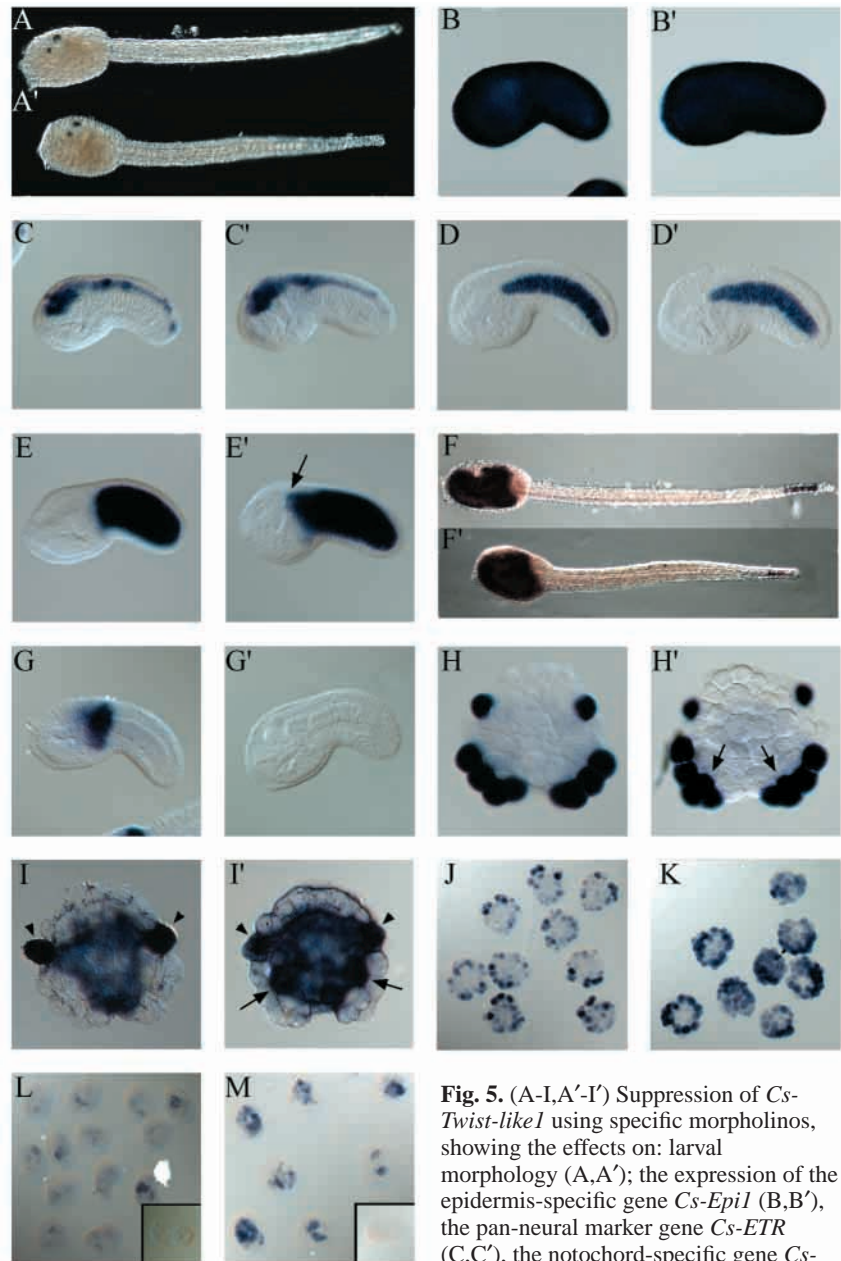


Fig. 5. (A-I,A'-I') Suppression of *Cs-Twist-like1* using specific morpholinos, showing the effects on: larval morphology (A,A'); the expression of the epidermis-specific gene *Cs-Epi1* (B,B'), the pan-neural marker gene *Cs-ETR* (C,C'), the notochord-specific gene *Cs-fibrn* (D,D'), and the muscle-specific actin gene *Cs-MAI* (E,E',H,H'); the endoderm-specific histochemical activity of alkaline phosphatase (F,F',I,I'); and the mesenchyme-specific histochemical activity of alkaline phosphatase (F,F',I,I'). (A-I) Control embryos. (A'-I') Embryos developed from eggs injected with the *Twist-like1*-morpholino. (H,H',I,I') Embryos arrested at the 110-cell stage. Arrows in E' and H' indicate the appearance of extra cells with *Cs-MAI* expression. Arrows in I' indicate the occurrence of extra cells with histochemically detected activity of alkaline phosphatase. Arrowheads in I and I' indicate pigmented cells that are not alkaline-phosphatase positive. (J,K) Effect of overexpression of *Cs-Twist-like1* mRNA as assessed by expression of *Cs-Mech1* (J) and *Cs-Twist-like2* (K). (L,M) Co-injection of the *Cs-FGF9/16/20* morpholino and *Cs-Twist-like1* mRNA resulted in the recovery of *Cs-Mech1* (L) and *Cs-Twist-like2* (M) expression. The insets in L and M are control embryos injected with the *Cs-FGF9/16/20* morpholino and examined for *Cs-Mech1* (L) and *Cs-Twist-like2* (M) expression.

cells were trans-differentiated in the *Cs-Twist-like1* morpholino-injected embryos, we again took advantage of cleavage-arrested embryos. In control 110-cell-arrested

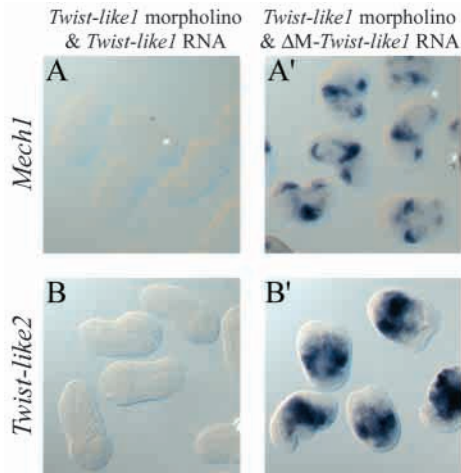


Fig. 6. Specificity of *Cs-Twist-like1* morpholino as assessed by the expression of *Cs-Mech1* (A,A') and *Cs-Twist-like2* (B,B'). (A,B) Co-injection of the *Cs-Twist-like1* morpholino and *Cs-Twist-like1* mRNA with the morpholino recognition sequence resulted in loss of expression of *Cs-Mech1* (A) and *Cs-Twist-like2* (B). (A',B') Co-injection of the *Cs-Twist-like1* morpholino and *Cs-Twist-like1* mRNA lacking the morpholino recognition sequence resulted in recovery of the expression of *Cs-Mech1* (A') and *Cs-Twist-like2* (B').

embryos, *Cs-MAI* was expressed in 10 cells with a muscle fate (Fig. 5H). By contrast, *Cs-Twist-like1* morpholino-injected embryos expressed *Cs-MAI* in one extra pair of cells (B7.7; arrows in Fig. 5H'; 20/22). Together with the result shown in Fig. 5E', this result demonstrates that the fate of B7.7-line mesenchymal cells was changed to muscle. Similarly, the developmental fate of B8.5-line mesenchymal cells was changed to endoderm in embryos injected with the *Cs-Twist-like1* morpholino (arrows in Fig. 5I; 36/36). Because A7.6 cells could not be easily discriminated in the 110-cell-arrested embryos, we could not determine into what kind of cells A7.6 differentiated.

The specificity of the *Cs-Twist-like1* morpholino was confirmed by a rescue experiment. Co-injection of the morpholino and an in vitro synthesized *Cs-Twist-like1* mRNA with the morpholino recognition sequence resulted in the loss of expression of *Cs-Mech1* (Fig. 6A; 0/11) and *Cs-Twist-like2* (Fig. 6B; 0/10), as had occurred in the embryos injected with the morpholino only (Fig. 5G', Fig. 4A'). However, co-injection of the morpholino and *Twist-like1* mRNA without the morpholino recognition sequence (Δ M-*Cs-Twist-like1*) resulted in the recovery of *Cs-Mech1* (Fig. 6A'; 11/11) and *Cs-Twist-like2* expression (Fig. 6B'; 10/10). Therefore, these results strongly suggest that this morpholino works as a specific inhibitor of *Cs-Twist-like1* mRNA.

Next, we examined the effects of *Cs-Twist-like1* mRNA overexpression. Microinjection of synthetic *Cs-Twist-like1* mRNA resulted in the ectopic differentiation of mesenchymal cells, as revealed by the expression of *Cs-Mech1* (25/28) and *Cs-Twist-like2* (18/18). The 110-cell-arrested embryos showed ectopic expression of *Cs-Mech1* (Fig. 5J; 21/21) and *Cs-Twist-like2* (Fig. 5K; 20/20), mainly in vegetal cells and a-line cells, but not in b-line cells.

If *Twist-like1* plays a central role in mesenchyme determination after induction by *Cs-FGF9/16/20*, *Twist-like1*

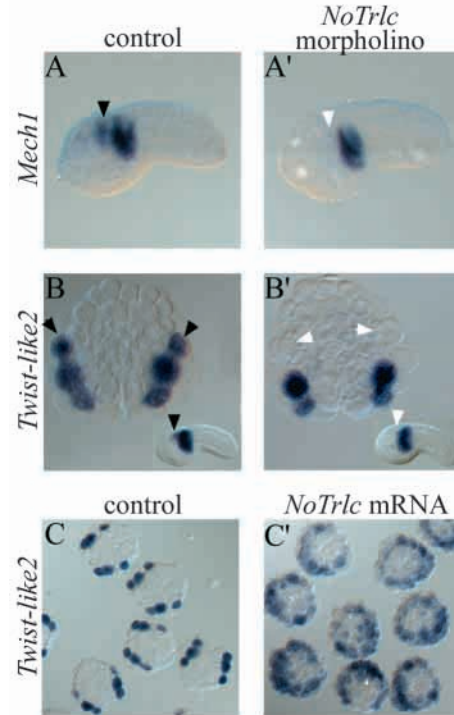


Fig. 7. Effects of suppression by the *Cs-NoTrlc* morpholino on the mesenchyme-specific gene *Cs-Mech1* (A,A') and *Cs-Twist-like2* (B,B'). (A,B) Control embryos. (A',B') Embryos developed from eggs injected with the *Cs-NoTrlc* morpholino. (B,B') Embryos arrested at the 110-cell stage and (B and B' insets) tailbud embryos. White arrowheads in A' and B' indicate loss of expression of *Cs-Mech1* and *Cs-Twist-like2*, respectively, in TLCs (compare with expression shown by black arrowheads in A and B). (C,C') Expression of *Cs-Twist-like2* in control (C) and *Cs-NoTrlc*-mRNA overexpressing (C') embryos.

overexpression in the *Cs-Fgf9/16/20* suppressed embryos should cause recovery of the expression of mesenchyme-specific genes. Microinjection of *Twist-like1* mRNA with the *Cs-Fgf9/16/20*-morpholino into fertilized eggs resulted in recovery of *Cs-Mech1* (13/15) and *Cs-Twist-like2* (15/15) expression at the tailbud stage (Fig. 5L,M), although the expression was not as strong as that seen when *Cs-Twist-like1* mRNA was injected alone. These results suggest that *Twist-like1* acts as a 'key regulatory gene' in the differentiation of ascidian mesenchymal cells.

NoTrlc is essential for the determination of trunk lateral mesenchyme cells

Microinjection of the *Cs-NoTrlc* morpholino into fertilized eggs resulted in larvae whose morphology appeared normal (data not shown). The differentiation of major tissues was examined with molecular markers. The expression of marker genes for epidermis (*Cs-Epi1*), nervous system (*Cs-ETR*), notochord (*Cs-fibrn*) and muscle (*Cs-MAI*) was normal in the experimental tailbud embryos (data not shown). Normal differentiation of endoderm was also confirmed by histochemical staining of AP in larvae (data not shown). However, the differentiation marker of mesenchyme (*Cs-Mech1*) was lost in TLCs of the experimental embryos (Fig. 7A,A'; 0/18), suggesting an

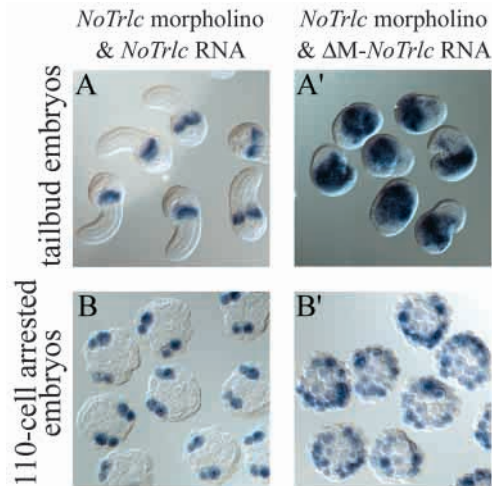


Fig. 8. Specificity of *Cs-NoTrlc* morpholino as assessed by the expression of *Cs-Twist-like2* in tailbud (A,A') and 110-cell-arrested tailbud (B,B') embryos. (A,B) Co-injection of the *Cs-NoTrlc* morpholino and *Cs-NoTrlc* mRNA with morpholino recognition sequence resulted in loss of expression of *Cs-Twist-like2* in TLCs. (A',B') Co-injection of the *Cs-NoTrlc* morpholino and *Cs-NoTrlc* mRNA without a morpholino recognition sequence resulted in recovery of the expression of *Cs-Twist-like2*.

essential role for *Cs-NoTrlc* in the specification of TLCs. This was consistent with the loss of *Cs-Twist-like2* expression in TLCs of the *NoTrlc* morpholino-injected embryos (Fig. 7B,B'; 0/9 for tailbud embryos and 0/27 for the 110-cell-arrested embryos). Because *Cs-Twist-like2* is expressed more strongly in TLCs than *Cs-Mech1*, we used *Cs-Twist-like2* as a marker for TLCs in the subsequent experiments.

Conversely, overexpression of *Cs-NoTrlc* by microinjection of its synthetic mRNA led to ectopic expression of *Cs-Twist-like2* in experimental tailbud embryos (data not shown; 10/10) and in embryos arrested at the 110-cell stage (Fig. 7C,C'; 27/27), suggesting that *Cs-NoTrlc* overexpression promotes the ectopic differentiation of mesenchyme.

The specificity of the morpholino was confirmed by a rescue experiment. Co-injection of the morpholino and in vitro synthesized *Cs-NoTrlc* mRNA with the morpholino recognition sequence resulted in the loss of *Cs-Twist-like2* expression in TLCs of experimental tailbud embryos (Fig. 8A; 0/8) and embryos arrested at the 110-cell stage (Fig. 8B; 0/16), as it did in embryos injected with the morpholino alone (Fig. 7B'). However, co-injection of the morpholino and *Cs-NoTrlc* mRNA without the morpholino recognition sequence (ΔM -*NoTrlc*) resulted in the recovery of *Cs-Twist-like2* expression in the experimental tailbud embryos (Fig. 8A'; 9/9) and in the 110-cell arrested embryos (Fig. 8B'; 18/18), suggesting that this morpholino works as a specific inhibitor of *Cs-NoTrlc* mRNA.

FoxD is necessary for specification of trunk lateral cells and partially regulates *NoTrlc*

Previously, we showed that *Cs-FoxD* is an essential factor for notochord specification (Imai et al., 2002b). *Cs-FoxD* is transiently expressed in A5.2 blastomeres of the 16-cell embryo and A6.3 blastomeres of the 32-cell embryo. A5.2 and

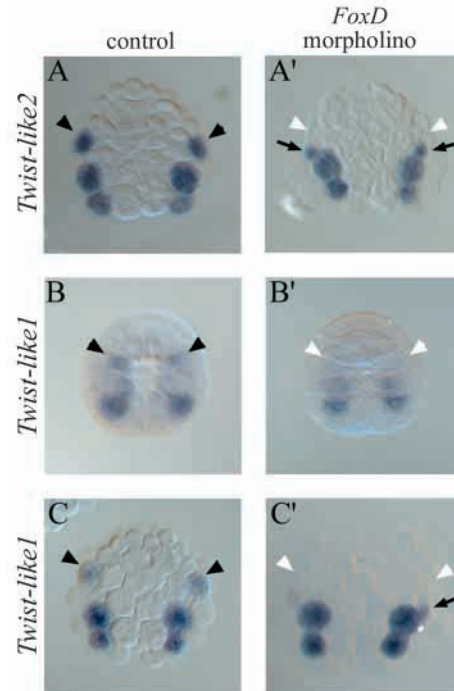


Fig. 9. Effect of functional suppression of *Cs-FoxD* on the differentiation of TLCs. Expression of *Cs-Twist-like2* (A,A') and *Cs-Twist-like1* (B,B',C,C') in control embryos (A-C) and embryos developed from eggs injected with the *Cs-FoxD* morpholino (A'-C'). The loss of expression of the genes in TLCs is shown by white arrowheads. (A,A',C,C') Embryos arrested at the 110-cell stage, in which ectopic expression of *Cs-Twist-like1* and *Cs-Twist-like2* was evident in B-line notochord cells (black arrows).

A6.3 are progenitors of A7.6 TLCs (see Fig. 2A). Therefore, *Cs-FoxD* is a potential regulator of TLC specification. In the previous study, the differentiation of mesenchyme was assessed by examining the expression of *Cs-Mech1*, and expression of *Cs-Mech1* was not lost in the *Cs-FoxD* knockdown embryos. However, it was not determined whether all mesenchyme cells, including TLCs, were differentiated. Therefore, we re-examined this issue using cleavage-arrested embryos. As shown in Fig. 9A,A', in *Cs-FoxD* knockdown embryos, *Cs-Twist-like2* was not expressed in TLCs (white arrowheads in Fig. 9A'), although B-line notochord cells expressed *Cs-Twist-like2* (arrows in Fig. 9A'). Therefore, *Cs-FoxD* is essential for specification of TLCs.

Next, the expression of *Cs-Twist-like1* and *Cs-NoTrlc* was examined in *Cs-FoxD* knockdown embryos, because these genes are the upstream factors of *Cs-Twist-like2*. Suppression of *Cs-FoxD* resulted in loss of *Cs-Twist-like1* expression in TLCs of early gastrulae (Fig. 9B,B'; 0/31) and of cleavage-arrested embryos (Fig. 9C,C'; 0/14). In the case of *Cs-NoTrlc*, 74% of experimental embryos lost expression, whereas the remaining 26% expressed *Cs-NoTrlc* weakly ($n=26$; data not shown). To confirm this result, quantitative RT-PCR was performed. Template RNA was extracted from 25 embryos and reverse-transcribed. Then real-time PCR was performed using a quantity of cDNA that was equivalent to one embryo. A 56% and 67% reduction of the amount of *Cs-NoTrlc* mRNA was observed in 64-cell and 110-cell stage embryos, respectively,

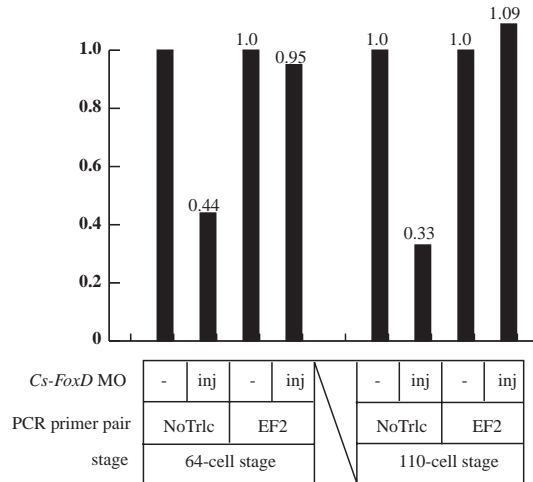


Fig. 10. Quantification of the amounts of *Cs-NoTrlc* and *EF2* mRNA in control embryos (-), and in experimental embryos developed from eggs injected with the *Cs-FoxD* morpholino (inj). Quantification was performed with 64-cell embryos and 110-cell embryos by real-time RT-PCR. The relative amounts of mRNA compared with those in control embryos are shown.

when compared with uninjected control embryos (Fig. 10). The amount of *EF2* mRNA, used as a control, was not changed (Fig. 10). Therefore, *Cs-FoxD* is highly likely to be one of the upstream factors of *Cs-NoTrlc*, and some other unknown factors may also regulate *Cs-NoTrlc* expression.

DISCUSSION

Genetic cascades for specification of mesenchyme

In the present and previous studies, we identified key players for specification, determination and differentiation of ascidian larval mesenchyme. The present study also disclosed intrinsic differences among different types of mesenchyme. Fig. 11 is an outline of molecular cascades for the specification of ascidian mesenchyme.

B8.5-line mesenchyme (Fig. 11A)

Previous studies demonstrated that the presumptive endoderm cells induce the B-line mesenchyme (Kim and Nishida, 1999) and that *Cs-Fgf9/16/20*, which is expressed in endodermal cells under regulation of maternally supplied β -catenin, is an inducer of this mesenchyme (Imai et al., 2002a). As shown in Fig. 3A, this induction is essential for the expression of *Twist-like1*. *Twist-like1* then triggers the differentiation of this line of mesenchyme, which leads to expression of *Twist-like2*, *Hex* and *Mech1*. Without *Twist-like1* activity, B8.5-line cells differentiate into endoderm; however, they differentiate into muscle without induction through *FGF9/16/20*. The reason for this difference remains to be determined. What makes B8.5 cells competent to respond to *FGF9/16/20*? Our previous study showed that β -catenin is not required for B8.5 to acquire this competence, because mesenchyme is able to differentiate in embryos co-injected with β -catenin-morpholino and *FGF9/16/20* synthetic mRNA, although we did not determine which types of mesenchyme differentiated in the experimental

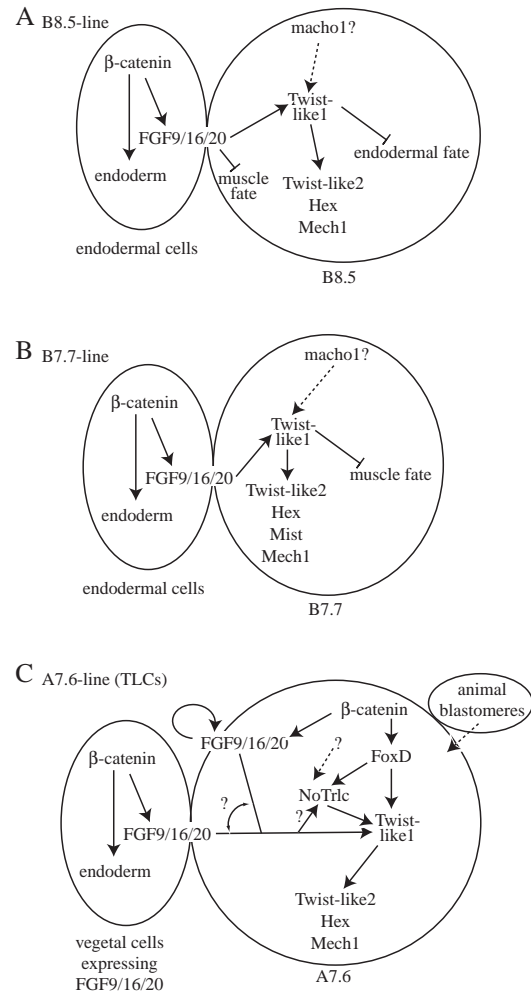


Fig. 11. Representation of the genetic cascades involved in the differentiation of B8.5-line (A), B7.7-line (B) and A7.6-line (C) mesenchyme (TLCs). Arrows indicate positive regulation of genes. Dotted arrows indicate a possible involvement that is suggested on the basis of results from another ascidian species. See details in text.

embryos (Imai et al., 2002a). In *Halocynthia roretzi*, it has been demonstrated that posterior vegetal cytoplasm containing a muscle determinant, *macho1*, is essential for differentiation of mesenchyme (Kim et al., 2000; Nishida and Sawada, 2001). This may be the case with *Ciona*, although it has not yet been proven.

B7.7-line mesenchyme (Fig. 11B)

This line of mesenchyme is probably determined in a similar way to the B8.5-line. The differences are (1) that *Twist-like1* is expressed earlier in B7.7 than in B8.5, (2) that suppression of *Twist-like1* results in ectopic differentiation of muscle cells, and (3) that *Mist* is expressed in this line of mesenchyme under the control of *Twist-like1*.

A7.6-line mesenchyme (TLCs; Fig. 11C)

Twist-like1 is also essential for the determination of this lineage. As in the other two lines of mesenchyme, *Fgf9/16/20* is required for expression of *Twist-like1* and, thus,

differentiation of TLCs. At present, we do not know when and from where this FGF signal is emitted. *Fgf9/16/20* is also expressed transiently in the presumptive TLC blastomeres (A5.2 at the 16-cell stage and A6.3 at the 32-cell stage). Therefore it is also possible that the presumptive TLC blastomeres induce themselves during this period through an autocrine loop. In addition, *NoTrlc* and *FoxD* are also intrinsically required for expression of *Twist-like1* in this lineage. *NoTrlc* is also partially regulated by FGF9/16/20, whereas *FoxD* is independent of FGF9/16/20 (Imai et al., 2002b). Injection of the *FoxD* morpholino results in a complete loss of expression of *Twist-like1* in this line, although *NoTrlc* expression is not completely lost in experimental embryos. Therefore, *NoTrlc* and *FoxD* govern *Twist-like1* expression, and *NoTrlc* is partially regulated by *FoxD*. Because *FoxD* regulation of *NoTrlc* is partial, it would appear that there is an unknown factor that regulates *NoTrlc*. Kawaminani and Nishida showed by embryological manipulation of *Halocynthia* embryos that induction from animal blastomeres is required for differentiation of TLCs (Kawaminani and Nishida, 1997). If this is the case with *Ciona*, then this signal might regulate *NoTrlc*.

In the *FoxD*-knockdown embryos, the presumptive B-line notochord blastomeres did not differentiate into notochord (Imai et al., 2002b) (this study), but instead expressed *Cs-Twist-like1* and *Cs-Twist-like2*, suggesting that the B-line notochord precursors trans-differentiated into the mesenchyme (Fig. 9A',C'). Because *FoxD* is essential for notochord (Imai et al., 2002b) and TLCs, the differentiated mesenchyme in the *FoxD*-knockdown embryos is likely to be B-line mesenchyme and not TLCs. For example, the B-line notochord precursors (B8.6) may trace the same developmental fate as their sibling mesenchymal blastomeres (B8.5) in the *FoxD*-knockdown embryos. Nevertheless, it is also possible that the B-line notochord precursors trans-differentiated into TLCs. At present, we do not have any good marker for discriminating the B-line mesenchyme from the TLCs; this will be one of the subjects for future research.

Determination of mesenchyme

In *Halocynthia* embryos, B-line (B8.5 and B7.7) mesenchyme is specified or committed to mesenchyme fate at the late 64-cell stage, and TLCs are specified at the 44-cell stage when A6.3 cleaves into A7.6 (TLC precursor) and A7.5 (endoderm precursor) (Kawaminani and Nishida, 1997; Kim and Nishida, 1999). The expression of *Twist-like1* begins after the specification of these precursors, and all known mesenchymal genes in *Ciona* are completely regulated by *Twist-like1*. Therefore, it is likely that *Twist-like1* is a transcription factor that determines the mesenchymal fate, triggering a final step for the differentiation of mesenchyme.

In *Ciona* embryos, *Cs-FGF9/16/20* and a Notch signaling pathway are potential inducers of notochord (Corbo et al., 1998; Imai et al., 2002a). After induction, the notochord precursors express *Brachyury*, which triggers differentiation of the notochord. Therefore, a genetic cascade for notochord differentiation converges on *Brachyury*, which then activates expression of a variety of notochord-specific genes. This scheme is probably applicable to mesenchyme. That is, the genetic cascade for mesenchyme differentiation converges on *Twist-like1*.

Conserved and non-conserved mechanisms for mesoderm induction within chordates

Twist-like1 and *Twist-like2* are bHLH factor genes that have recently duplicated in the ascidian lineage (Fig. 1B). A Blast search revealed the factors to have the closest relationship with *Twist*. However, a detailed phylogenetic analysis did not support this orthology with a high bootstrap value, although *Twist* proteins have been identified in a wide range of animals from jellyfish to vertebrates. In addition to the bHLH domains, vertebrate and jellyfish *Twist* proteins have a conserved motif called the WR motif (Castanon and Baylies, 2002). The WR motif was not found in *Twist-like1* or *Twist-like2*. In spite of the lack of supporting evidence for their orthology, the *Ciona intestinalis* genome does not contain any genes closer to *Twist* than *Twist-like1* and *Twist-like2* (Satou et al., 2003). Therefore, if genes orthologous to *Twist* have been maintained in the *Ciona* genome, *Twist-like1* and *Twist-like2* are the most likely candidates.

Twist can be found across various species in cells contributing to mesoderm and/or its derivatives, suggesting that *Twist* may have a common role in mesoderm differentiation (reviewed by Castanon and Baylies, 2002). In *Drosophila*, *Twist* is required for the induction of mesoderm and gastrulation, and subsequently acts as an activator in the process of somatic muscle development. In mice, *Twist1* is first uniformly expressed in the early somites, and is then expressed in the sclerotome and the dermatome, but not in the myotome, after the somites are compartmentalized. The myotome expresses other bHLH genes called myogenic factors, including *MyoD* and *Myf5*. Indeed, *Twist1* has an inhibitory role in muscle differentiation.

If *Twist-like1* and *Twist-like2* are orthologs of *Twist*, their roles, especially the role of *Twist-like1*, appear to be similar to those of vertebrate *Twist*. *Twist-like1* and *Twist-like2* are not expressed in muscle cells. In ascidian larval muscle cells, a myogenic factor (AMD1 in *Halocynthia* and Ci-MDF in *Ciona intestinalis*), which represents an ancestral form of four vertebrate myogenic factors (*MyoD*, *Myf5*, myogenin and MRF4), is expressed (Satoh et al., 1996; Meedel et al., 1997). Without induction by *Cs-FGF9/16/20*, mesenchyme precursors fail to express *Twist-like1* and differentiate into muscle cells. These ectopic muscle cells express a myogenic factor (K.S.I., unpublished). This mutually exclusive expression of *Twist-like1* and the myogenic factor is closely comparable to the vertebrate situation.

In the *Ciona intestinalis* genome, there are two orthologous genes for *Twist-like1* and one orthologous gene for *Twist-like2*. These three genes are aligned in tandem within 15 kb of the genome. The sequence similarity shown in Fig. 1, and the genomic organization strongly suggests that these genes occurred recently by gene duplications after the divergence of ascidians and vertebrates. The phylogenetic tree shown in Fig. 1B also shows that *Twist-like1* was further duplicated in the *Ciona intestinalis* lineage. Although the function of *Twist-like2* has not yet been determined, *Twist-like2* may have acquired a novel function during ascidian evolution, if an ancestral function of *Twist-like1* and *Twist-like2* is the specification of mesoderm (mesenchyme), the common function for *Twist* among other organisms.

The phylogenetic relationship of another bHLH gene,

NoTrlc, with other known bHLH genes is also not clear. Although a Blast search suggests that *NoTrlc* is evolutionarily close to *dHand* and *eHand*, the *Ciona* genome contains a clear ortholog of vertebrate *eHand* and *dHand* (Satou et al., 2003). Therefore, *NoTrlc* characterized in the present study is not likely to be an ortholog of vertebrate *dHand* and *eHand*. The molecular evolution of the ascidian bHLH genes will be an intriguing subject for future research.

Intrinsic differences among types of ascidian larval mesenchyme cells

Ascidian larval mesenchymal cells express many genes for proteins that function in protein synthesis and RNA metabolism (Satou et al., 2001; Kusakabe et al., 2002). The mesenchymal cells also have developmental competency as primordia for adult tissues and organs. Hirano and Nishida examined the developmental fates of *Halocynthia* larval mesenchyme cells (Hirano and Nishida, 1997). They reported that B7.7- and B8.5-line mesenchymal cells mainly give rise to tunic cells, whereas TLCs give rise to blood cells, latitudinal mantle muscle and ciliary epithelium of gill-slits. In the present study, we identified three transcription factor genes, *Twist-like2*, *Hex* and *Mist*, that are expressed during the late embryogenesis. A preliminary experiment using a morpholino against *Twist-like2* did not show any defect in larvae injected with the morpholino. However, it is possible that these larvae eventually show defects in the formation of adult organs or tissues after metamorphosis. *Mist* is expressed only in the B7.7-line mesenchyme, suggesting that this line of mesenchymal cells is intrinsically different from the B8.5-line mesenchyme. Hirano and Nishida reported that, in a few specimens examined, B7.7-line mesenchymal cells give rise to blood cells and B8.5-line mesenchymal cells give rise to granules near the holdfast (Hirano and Nishida, 1997). If this is the case with *Ciona*, the B7.7- and B8.5-line mesenchymes may have intrinsically different potentials to construct adult organs during larval development.

Ciona intestinalis has only ~16,000 protein-coding genes in its genome (Dehal et al., 2002), and an EST/cDNA project has collected numerous cDNAs of this animal (Satou et al., 2002b). We now have a cDNA collection covering more than 85% of *Ciona* genes and more than 90% of developmental genes (Satou and Satoh, 2003; Satoh et al., 2003). The draft genome sequence and this cDNA collection open the door to a comprehensive and integrated analysis of developmental mechanisms at the molecular level. In the present study, we have shown that comprehensive screening is one of the most powerful approaches to elucidating the molecular mechanisms of development in the *Ciona* system.

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