The ascidian *Mesp* gene specifies heart precursor cells

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Accepted 17 February 2004

Development 131, 2533-2541
Published by The Company of Biologists 2004
doi:10.1242/dev.01145

Summary

Understanding the molecular basis of heart development is an important research area, because malformation of the cardiovascular system is among the most frequent inborn defects. Although recent research has identified molecules responsible for heart morphogenesis in vertebrates, the initial specification of heart progenitors has not been well characterized. Ascidians provide an appropriate experimental system for exploring this specification mechanism, because the lineage for the juvenile heart is well characterized, with B7.5 cells at the 110-cell stage giving rise to embryonic trunk ventral cells (TVCs) or the juvenile heart progenitors. Here, we show that *Cs-Mesp*, the sole ortholog of vertebrate *Mesp* genes in the ascidian *Ciona savignyi*, is specifically and transiently expressed in the embryonic heart progenitor cells (B7.5 cells). *Cs-Mesp* is essential for the specification of heart precursor cells, in which *Nkx*, *HAND* and *HAND-like* (*NoTrlc*) genes are expressed. As a result, knockdown of *Cs-Mesp* with specific morpholino antisense oligonucleotides causes failure of the development of the juvenile heart. Together with previous evidence obtained in mice, the present results suggest that a mechanism for heart specification beginning with *Mesp* through *Nkx* and *HAND* is conserved among chordates.

Movies available online

Key words: Chordate, *Ciona savignyi*, *Mesp*, Heart development

Introduction

Ascidians are primitive chordates that share some developmental features with vertebrates (Satoh, 1994). The trunk of the tadpole larva contains a dorsal central nervous system (CNS) with two sensory organs (otolith and ocellus), endoderm, mesenchyme including trunk lateral cells (TLCs), and trunk ventral cells (TVCs). The larval tail contains the notochord, 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; Corbo et al., 2001; Satoh, 2003).

Recent studies have identified genes and molecules responsible for specification of endomesodermal cells of the ascidian embryo. Muscle cells are specified primarily by maternal transcripts of the *macho-1* gene, which encodes a Zic-like zinc finger protein (Nishida and Sawada, 2001; Satou et al., 2002a). Macho-1 is also involved in the antero-posterior patterning of *Halocynthia roretzi* embryos (Kobayashi et al., 2003). Endodermal cells are specified primarily by maternally provided β-catenin (Imai et al., 2000), and *Lhx3*, which is expressed in the endodermal lineage under the control of β-catenin, is essential for the differentiation of endodermal cells (Satou et al., 2001a). A key gene for notochord differentiation is *Brachyury* (Yasuo and Satoh, 1998; Corbo et al., 1997). In *Ciona* embryos, *Fgf9/16/20* (Imai et al., 2002a), *ZicL* (Imai et al., 2002c; Yagi et al., 2004) and *FoxD* (Imai et al., 2002b) act in the upstream genetic cascade leading to *Ci-Bra* expression in notochord cells. Mesenchyme cells, including TLCs, are specified by cellular interaction, and Fgf9/16/20 plays a pivotal role in this interaction (Imai et al., 2002a) and activates *Twist-like1*, a key gene for the differentiation of mesenchyme cells and TLCs (Imai et al., 2003). However, no genes involved in the specification of TVCs have been identified yet. Therefore, identification and characterization of genes responsible for TVC specification will be required for a complete understanding of the molecular mechanisms of endomesoderm specification in the ascidian embryo.

In *Halocynthia*, TVCs give rise to heart, latitudinal mantle and atrial siphon muscle in the adult (Hirano and Nishida, 1997). The ascidian heart first appears after metamorphosis as a tube with a single layered myoepithelium that is continuous to a single layered pericardial wall (Ichikawa and Hoshino, 1967; Satoh, 1994; Davidson and Levine, 2003). The ascidian has an open blood-vascular system, and its blood flow is regularly reversed. Despite its structural primitiveness, the ascidian heart undergoes morphogenesis in a similar manner to the vertebrate heart (Davidson and Levine, 2003). As shown in Fig. 1A-D, in ascidians a pair of B7.5 cells of the bilaterally symmetrical 110-cell embryo gives rise to TVCs and a pair of anterior muscle cells in the larva. The TVCs differentiate on both sides of the trunk of the tailbud embryo, and after hatching they migrate and fuse along the ventral midline of the larva. After metamorphosis, the majority of these cells are thought to differentiate to form the heart.

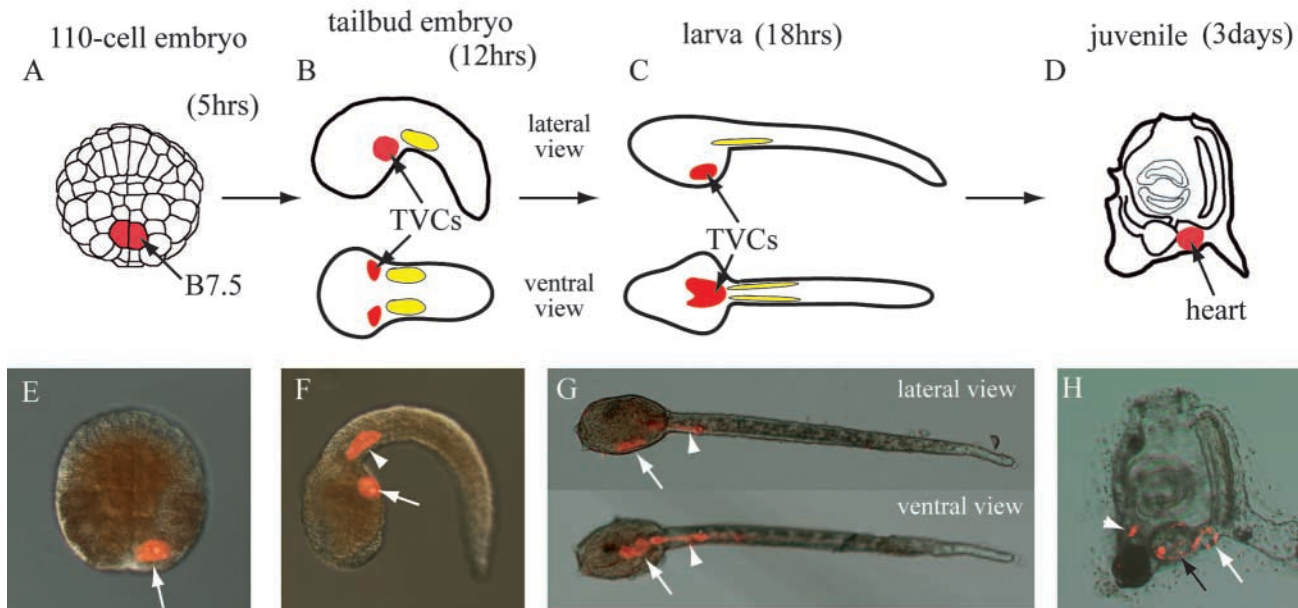

Fig. 1. (A-D) Schematic representation of ascidian heart development. (A) A pair of heart lineage cells (B7.5) in the bilaterally symmetrical 110-cell embryo. (B) Heart progenitors called trunk ventral cells (TVCs) in the tailbud embryo, which differentiate first on each lateral side of the embryo and (C) then fuse ventrally in the swimming larva. In (B,C), upper panels show lateral views and lower panels show ventral views. A pair of anterior muscle cells is also derived from B7.5 (yellow). (D) The heart in the juvenile. (E-H) The developmental fate of the B7.5 blastomere. (E) One of the B7.5 blastomeres is labeled with DiI (arrow). (F) A tailbud embryo and (G) a larva in which DiI was injected into B7.5 at the 110-cell stage. The TVCs are labeled with DiI (arrows), as well as two anterior muscle cells (arrowheads). (H) A juvenile in which DiI was injected into B6.3 at the 32-cell stage. The heart (white arrow), germline cells (black arrow) and degenerated larval tail muscle cells (white arrowhead) are labeled.

In vertebrates, BMP, Wnt-11 and FGF signaling promote the restricted expression in the heart field of genes including *Nkx* and *HAND*, which play essential roles in heart development (reviewed by Harvey, 2002; Cripps and Olson, 2002). However, the initial mechanism of specification of heart progenitors has not yet been clarified. Mouse *Mesp1* and *Mesp2* are candidates for genes with expression that specifies heart progenitors, because these genes are known to be expressed in the ingressing mesoderm fated to become extra-embryonic and cranial-cardiac mesoderm (Saga et al., 2000). *Mesp1* knockout causes abnormal heart development and a *Mesp1*/*Mesp2* double-knockout mouse was found to lack any cranio-cardiac mesoderm. Therefore, the precise function of *Mesp1* and *Mesp2* in heart development is unclear, although the genes may be involved in the first step of heart development.

The draft genome sequence of *Ciona intestinalis* suggests that the ascidian has a much simpler genome than those of vertebrates (Dehal et al., 2002), due primarily to carrying fewer paralogous genes and partly to the compactness of its intergenic regions (e.g. Satou et al., 2003; Wada et al., 2003). This simplicity greatly facilitates studies of genetic networks. In the present study, we tried to identify genes involved in ascidian heart formation, focusing especially on the mechanism by which early heart progenitor cells are specified, a process that is rather difficult to study in higher vertebrates.

Materials and methods

Ascidian eggs and embryos

Ciona savignyi adults were obtained from the Maizuru Fisheries Research Station of Kyoto University and the International Coast Research Centre of the Ocean Research Institute, 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 gonoducts. 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 a '*Ciona intestinalis* gene collection' (Satou et al., 2002b). Their *C. savignyi* counterparts were first searched for in genome sequences that have been produced by whole-genome shotgun sequencing and 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 distributions 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).

Microinjection of morpholino oligos and DiI

In the present study, we used 25-mer morpholino oligos (hereafter referred to as 'morpholinos'; Gene Tools, LLC). The sequences of the morpholinos against *Cs-Mesp* were as follows: Cs-Mesp-MO1, 5′-CATGAATACGTTTCCAGGTAAAAAT-3′; and Cs-Mesp-MO2, 5′-AGATTTAAGCAAATATCGTTGCCGA-3′. The morpholinos against *β-catenin* and *Cs-macho1* are described in previous reports

and their specificities have been demonstrated (Satou et al., 2001b; Satou et al., 2002a).

After insemination, fertilized eggs were microinjected with 15 pmole of morpholinos and/or synthetic capped mRNAs in 30 pl of solution using a micromanipulator (Narishige Scientific Instrument Lab, Tokyo) as described (Imai et al., 2000). Injected eggs were reared at about 18°C in MFSW containing 50 μg/ml streptomycin sulfate.

DiI (CellTracker CM-DiI, Molecular Probes) was dissolved in soybean oil at the concentration of 1 mg/ml. We injected the DiI-solution into the B6.3 blastomere of the 32-cell embryo with intact chorion to trace its lineage after metamorphosis and into the B7.5 blastomere of the 110-cell dechorionated embryo to trace its lineage to the larva. DiI-labeled embryos and juveniles were observed using fluorescent microscopy.

Detection of differentiation markers

The following cell-specific markers were used to assess the differentiation of embryonic cells: a larval muscle-specific actin gene (*Cs-MA1*) (Chiba et al., 1998), an epidermis-specific gene (*Cs-Epi1*) (Chiba et al., 1998), a notochord-specific gene (*Cs-fibrinogen-like* or *Cs-fibrn*) (Imai et al., 2002a), a mesenchyme-specific gene (*Cs-Mech1*) (Imai et al., 2002a) and a pan-neural marker gene (*Cs-ETR*) (Imai et al., 2002a). The marker genes were detected by whole-mount in-situ hybridization. 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

In total 25 embryos 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 was prepared. The RNA was then used for cDNA synthesis with oligo(dT) primers as described by Imai (2003). Real-time RT-PCR was performed using SYBR Green PCR Master Mix and an ABI prism 7000 (Applied Biosystem). One-embryo-equivalent 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.

Results

Trunk ventral cells of *Ciona* embryos are precursors for the adult heart

To confirm that *Ciona* TVCs are derived from B7.5 blastomeres, one of the B7.5 blastomeres of the dechorionated 110-cell embryo was labeled by DiI injection (Fig. 1E). The labeled embryos were incubated and fixed at the middle tailbud stage. It was observed that TVCs and two muscle cells at the anterior part of the tail were labeled, indicating that the B7.5 blastomere in *Ciona* embryos has the same fate as that in *Halocynthia* embryos up to the tailbud embryo stage (Fig. 1F). Then the TVCs migrate along the ventral midline of the larva (Fig. 1G), as shown previously (Davidson and Levine, 2003).

Next, to confirm that *Ciona* TVCs give rise to adult heart, another DiI-labeling experiment was performed. Because it is difficult to inject DiI into the B7.5 blastomere of the 110-cell embryo with intact chorion, which is required for metamorphosis, we injected DiI into B6.3, which is a parental blastomere of B7.5 and B7.6, of the 32-cell embryo with

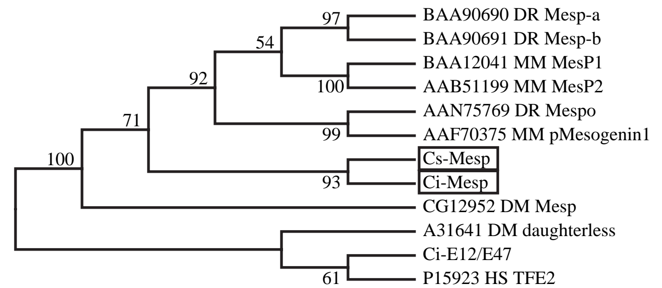

Fig. 2. *Ciona Mesp* gene. A molecular phylogenetic tree generated by the neighbor-joining method based on the alignment of the bHLH domain indicating orthology between Mesp proteins of two *Ciona* species (enclosed by boxes) and other animals. The number on each node indicates the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. The tree is shown as a bootstrap consensus tree (cut-off value=50%). E proteins of *Drosophila* (daughterless), *C. intestinalis* (Ci-E12/E47) and humans (TFE2) are used as outgroups. Proteins of animals other than ascidians are shown by their accession number, species abbreviation and protein name. Species abbreviations are as follows: HS, *Homo sapiens*; MM, *Mus musculus*; DR, *Danio rerio*; DM, *Drosophila melanogaster*; Ci, *Ciona intestinalis*; Cs, *Ciona savignyi*.

chorion. The injected embryos were incubated until they become juveniles with two gill-slits. As shown in Fig. 1H, the heart of the resultant juvenile was labeled with DiI, as well as several cells along the ventral side of the stomach and degenerated larval tail muscle. Because one of the B6.3 daughter cells, B7.6, was shown to become a germ cell, and the primordial germ cells are aligned toward the ventral side of the stomach (Takamura et al., 2002), the labeled cells along the ventral side of the stomach are probably the primordial germ cells derived from B7.6. Therefore, the juvenile heart is highly likely to be derived from embryonic B7.5. In *Halocynthia*, B7.5 gives rise to latitudinal mantle muscle and atrial muscle in addition to the heart in juveniles. However, we could not observe the labeling of these muscles with DiI.

Identification of *Cs-Mesp* and its embryonic expression

In a comprehensive in-situ hybridization study of *C. intestinalis* genes (Satou et al., 2002c), we found that a sole ortholog of the vertebrate *Mesp* genes is expressed specifically and transiently in heart progenitor cells at the beginning of and during gastrulation. Because embryos of a closely related species, *C. savignyi*, are more amenable to embryological manipulations, we used *C. savignyi* embryos in the following studies. First, we obtained cDNA spanning the entire coding sequence of the *C. savignyi Mesp* gene (*Cs-Mesp*) by 5′ and 3′ RACE reactions (DDBJ/EMBL/GENBANK accession number AB125640). The deduced amino acid sequence indicated the presence of a basic helix-loop-helix (bHLH) region that is highly conserved among Mesp family members, including mouse Mesp1, Mesp2 and pMesogenin1. A phylogenetic tree constructed by a neighbor-joining method revealed that the ascidian Mesp is orthologous to all known Mesp family proteins (Fig. 2), suggesting that the last common ancestor of ascidians and vertebrates had one gene in this family.

The expression pattern of *Cs-Mesp* during embryogenesis was examined by whole-mount in-situ hybridization. Neither

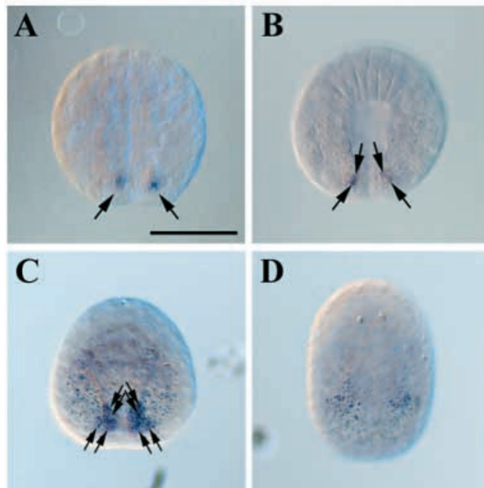

Fig. 3. *Cs-Mesp* is expressed transiently and specifically in (A) B7.5 blastomeres at the 110-cell stage, (B) their daughter cells (B8.9 and B8.10) at the early to mid-gastrula stage, and (C) their granddaughter cells (B9.17, B9.18, B9.19 and B9.20) at the late gastrula stage. (D) The expression is not observed at the neurula stage. The signals are shown by arrows. Scale bar represents 100 μm.

maternal nor zygotic transcript was detected during cleavage stages. At the beginning of gastrulation (the 110-cell stage), *Cs-Mesp* was expressed in B7.5 blastomeres (Fig. 3A), cells that eventually give rise to TVCs and anterior muscle cells of the larva (Fig. 1). At the early to mid-gastrula stage, *Cs-Mesp* was expressed in the daughter cells of the B7.5 blastomeres (B8.9 and B8.10) (Fig. 3B). At the late gastrula stage, a pair of four B7.5-descendant cells expressed *Cs-Mesp* (Fig. 3C). The *Cs-Mesp* expression was very transient and disappeared by the beginning of neurulation (Fig. 3D).

Characterization of genes expressed in TVCs

To analyze the function of *Cs-Mesp*, several cDNA clones were isolated, including those for the *Nkx* and *HAND* genes (*Cs-Nkx* and *Cs-HAND*), which are known to encode key transcription factors in vertebrate heart development (Harvey, 2002). Genome-wide phylogenetic analyses showed that the completely sequenced genome of *C. intestinalis* contains one ortholog of NK4 subfamily genes, including *Nkx2.5*, and one for *HAND* (Satou et al., 2003; Wada et al., 2003). This genomic organization was expected for *C. savignyi* as well. A cDNA clone for *Cs-Nkx* was 2263 bp in length and encoded a polypeptide of 595 amino acid residues (DDBJ/EMBL/GENBANK accession number AB012666). Whole-mount in-situ hybridization revealed that *Cs-Nkx* begins to be expressed at the neurula stage and is expressed in TVCs and, to some extent, in epidermis and endoderm at the tailbud stage (Fig. 4A). This embryonic expression pattern of *Cs-Nkx* is almost the same as that of its *C. intestinalis* counterpart (*Ci-Nkx*) (Davidson and Levine, 2003).

The cDNA clone isolated for *Cs-HAND* was 1325 bp in length and encoded a polypeptide of 346 amino acid residues (DDBJ/EMBL/GENBANK accession number AB125641). Whole-mount in-situ hybridization showed that *Cs-HAND* is not zygotically activated until the neurula stage but is expressed exclusively in TVCs at the tailbud stage (Fig. 4B).

Table 1. Effects of morpholinos against *Cs-Mesp* on the expression of genes in TVCs

Gene examined	Number of embryos with the gene expression/number of embryos examined: Cs-Mesp-MO1	Cs-Mesp-MO2
Cs-Nkx	1/16	0/5
Cs-HAND	0/21	0/6
NoTrlc	0/25	0/5
00152	0/8	0/5
ATP2A1/2/3	0/12	0/5

In addition, the *C. intestinalis* genome contains one more *HAND*-like gene. Its *C. savignyi* ortholog was analyzed in detail and was named *NoTrlc* ('no trunk lateral cells') after its function. As was shown previously (Imai et al., 2003), this gene is also expressed in TVCs, as well as in the brain and in the tip of the tail at the tailbud stage (Fig. 4C).

Furthermore, two cDNA clones for *C. savignyi* genes, which are known to be expressed in the TVCs of *C. intestinalis* embryos (Satou et al., 2001c), were also isolated as molecular markers for TVCs. One gene (known by an ID number, 00152; DDBJ/EMBL/GENBANK accession number AB125643) encodes a protein with no significant similarity to any known proteins. This gene is expressed in TVCs, endoderm, notochord and tail muscle (Fig. 4D). The other gene (ID 02049; DDBJ/EMBL/GENBANK accession number AB125642), which encodes a Ca^{2+} transporting ATPase (ATP2A1/2/3), is expressed in TVCs and tail muscle (Fig. 4E).

Cs-Mesp is essential for specification of TVCs

Cs-Mesp is the first gene among the four transcription factor genes *Cs-Mesp*, *NoTrlc*, *Cs-Nkx* and *Cs-HAND* to be expressed in the TVC lineage. Therefore, the function of *Cs-Mesp* was examined. To suppress the function of *Cs-Mesp*, specific morpholinos were microinjected into fertilized eggs. Embryos injected with one such morpholino, Cs-Mesp-MO1, developed and hatched normally, similarly to control uninjected embryos. The morpholino-injected larvae were also similar to controls in their general morphology (Fig. 5A) and swimming behavior. We confirmed normal differentiation of the endoderm (Fig. 5B), epidermis (Fig. 5C), nervous system (Fig. 5D), mesenchyme (Fig. 5E), notochord (Fig. 5F) and muscle (Fig. 5G) in the morpholino-injected embryos by histochemical staining and whole-mount in-situ hybridization of the marker genes.

However, these *Cs-Mesp* knockdown embryos displayed the suppression of expression in TVCs of the *Cs-Nkx* (Fig. 4A′), *Cs-HAND* (Fig. 4B′) and *NoTrlc* (Fig. 4C′), despite their normal expression in tissues other than TVCs. As shown in Table 1, all the experimental embryos except for one examined with *Cs-Nkx* showed suppression of the marker gene expression. In addition, it was noticed that the morpholino affected the marker gene expression only in TVCs. For example, the *NoTrlc* gene is expressed in TVCs as well as in the brain and in the tip of the tail (Fig. 4C). Knockdown of *Cs-Mesp* with the morpholino resulted in the failure of the gene expression only in TVCs but not in the brain and in the tip of the tail (Fig. 4C′). That is, knockdown of *Cs-Mesp* resulted in failure of the differentiation of TVCs, implying that this gene

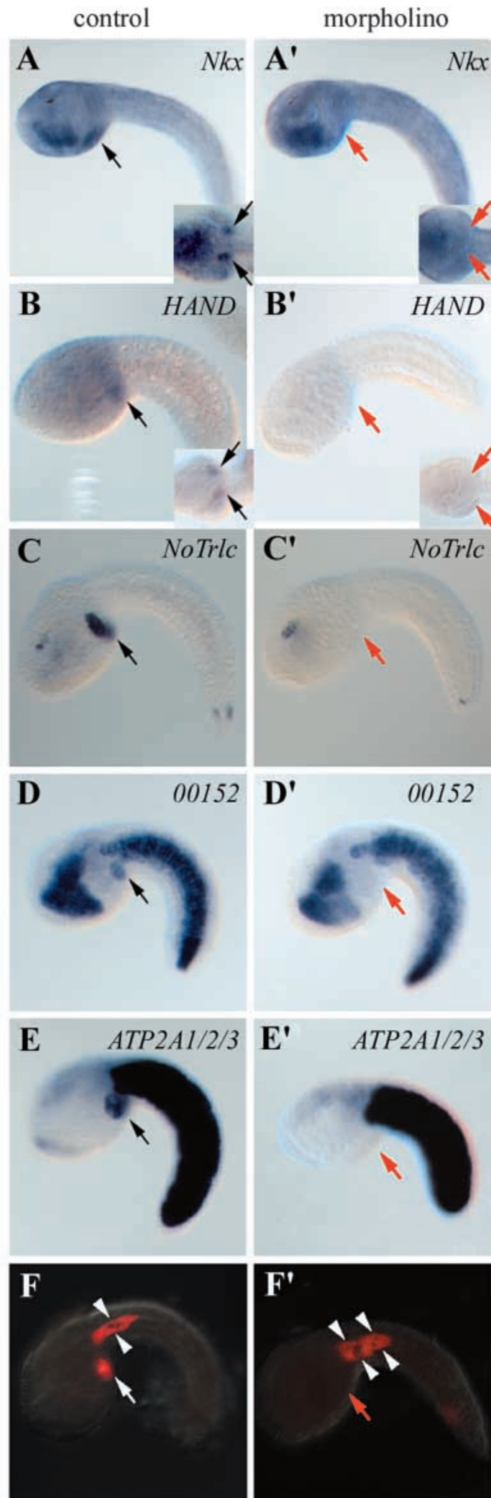

Fig. 4. *Cs-Mesp* is essential for specification of TVCs. (A-E) Expression of genes in TVCs of control and (A′-E′) Cs-Mesp-MO1-injected embryos. (A,A′) *Cs-Nkx*; (B,B′) *Cs-HAND*; (C,C′) *NoTrlc*; (D,D′) a gene orthologous to the *C. intestinalis* ID00152 gene; and (E,E′) ATP2A1/2/3. Inserts in (A,A′,B,B′) are ventral views of the trunk region showing *Cs-Nkx* and *Cs-HAND* expression. Arrows in (A-E) indicate TVCs. Red arrows in A′-E′ indicate suppressed expression of downstream genes in TVCs. (F,F′) Tailbud embryos in which the B7.5-lineage cells are labeled with DiI. (F) In a control embryo, TVCs (white arrow) and two anterior muscle cells (arrowheads) are labeled. (F′) In a Cs-Mesp-MO1-injected embryo, labeled cells are not observed in the region in which TVCs should be located (a red arrow). Instead, four labeled cells are observed in the tail (arrowheads).

plays a critical role in the specification of TVCs. The expression in TVCs of *00152* (Fig. 4D′) and *ATP2A1/2/3* genes (Fig. 4E′) were also not observed (Table 1). However, these two genes are expressed in TVCs and muscle cells, and B7.5 cells give rise to TVCs and muscle cells. Therefore, it is possible that TVCs lose their distinctive position to change their fate into muscle and that they still express *00152* and *ATP2A1/2/3*. We examined this possibility in the following experiments.

First, we examined whether B7.5-derived muscle cells are differentiated in *Cs-Mesp* knockdown embryos. 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. As in the control embryos (Fig. 5H), muscle actin gene is expressed in B7.5 blastomeres of the experimental embryos (Fig. 5I). Thus, knockdown of *Cs-Mesp* did not disturb the differentiation of B7.5-derived muscle cells.

Next, we examined whether TVCs retain their distinctive position despite losing expression of these TVC genes in the *Mesp*-knockdown embryo. The B7.5 cell of the morpholino-injected embryos was labeled with DiI at the 110-cell stage and the resultant embryos were observed under a fluorescent microscope. The B7.5 cell of the control embryo gave rise to TVCs and two muscle cells located in the anterior part of the tail (Fig. 1F, Fig. 4F). However, in the experimental embryo any B7.5-derived cells were not observed in the region where TVCs should be located (Fig. 4F′). Instead, four DiI-labeled cells were observed in the anterior part of the tail when *Cs-Mesp* function is suppressed. This suggests that B7.5 descendants with TVC fate would change their fate to that of embryonic muscle cells.

To examine the specificity of Cs-Mesp-MO1, another morpholino, designated as Cs-Mesp-MO2, was designed to bind a different region of the *Mesp* mRNA than does Cs-Mesp-MO1. Experiments using Cs-Mesp-MO2 yielded a similar suppression of TVC-specific gene expression (Table 1). These experiments, in which two independent morpholinos for one gene gave the same result, provide strong support for the specificity of these morpholinos (Heasman, 2002).

In addition, we examined whether overexpression of *Cs-Mesp* mRNA leads to ectopic differentiation of TVCs. The microinjection of 60 pg of *Cs-Mesp* mRNA affected normal embryogenesis but did not induce any ectopic expression of *HAND* gene (n=7; data not shown), suggesting that *Cs-Mesp* alone is not sufficient for turning on the TVC program in cells other than the TVC lineage.

Cs-Mesp is essential for juvenile heart development

The failure of differentiation of the TVCs caused by *Cs-Mesp* knockdown may lead to failure of differentiation of the juvenile heart after metamorphosis. To test this possibility, we further

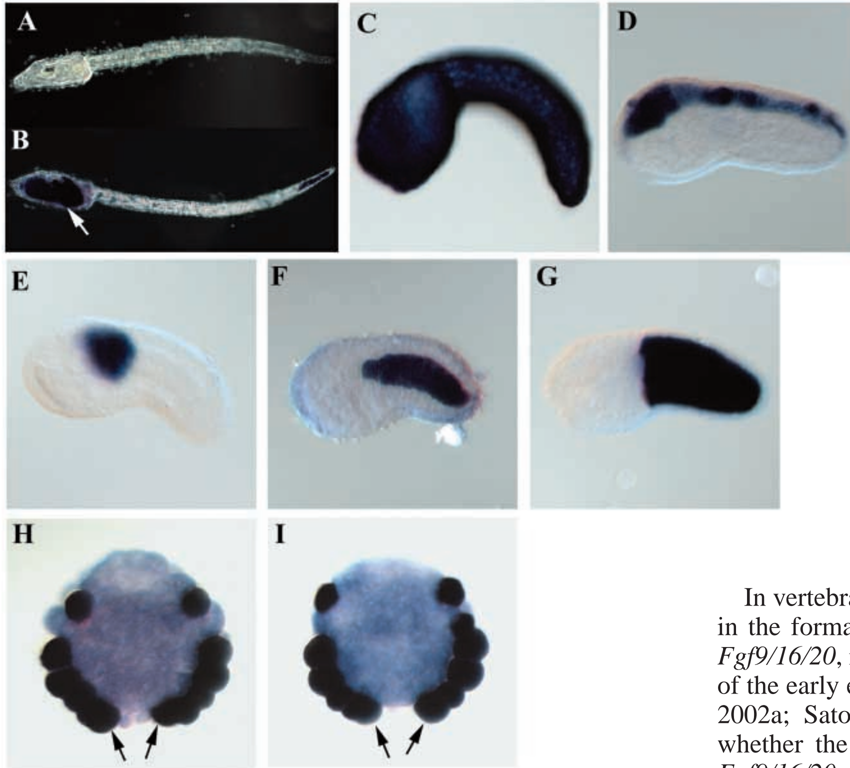

Fig. 5. Differentiation of tissues other than TVC in *Cs-Mesp* knockdown embryos. (A) Suppression of *Cs-Mesp* with specific morpholino did not affect larval morphology or the expression of (B) endoderm-specific histochemical activity of alkaline phosphatase (arrow), (C) epidermis-specific gene *Cs-Epi1*, (D) pan-neural marker gene *Cs-ETR*, (E) mesenchyme-specific gene *Cs-Mech1*, (F) notochord-specific gene *Cs-fibrn*, and (G) a muscle-specific actin gene *Cs-MA1*. (H,I) Expression of *Cs-MA1* in (H) control and (I) *Cs-Mesp* knockdown embryos, which are arrested at the 110-cell stage. As indicated by arrows, a pair of B7.5 blastomeres expresses *Cs-MA1* both in control and *Cs-Mesp* knockdown embryos.

examined *Cs-Mesp* knockdown embryos, which hatch normally. The resulting larvae could swim and then metamorphosed into juveniles, but all of the experimental organisms died within 2 weeks after metamorphosis. As shown in Fig. 6A, the heart in control juveniles can be easily recognized as a tube-like structure due to the transparency of the juvenile body (see Movie 1 at http://dev.biologists.org/supplemental/). By contrast, all of the experimental juveniles lacked a heart completely. That is, in the experimental juveniles, no tube-like structure could be recognized (Fig. 6A′; see Movie 2 http://dev.biologists.org/supplemental/) (*n*=10 with Cs-Mesp-MO1 and *n*=9 with Cs-Mesp-MO2).

Cs-Mesp is under the control of maternal β-catenin and Cs-macho1

The results described above demonstrated that the expression of *Cs-Mesp* is the first trigger for the specification of embryonic TVCs and subsequent differentiation of the juvenile heart. We then examined whether *Cs-Mesp* is under the control of β-catenin and Cs-macho1, because these are two maternal factors known to be important for determination of the posterior and vegetal region of the ascidian embryo (Imai et al., 2000; Nishida and Sawada, 2001; Satou et al., 2002a).

Forty-five percent of *β-catenin*-morpholino-injected mid-gastrula embryos lacked the expression of *Cs-Mesp*, while the remaining embryos did express *Cs-Mesp* (Fig. 7B) (*n*=40). *Cs-Mesp* mRNA was not detected in *Cs-macho1*-morpholino injected embryos (Fig. 7C) (0%, *n*=24). These results were further confirmed by measuring the relative amounts of *Cs-Mesp* mRNA using real-time PCR. The quantities of *Cs-Mesp* mRNA in the *β-catenin*- and *Cs-macho1*-morpholino-injected embryos were 37% and 2% of the quantity in control embryos, respectively (Fig. 7D). Therefore, both *β-catenin* and *Cs-macho1* are essential for *Cs-Mesp* transcription.

In vertebrates, FGF, BMP and Wnt11 signaling are involved in the formation of heart field. One of the *Ciona* Fgf genes, *Fgf9/16/20*, is known to be expressed in mesoendodermal cells of the early embryo under the control of β-catenin (Imai et al., 2002a; Satou et al., 2002d). Therefore, we also examined whether the regulation of *Cs-Mesp* by β-catenin is through *Fgf9/16/20*. Microinjection of the morpholino against *Fgf9/16/20* did not inhibit the expression of *Cs-Mesp* at the mid-gastrula stage (*n*=20; data not shown), indicating that this Fgf is not involved in the regulation of *Cs-Mesp* expression.

Discussion

A possible conserved mechanism of heart specification among chordates

Here we demonstrated that the ascidian *Mesp* gene is specifically and transiently expressed in B7.5 blastomeres and their descendants, and that the gene specifies embryonic TVCs or progenitor cells of the juvenile heart. Two lines of evidence support this notion: first, we observed that *Mesp* governs the expression of downstream transcription factor genes, including *Nkx*, *HAND* and *NoTrlc*; and second, *Cs-Mesp*-knockdown embryos did not develop heart primodia or TVCs upon maturing to larvae, and juvenile animals had no heart. B7.5 blastomeres also give rise to a pair of anterior muscle cells of the larva. But *Cs-Mesp* is not required for differentiation of these muscle cells,

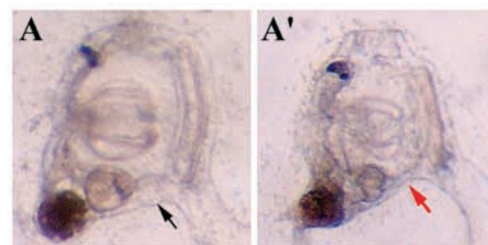

Fig. 6. *Cs-Mesp* is essential for juvenile heart development. (A) A juvenile with two gill-slits developed from a control egg and (A′) that from a Cs-Mesp-MO1-injected egg. Black and red arrows indicate the heart and the region in which the heart should be found.

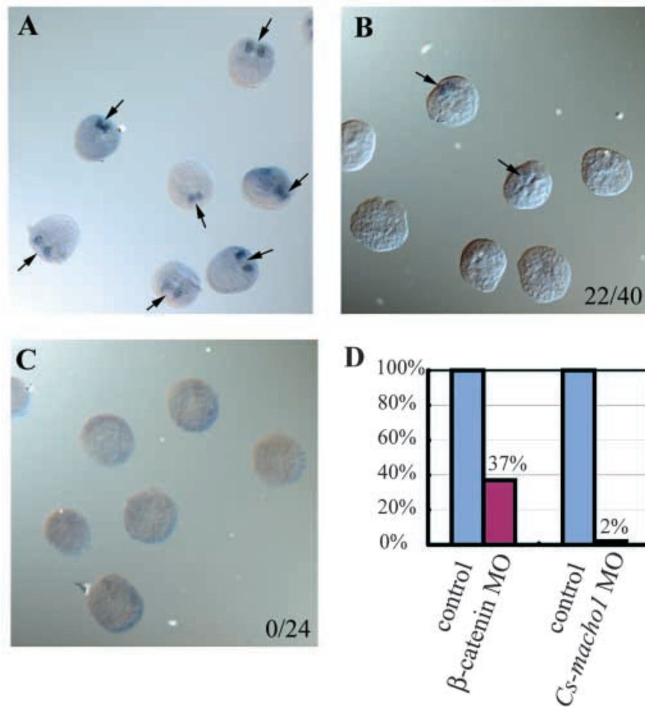

Fig. 7. Expression of *Cs-Mesp* is under the control of β-catenin and *Cs-macho1*. (A-C) Expression of *Cs-Mesp* at the late gastrula stage. (A) Control embryos and embryos injected with (B) *β-catenin* and (C) *Cs-macho1* morpholino. (D) Quantification of the amount of *Cs-Mesp* mRNA in control embryos and experimental embryos developed from eggs injected with *β-catenin* and *Cs-macho1* morpholino. Quantification was performed by real-time RT-PCR. The relative amounts of mRNA compared with those in control embryos are shown.

because the B7.5-derived muscle cells are differentiated in the *Cs-Mesp*-suppressed embryo. Vertebrates have at least three paralogs of *Mesp* (*pMesogenin1*, *Mesp1* and *Mesp2*), the functions of which have been analyzed mainly in mouse embryos. *pMesogenin* is expressed in the caudal domain of the presomitic mesoderm (Yoon et al., 2000) and is required for maturation and segmentation of paraxial mesoderm (Yoon and Wold, 2000). *Mesp1* and *Mesp2* are known to be expressed in the early ingressing part of the mesoderm that is fated to become extra-embryonic and cranio-cardiac mesoderm (Saga et al., 2000). An *Mesp1* knockout mouse was reported to show abnormalities in heart development, and an *Mesp1*/*Mesp2* double-knockout mouse died around embryonic day 9.5 and lacked any cranio-cardiac mesoderm (Saga et al., 1999; Kitajima et al., 2000). Therefore, the mouse *Mesp* genes are thought to be important for proper differentiation of the mesoderm, although the precise reason why heart progenitors cannot be formed properly in the double-knockout mice is not fully understood. Functional redundancies of *Mesp* genes and the complexity of mammalian embryonic development have rendered further analysis of these genes difficult. The results of the present study using the ascidian embryo strongly indicate that the mechanism of heart development, beginning with the expression of *Mesp* through the expression of *Nkx* and *HAND*, is conserved among chordates. If so, vertebrate *Mesp* genes should also play a key role in the specification of heart progenitors.

How are key genes such as *Nkx* and *HAND* regulated by *Mesp*? *Cs-Mesp* expression terminates before the initiation of the expression of *Cs-Nkx*, *Cs-HAND* and *NoTrlc*, although it has not yet been determined how long Cs-Mesp protein is retained. Therefore, Cs-Mesp may indirectly regulate the expression of *Cs-Nkx*, *Cs-HAND* and *NoTrlc*. In the *Cs-Mesp*-suppressed embryos, cells fated to be TVCs cannot migrate precisely to the ventral trunk region but migrate to the tail region together with their sister cells fated to be anterior muscle cells. In vertebrates, cells expressing *Mesp* genes receive positive (BMP and FGF) and negative (Wnt) signals, and these signals activate transcription of the key genes in the heart precursors (Andree et al., 1998; Reifers et al., 2000; Pandur et al., 2002). Similarly, *Ciona* TVCs may require such signals to express *Cs-Nkx*, *Cs-HAND* and *NoTrlc* genes. In this case, the failure of migration of the TVCs in the *Cs-Mesp*-suppressed embryos may disrupt cell–cell interaction between the TVCs and cells expressing such signals, although it should be determined whether or not these signals are also required for ascidian heart development. Two lines of experiments will reveal the links between *Mesp* and the other key regulatory genes in the ascidian embryo. One such line of experiments is analyzing the cis-regulatory system of *Nkx* and *HAND* genes. *Ciona* embryos provide an ideal system for this kind of assay (Corbo et al., 2001; Satoh et al., 2003) and, actually, the cis-elements of *NoTrlc* have been analyzed in *C. intestinalis* (Davidson and Levine, 2003). The other line of experiments is identification of *Mesp*-downstream genes. This can be easily realized on a genome-wide scale, using a microarray covering almost all *Ciona* genes (Azumi et al., 2003). This information will also illuminate the core system of vertebrate heart development, which is expected to be conserved among chordates.

Genes specifying the ascidian endomesoderm

The organization of the ascidian embryonic endomesoderm is simple compared with that of other chordates. In the trunk, endoderm is developed ventrally, and TVCs and mesenchyme, including TLCs, differentiate in the lateral region. In the tail, the axial notochord is flanked laterally by muscle cells. In previous studies, we demonstrated that maternal β-catenin is essential for specification of this endomesoderm, except for muscle (Imai et al., 2000). Maternal β-catenin, when it translocates from the cytoplasm to the nuclei of vegetal blastomeres, activates key genes directly or indirectly, one or a few of which is essential and sufficient for differentiation of each tissue. These are *Lhx3* for endodermal cells (Satou et al., 2001a), *Twist-like1* for mesenchymal cells (Imai et al., 2003) and *Brachyury* for notochord cells (Yasuo and Satoh, 1998; Corbo et al., 1997). In the present study, we demonstrated that *Cs-Mesp* is such a key gene for specification of TVCs. While muscle cells are specified and determined by the maternal *macho1* gene, each of the endomesodermal tissues other than muscle is specified through one zygotically expressed key gene. Therefore, identification of upstream and downstream factors of these key genes will reveal the complete gene circuits behind the ascidian larval endomesoderm specification (Imai et al., submitted).

For understanding the entire genetic pathway from maternal information to the final heart differentiation, it is also important to analyze the upstream mechanism regulating the initiation of

Cs-Mesp expression. In the present study, we showed that both Cs-macho1 and β-catenin are required for the expression of *Cs-Mesp*. Cs-macho1 and β-catenin are thought to determine the posteriormost axis and vegetal axis, respectively. The TVC precursors are located in the posteriormost and vegetal regions of the embryo (Fig. 1). As expected, transcription of *Cs-Mesp* is regulated by Cs-macho1 and β-catenin, although the *β-catenin* knockdown is less effective than the *Cs-macho1* knockdown. Because the morpholino against *β-catenin* has been repeatedly used in previous studies to confirm the suppression of its function effectively (Satou et al., 2001b; Imai et al., 2002a; Imai et al., 2002b; Imai, 2003), the low effectiveness may indicate that β-catenin is not always essential for the initiation of expression of *Cs-Mesp* but is required for reinforcement or maintenance of the *Cs-Mesp* expression. The parental blastomere of B7.5, i.e. B6.3, has developmental fates of germ cells, TVCs and muscle cells, and the cell division at the 64-cell stage restricts one pair of its daughter cells (B7.5) to the TVC and muscle fates (B7.5 is born at the 64-cell stage and is retained at the 110-cell stage). Therefore, the most attractive hypothesis is that release from germline repression initiates *Cs-Mesp* expression at the 110-cell stage, as previously suggested by Davidson and Levine (2003). In this case, Cs-macho1 and β-catenin may directly activate *Cs-Mesp*.

The developmental fate of TVCs

It has been shown in another ascidian, *H. roretzi*, that B7.5-derived cells give rise to latitudinal mantle muscle and atrial muscle in addition to the heart in juveniles (Hirano and Nishida, 1997). The adult body plan of *Ciona* is somewhat different from that of *Halocynthia* (Satoh, 1994). They are evolutionarily distant, because *Halocynthia* is an Enterogona ascidian and *Ciona* is a Pleurogona ascidian, these two being major 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*. For this purpose, we labeled a TVC precursor cell with DiI and confirmed that TVCs give rise to the juvenile heart. However, we could not observe DiI-labeling of latitudinal mantle muscle and atrial muscle in the experimental juveniles. This is probably due to species differences, although there is a possibility that it is due to the labeling method because Hirano and Nishida (1997) labeled the blastomere with HRP. However, even if DiI-labeling was not as sensitive as HRP, and *Ciona* TVCs also gave rise to these adult muscles, external stimuli were found to cause our morpholino-injected animals to contract normally, indicating that the mantle and atrial muscles had differentiated normally even in the *Cs-Mesp* knockdown animals. Therefore, it is highly likely that *Cs-Mesp* plays a specific role in heart development.

Conclusion

As shown in the present study, ascidians have advantages for studying heart development during chordate embryogenesis. The development of the heart in ascidians appears to share a common mechanism with that in all chordates. The specification of heart progenitors occurs in the 110-cell embryo, which enables us to analyze the process at the single-cell level. The ascidian genome contains fewer genetic redundancies, represented by the presence of only one *Mesp* gene. Heart morphology can easily be observed because the ascidian juvenile is transparent. Ascidian development is rapid, and thus heartbeats can be observed within 3 days after fertilization. In addition, the juvenile can survive for about 2 weeks without the heart. Therefore, the ascidian provides a powerful system for studying chordate heart development and this system may enable us to study the functions of genes that are difficult to analyze in vertebrates.

We thank Kazuko Hirayama and all the staff members of the Maizuru Fisheries Research Station of Kyoto University and the International Coast Research Centre of the Ocean Research Institute, University of Tokyo, for their help in collection of ascidians. This research was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, Culture, and Technology of Japan and the Japan Society for the Promotion of Science (JSPS) to Y.S. (14704070 and 13044001). K.S.I. was supported by a Postdoctoral Fellowship from JSPS with Research Grant 15004751. This research was also supported in part by the CREST project of JST to N.S. and Grants for the Biodiversity Research of the 21st Century COE (A14) to Y.S. and N.S.

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