Development 128, 3559-3570 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV3505

Early embryonic expression of a LIM-homeobox gene *Cs-lhx3* is downstream of β-catenin and responsible for the endoderm differentiation in *Ciona savignyi* embryos

Yutaka Satou*,‡, Kaoru S. Imai* and Nori Satoh

Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
*These authors contributed equally to this work
‡Author for correspondence (e-mail: yutaka@ascidian.zool.kyoto-u.ac.jp)

Accepted 20 June 2001

SUMMARY

In early *Ciona* embryos, nuclear accumulation of β-catenin is most probably the first step of endodermal cell specification. If β-catenin is mis- and/or overexpressed, presumptive notochord cells and epidermal cells change their fates into endodermal cells, whereas if β-catenin nuclear localization is downregulated by the overexpression of cadherin, the endoderm differentiation is suppressed, accompanied with the differentiation of extra epidermal cells (Imai, K., Takada, N., Satoh, N. and Satou, Y. (2000) *Development* 127, 3009-3020). Subtractive hybridization screens of mRNAs between β-catenin overexpressed embryos and cadherin overexpressed embryos were conducted to identify potential β-catenin target genes that are responsible for endoderm differentiation in *Ciona savignyi* embryos. We found that a LIM-homeobox gene (*Cs-lhx3*), an *otx* homolog (*Cs-otx*) and an *NK-2* class gene (*Cs-ttf1*) were among β-catenin downstream genes. In situ hybridization signals for early zygotic expression of *Cs-lhx3* were evident only in the presumptive endodermal cells as early as the 32-cell stage, those of *Cs-otx* in the mesoendodermal cells at the 32-cell stage and those of *Cs-ttf1* in the endodermal cells at the 64-cell stage. Later, *Cs-lhx3* was expressed again in a set of neuronal cells in the tailbud embryo, while *Cs-otx* was expressed in the anterior nervous system of the embryo. Expression of all three genes was upregulated in β-catenin overexpressed embryos and downregulated in cadherin overexpressed embryos. Injection of morpholino oligonucleotides against *Cs-otx* did not affect the embryonic endoderm differentiation, although the formation of the central nervous system was suppressed. Injection of *Cs-ttf1* morpholino oligonucleotides also failed to suppress the endoderm differentiation, although injection of its synthetic mRNAs resulted in ectopic development of endoderm differentiation marker alkaline phosphatase. By contrast, injection of *Cs-lhx3* morpholino oligo suppressed the endodermal cell differentiation and this suppression was rescued by injection of *Cs-lhx3* mRNA into eggs. In addition, although injection of *delE-Ci-cadherin* mRNA into eggs resulted in the suppression of alkaline phosphatase development, injection of *delE-Ci-cadherin* mRNA with *Cs-lhx3* mRNA rescued the alkaline phosphatase development. These results strongly suggest that a LIM-homeobox gene *Cs-lhx3* is one of the β-catenin downstream genes and that its early expression in embryonic endodermal cells is responsible for their differentiation.

Key words: Ascidians, β-Catenin, Target genes, Subtraction screening, *Cs-lhx3, Cs-otx, Cs-ttf1,* Early expression, Endoderm differentiation, *Ciona savignyi*

INTRODUCTION

Recently convincing evidence has been accumulated to show an involvement of β-catenin in axis determination and embryonic cell specification in a wide range of organisms from cnidarians to vertebrates (reviewed by Cadigan and Nusse, 1997; Moon and Kimelman, 1998; Sokol, 1999). In *Xenopus* and zebrafish, β-catenin is concentrated in nuclei of dorsal cells of blastula, and is involved in an establishment of the dorsoventral axis of the embryo (Heasman et al., 1994; Funayama et al., 1995; Guger and Gumbiner, 1995; Kelly et al., 1995; Kelly et al., 2000; Schneider et al., 1996; Larabell et al., 1997; Miller et al., 1999). In sea urchins, the nuclear localization of β-catenin is seen in vegetal blastomeres, which is essential for vegetal fate determination of embryonic cells (Wikramanayake et al., 1998; Logan et al., 1999). β-catenin is also involved in the specification of the endoderm in early *C. elegans* embryos (Thorpe et al., 1997; Rocheleau et al., 1997). In addition, a recent study demonstrated that β-catenin also works in the axis determination of hydra (Hobmayer et al., 2000). To exert its function, β-catenin enters the nucleus and activates downstream genes together with TCF/LEF1, which is a transcriptional factor with an HMG box (reviewed by Willert and Nusse, 1998). In vertebrates, several genes have been

identified as factors downstream of β-catenin, including *siamois* (Brannon et al., 1997; Fan and Sokol, 1997), *twins* (Laurent et al., 1997) and *fibronectin* (Gradl et al., 1999) in *Xenopus* embryos, *boz/dharma* in zebrafish embryos (Fekany et al., 1999), and *Nodal-related3* (McKendry et al., 1997) and *Brachyury* (Yamaguchi et al., 1999; Arnold et al., 2000) in mouse embryos.

The ventral trunk region of an ascidian tailbud embryo is occupied by the endoderm that extends ventrally as the endodermal strand along the notochord of the tail region (reviewed by Satoh, 1994). This tissue is composed of about 500 cells, and its embryonic lineage is well documented (Conklin, 1905; Nishida, 1987). The endodermal cells are derived from the vegetal A4.1 and B4.1 blastomeres of the 8-cell stage embryo, and A5.1, A5.2, B5.1 and B5.2 of the 16-cell stage (see Fig. 1). As early as the 32-cell stage, two pairs of vegetal blastomeres, A6.1 and B6.1, become restricted to generate endoderm only, and at the 64-cell stage, five pairs of vegetal blastomeres (A7.1, A7.2, A7.5, B7.1 and B7.2) become endoderm restricted. These primordial endodermal cells proliferate to form about 500 endodermal cells.

Reflecting such an early fate restriction, presumptive endodermal blastomeres show a high potential for autonomous differentiation when they are isolated from early embryos (e.g. Whittaker, 1990; Nishida, 1992). This autonomy is dependent on maternal factors or determinants that are pre-localized in the endoplasm of eggs and early embryos (Nishida, 1993). In a previous study, we have shown that during cleavages of the ascidians *Ciona intestinalis* and *C. savignyi*, β-catenin accumulates in the nuclei of vegetal blastomeres by the 32-cell stage, that mis- and/or overexpression of β-catenin induces the development of an endoderm-specific alkaline phosphatase (AP) in presumptive notochord cells and epidermal cells without affecting differentiation of primary lineage muscle cells, and that down regulation of nuclear β-catenin induced by the overexpression of cadherin results in the suppression of endodermal cell differentiation (this suppression was compensated for by the differentiation of extra epidermal cells; Imai et al., 2000). The accumulation of β-catenin in the nuclei of endoderm progenitor cells is therefore most probably the first step in the process of ascidian embryonic endoderm specification.

To understand the molecular mechanisms of endoderm differentiation in ascidian embryos, it is important to identify the genes that act as direct targets and/or downstream of β-catenin. Because in ascidians β-catenin plays a pivotal role in the endodermal cell specification, it may regulate the expression of genes other than *siamois*, *twins* and *boz/dharma,* these genes being involved in dorsoventral axis determination. In addition, because the ascidian endoderm functions as an inductive source for the differentiation of notochord cells (Nakatani and Nishida, 1994) and mesenchymal cells (Kim et al., 2000), the identification of β-catenin downstream genes will also lead to an elucidation of these induction mechanisms. We took advantage of β-catenin overexpressed embryos and cadherin overexpressed embryos; in the former, β-catenin targets may be upregulated, and in the latter, β-catenin targets may be downregulated. We found that a LIM-homeobox gene *Cs-lhx3*, an *otx* homolog *Cs-otx* and an *NK-2* class homeobox gene *Cs-ttf1* are downstream genes of β-catenin. Of these genes, an inhibition of possible early embryonic function of *Cs-lhx3* resulted in the suppression of endoderm differentiation.

MATERIALS AND METHODS

Ascidian eggs and embryos

Adults of *Ciona savignyi* were collected near the Otsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo (Iwate, Japan) and maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, embryos were reared at about 18°C in Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate.

Isolation and characterization of candidate cDNA clones

β-catenin overexpressed embryos and cadherin overexpressed embryos were prepared by microinjection of synthetic mRNAs as described previously (Imai et al., 2000). One hundred and twenty former embryos and 129 latter embryos, both at the 110-cell stage, were lysed in 400 μl of GTC solution (4 M guanidinium thiocyanate, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2% sarkosyl, 1% β-mercaptoethanol). Total RNA was isolated from the lysates by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). cDNA was synthesized from 0.3 μg of total RNA using a SMART PCR cDNA Synthesis kit (Clontech).

The subtraction procedure of Wang and Brown (Wang and Brown, 1991) was adopted with several modifications. Each of the cDNA pools was digested completely with *Rsa*I, ligated with double-stranded linkers prepared from dCTCTTGCTTGAATTAGGACTA and dTAGTCCGAATTCAAGCAAGAGCACA, and electrophoresed through a 1.5% low melting temperature agarose gel to remove the unligated linkers. 0.01 μg of cDNA fragments recovered from the gel were amplified by PCR with dCTCTTGCTTGAATTAGGACTA primer. The optimal number of PCR cycles was determined by electrophoresis of PCR products from every five additional cycles so that the double-stranded cDNA would remain in the exponential phase of amplification. The PCR products were the starting material for subtractive hybridization. The PCR products were divided into the 'tracer' fraction and the 'driver' fraction. *Cs-β-catenin* and *Ci-cadherin* in pBluescript RN3 vector (Lemaire et al., 1995) were amplified by PCR and digested with *Rsa*I. These PCR products were also used as 'driver' in order to exclude the specific cDNAs that were derived from injected mRNAs. 20 μg of the 'driver' fraction digested with *Eco*RI and 5 μg of the 'injected cDNA driver' were photobiotinylated using a Photoprobe biotin (Vector). This was mixed with 1 μg of the 'tracer', denatured and hybridized at 68°C for 20 hours (long hybridization). Then the biotin-containing cDNAs were removed by four cycles of streptavidin (Vector) and phenol-chloroform extraction. The remaining cDNAs were hybridized again with 10 μg of the biotinylated 'driver' and 2.5 μg of the biotinylated 'injected cDNA driver' for 2 hours (short hybridization). The biotinylated cDNAs were again removed, and the first subtraction cycle was completed. This cycle was repeated three times. The product of the first subtraction cycle was again PCR amplified with the optimal number of PCR cycles determined as previously described and subtracted as the same way as the first cycle. The long hybridization in the second and third cycle used the second and third PCR product, respectively, and the short hybridization in the second and third cycle used the first PCR product. The cDNA fragments amplified by PCR after three subtraction cycles were inserted into pGEM-T vector (Promega).

Isolation of cDNA clones containing the entire coding region and determination of the nucleotide sequences

cDNA clones containing the entire coding region were isolated from

Table 1. Morpholino oligonucleotides used in the present study

Morpholino oligos	Sequence
Cs-otx	5′-AGTGTGGAGATTTCAAGTATGA<u>CAT</u>-3′
Cs-ttf1	5′-CGAATGCTTTGGACTTACTGA<u>CAT</u>C-3′
Cs-lhx3	5′-ATTGGTAGAATCGCAAAGCGGGCT-3′
lacZ	5′-TACGCTTCTTCTTTGGAGCAGT<u>CAT</u>-3′

The underlined nucleotides correspond to the initiation codons of the genes. The morpholino oligo against *Cs-lhx3* corresponds to the 5′-UTR region of the gene.

a gastrula stage cDNA library by probes derived from the cDNA fragments. Nucleotide sequences were determined for both strands using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit and ABI PRISM 377 DNA sequencer (Perkin Elmer).

Whole-mount in situ hybridization

To determine mRNA distributions in eggs and embryos, RNA probes were prepared using a DIG RNA-labeling kit (Boehringer-Mannheim, Heidelberg, Germany). Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense probes as described previously (Satou and Satoh, 1997). Control embryos that were hybridized with a sense probe did not show signals above background.

In order to examine the endodermal cell differentiation, probes were prepared from cDNA for an endoderm-specific thyroid hormone receptor gene of *C. savignyi*.

Morpholino oligos and synthetic capped mRNAs

In the present study, we used 24- or 25-mer morpholino oligonucleotides (Gene Tools LLC) for *Cs-lhx3*, *Cs-otx*, *Cs-ttf1* and *lacZ*. Nucleotide sequences of the morpholinos are listed in Table 1. Synthetic capped mRNA for *Cs-lhx3* was synthesized from *Cs-lhx3* cDNA cloned into pBluescript RN3 vector (Lemaire et al., 1995) using a Megascript T3 kit (Ambion, Austin, TX, USA). To obtain a capped mRNA, the concentration of GTP was lowered to 1.5 mM and the cap analog 7mGpppG was added at a final concentration of 6 mM. Capped mRNA for *lacZ*, *delE-Ci-cadherin* or *delN-β-catenin* was synthesized in the same way as *Cs-lhx3*.

After insemination, fertilized eggs, usually with intact chorion, were microinjected with 15 fmole of morpholinos and/or synthetic capped mRNAs. Each injection contained 30 pl of solution, and microinjection was carried out using a micromanipulator (Narishige Scientific Instruments Laboratory, Tokyo, Japan), as described by Imai et al. (Imai et al., 2000). Injected eggs were reared at about 18°C in Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate.

Histochemical staining for alkaline phosphatase (AP)

Differentiation of endodermal cells was monitored by histochemical detection of AP activity (Whittaker and Meedel, 1989). Embryos were fixed with 5% formaldehyde in seawater for 10 minutes at room temperature. Embryos were washed in AP staining buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) three times. For signal detection, specimens were incubated with NBT/BCIP/AP staining buffer (4.5 μl NBT/ml, 3.5 μl BCIP/ml).

RESULTS

Isolation of cDNA clones for β-catenin downstream genes

To identify genes functioning downstream of β-catenin in *Ciona* embryos, we made a subtractive cDNA library that contains mRNAs, the genes of which are upregulated in β-catenin overexpressed embryos relative to those downregulated in cadherin overexpressed embryos. A winged helix/forkhead gene *Cs-HNF3* is a β-catenin downstream gene (Y. S. and N. S., unpublished) and *Cs-Epi1* is an epidermis-specific gene of *C. savignyi* (Chiba et al., 1998). Enrichment of *Cs-HNF3* in β-catenin overexpressed library and that of *Cs-Epi1* in cadherin overexpressed library were confirmed by Southern blot hybridization (data not shown). We selected candidate cDNAs by the following three criteria. The average length of cDNA fragments obtained by PCR was about 300~400 bp. First, both the 5′-most and 3′-most ends of cDNAs were completely sequenced, and nucleotide sequence information was used to check the independency of clones and sequence similarity to reported genes. We are particularly interested in genes that encode for transcription factors.

Second, if cDNA clones represent the β-catenin downstream and endoderm-related genes, they may be expressed in the presumptive endodermal blastomeres. The lineage of endodermal cells in early ascidian embryos has been completely documented (e.g. Nishida, 1987). The endodermal cells are derived from the vegetal A4.1 and B4.1 blastomeres of the bilaterally symmetrical eight-cell stage embryo. The developmental potential to form endoderm is segregated into A5.1, A5.2, B5.1 and B5.2 cells of the 16-cell stage embryo (Fig. 1A). Because the B5.2 cell contributes only two or so endodermal strand cells, it is not discussed in detail in this report. At the 32-cell stage, A6.1, A6.3, and B6.1 cells are the major endoderm lineages (Fig. 1B), and as early as this stage A6.1 and B6.1 cells become restricted to generate endoderm only. At the 64-cell stage, not only A7.1 and A7.2 cells (A6.1 daughter cells) and B7.1 and B7.2 cells (B6.1 daughter cells) but also the A7.5 cell becomes endoderm-restricted (Fig. 1C). These primordial cells divide five or six times to form the endodermal tissue of about 500 cells (Fig. 1E). Therefore, we examined the embryonic expression of candidate cDNAs by whole-mount in situ hybridization, and selected those expressed in the endoderm lineage.

Third, if cDNA clones represent β-catenin downstream genes, their expression should be upregulated in β-catenin overexpressed embryos and downregulated in Ci-cadherin overexpressed embryos. This was also examined by whole-mount in situ hybridization. If cDNAs satisfy these three criteria, their entire nucleotide sequences were determined. The present screening has yielded cDNAs for twelve different genes including a LIM-homeobox gene (*Cs-lhx3*), an *otx* gene (*Cs-otx*), a *ttf1* gene (*Cs-ttf1*), two *forkhead* genes, a type II *cadherin* gene, a *protocadherin* gene, a *netrin* gene, a *frizzled* gene, a *lefty/antivin* homolog, an *EPH* gene and a gene encoding a protein with no known sequence similarity. The first three transcription genes were studied in detail.

Characterization of cDNA clones for *Cs-lhx3*, *Cs-otx* and *Cs-ttf1*

Cs-lhx3

A cDNA clone for *Cs-lhx3* gene was 1988 bp in length, and the predicted protein consists of 472 amino acids (DDBJ/GenBank/EMBL Accession Number, AB057733). The encoded protein contains two LIM domains and a homeodomain (Fig. 2A), and Cs-LHX3 sequence most resembles that of *Halocynthia* HrLIM homeoprotein (Wada et

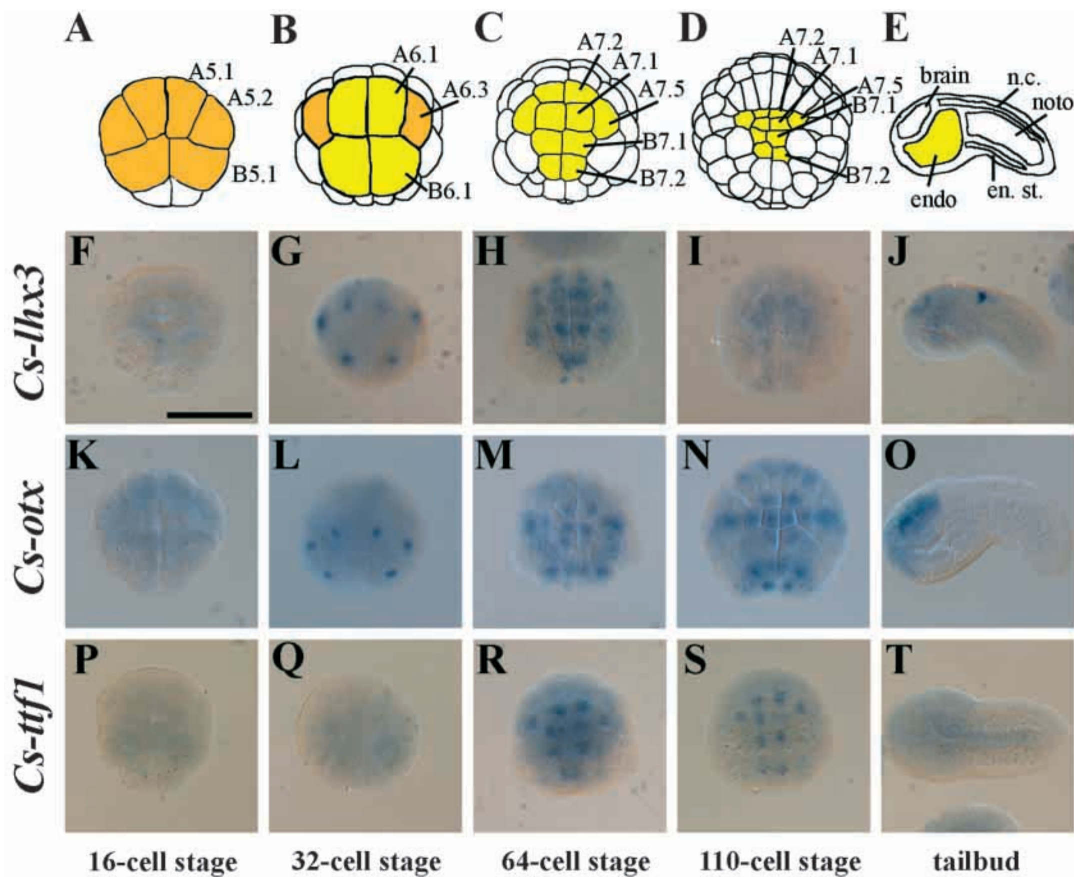

Fig. 1. Expression of genes downstream of β-catenin in *Ciona savignyi* embryos. (A-E) Lineage of endodermal cells at the 16-cell stage (A), 32-cell stage (B), 64-cell stage (C), 110-cell stage (D) and tailbud stage (E). A-D, vegetal view; E, lateral view. Blastomeres shown by orange color are presumptive endodermal cells, while those in yellow are primordial endodermal cells whose fates are restricted to endoderm. (F-J) The expression of *Cs-lhx3*, (K-O) *Cs-otx* and (P-T) *Cs-ttf1* gene, revealed by whole-mount in situ hybridization at the 16-cell stage (F,K,P), the 32-cell stage (G,L,Q), the 64-cell stage (H,M,R), the 110-cell stage (I,N,S) and the tailbud stage (J,O,T). Early embryos up to the 110-cell stage are viewed from the vegetal pole; anterior is upwards, posterior is downwards. The tailbud embryos in J and O are side views; T is a dorsal view. In ascidians, in situ signals for zygotic gene expression are first detected in nuclei of embryonic cells. Scale bar: 100 μm.

al., 1995). The LIM-homeobox genes are categorized, based on the sequence similarities, expression patterns and possible functions, into several groups, including LIN-11, Apterous, Lhx-3, islet, Lhx6/Lhx8 and Lmx groups (reviewed by Hobert and Westphal, 2000; Fig. 2B). The amino acid sequence of the homeodomain of Cs-LHX3 was compared with other LIM-homeodomains and subjected to molecular phylogenetic analysis using neighbor-joining method. The homeodomains of Cs-OTX and Cs-TTF1 were used as the outgroup. As shown in Fig. 2B, Cs-LHX3 was included within a group of LHX-3 members together with HrLIM. Vertebrate genes of this group are associated with the neuronal cell development (reviewed by Hobert and Westphal, 2000).

Embryonic expression of *Cs-lhx3* became evident as early as the 32-cell stage. As shown in Fig. 1G, the expression begins in A6.1, A6.3, and B6.1 cells of the 32-cell stage embryo. At the 64-cell stage, the expression was inherited to their daughter cells, A7.1, A7.2, A7.5, B7.1 and B7.2 with endodermal fate. In addition, A7.3, A7.7, B7.3 and B7.5 blastomeres of the 64-cell stage embryo expressed *Cs-lhx3* (Fig. 1H). This early expression of *Cs-lhx3* in endoderm precursor cells (A7.1, A7.2, A7.5, B7.1 and B7.2) persisted until the 110-cell stage (Fig. 1I) and was then downregulated. No detectable in situ hybridization signals were evident in gastrulae (data not shown). Later, neurulae and tailbud embryos expressed *Cs-lhx3* again, and one pair of cells in the nerve cord and a subset of brain cells expressed *Cs-lhx3*. This spatial expression pattern of *Cs-lhx3* resembles that of *Hrlim* (Wada et al., 1995). As shown in Fig. 3A,C, *Cs-lhx3* expression was upregulated in the β-catenin overexpressed embryos so that *Cs-lhx3* in situ hybridization signals were found in nuclei of almost all blastomeres including animal blastomeres at both the 32-cell (Fig. 3A) and 110-cell stages (Fig. 3C). By contrast, *Cs-lhx3* expression was downregulated in the cadherin overexpressed embryos, and no in situ hybridization signals were detectable in these embryos (Fig. 3B,D).

Cs-otx

A cDNA clone, which was 2393 bp in length and encoded a 422 amino acid polypeptide (DDBJ/GenBank/EMBL Accession Number, AB057732), predicted a *C. savignyi* homologue of *otx/orthodenticle* homeobox genes (Fig. 4A), and therefore this gene was named *Cs-otx*. The *otx* gene of ascidians was already identified in *H. roretzi* (*Hroth*; Wada et al., 1996), *Herdmania curvata* (Hinman and Degnan, 2000) and *C. intestinalis* (*Ci-otx;* Hudson and Lemaire, 2001). The

A

```
  1   MTSRAITSRV IASRYHPYKV SKPRRDSGLE FPPISAARPE VVGSIIPQTV PAEYEVERAE
 61   SMEDFTDILS VLTKIPKCTG CAQHIFDRFI LKVQDKPWHA QCLKCGDCGR QLTDKCFSRG
121   SFVYCKEDFF KRYGTKCAGC DEAIPPTEVV RRAQENVYHL ECFRCFMCND QLGTGDQFYL
181   LDDNRLVCKK DYETAKSRDI DMDNGIKRPR TTITAKQLET LKLAYNQSPK PARHVREQLS
241   SDTGLDMRVV QVWFQNRRAK EKRLKKDTSR QRWGEIFRSG APSSGPQMHA NPESPPSGGK
301   RRPGNHNSRK RPSSSPGGSR ISVPIPSSIQ SPVHAPPNGQ IEPGFLAPEH LLPPSDGIML
361   GESPCFTPGE LPYPQHPANP HFLSPGGIPD PMGPFHPQSY EYVDGPQMMG PGMAAMKLPV
421   NNTMHNYYVT HKQQQPAKND CDVISEGSGN LSDLSSSPRS WLGELDHVTH FQ (472)
```

B

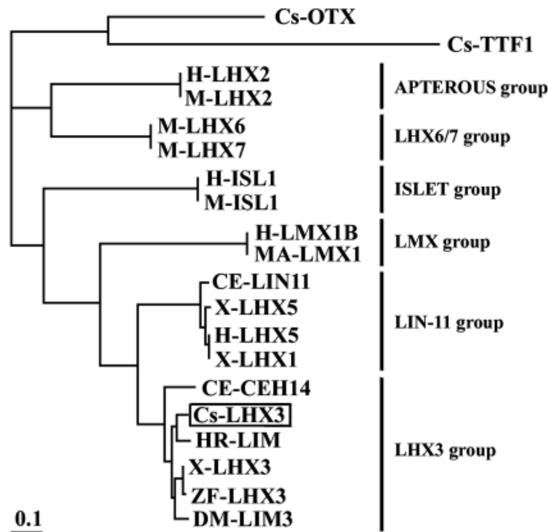

Fig. 2. The *Cs-lhx3* gene product of *Ciona savignyi*. (A) The predicted amino acid sequence. The LIM domains are shown by italic capitals and homeodomain is underlined. (B) Relationship of Cs-LHX3 with other LIM-homeodomain proteins. The phylogenetic tree of the homeodomains of the LIM-homeodomain proteins was constructed using the neighbor-joining method. The homeodomains of Cs-OTX and Cs-TTF1 were used as an outgroup. Branch length is proportional to the number of amino acid substitution; the scale bar indicates 0.1 amino acid substitution per position in the sequence.

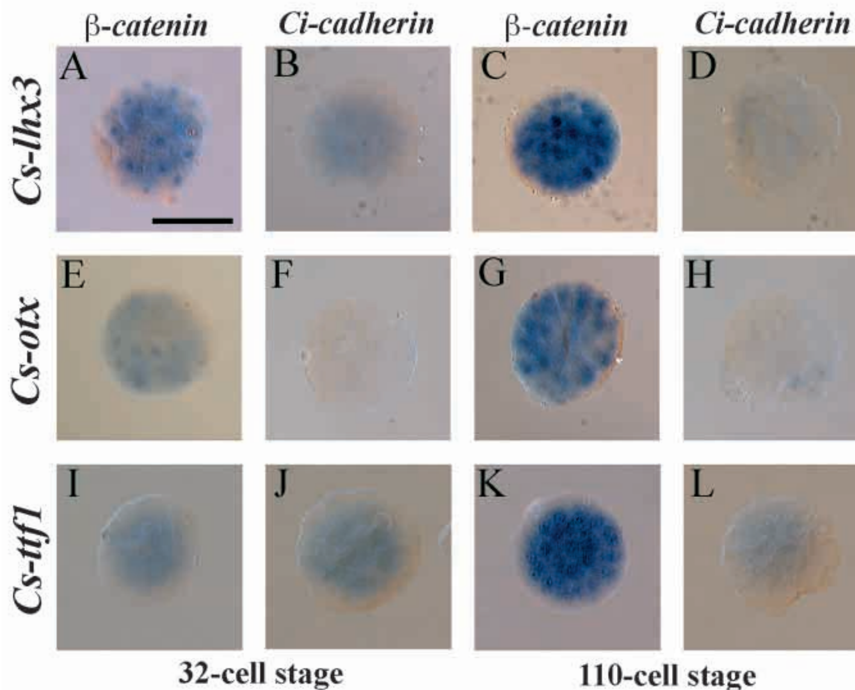

Fig. 3. Expression of *Cs-lhx3* (A-D), *Cs-otx* (E-H) and *Cs-ttf1* gene (I-L) in β-catenin overexpressed embryos (A, C, E, G, I, K) or in cadherin overexpressed embryos (B, D, F, H, J, L), at the 32-cell stage (A, B, E, F, I, J) and at the 110-cell stage (C, D, G, H, K, L). Vegetal pole view; anterior is upwards, posterior is downwards. Scale bar: 100 μm.

homeodomain amino acid residues were completely conserved between the ascidian Otx proteins, except for one residue between *Ciona* and *Halocynthia* (Fig. 4B).

Embryonic expression of *Cs-otx* also became evident as early as the 32-cell stage. As shown in Fig. 1L, the expression was evident in B6.1, B6.2 and B6.4 blastomeres of the 32-cell stage embryo. At the 64-cell stage, the expression was observed in endoderm precursor cells, A7.1, A7.2, A7.5, B7.1 and B7.2, trunk lateral precursor cells A7.6, presumptive notochord and mesenchyme cells B7.7 and B7.3, and presumptive muscle cells B7.5 (Fig. 1M). This complex spatial expression continued up to the early gastrula stage (Fig. 1N), and then this early embryonic *Cs-otx* expression in the vegetal blastomeres was downregulated. *Cs-otx* was also expressed in the neuronal cell lineage (A8.7 and A.8.8) at early gastrula and later stages (Fig. 1O). The embryonic expression pattern of *Cs-otx* was similar to that of *Hroth* (Wada et al., 1996) and *Ci-otx* (Hudson and Lemaire, 2001).

As in the case of *Cs-lhx3, Cs-otx* expression was upregulated in the β-catenin overexpressed embryos. In situ hybridization signals for *Cs-otx* expression were found in nuclei of every blastomere, including animal blastomeres of the 110-cell stage embryo (Fig. 3G), although at the 32-cell stage the upregulation of *Cs-otx* was not so conspicuous (Fig. 3E). By contrast, *Cs-otx* expression was downregulated in the cadherin overexpressed embryos, and no in situ signals were detectable in these embryos (Fig. 3F,H).

Cs-ttf1

A *C. savignyi* homolog of mammalian *ttf1* (thyroid transcription factor gene 1), which encodes an NK-2 class homeodomain protein, was also identified as a possible β-catenin downstream gene. A cDNA for this gene was 2357 bp in length, and the

predicted protein was 473 amino acids long (Fig. 5A) (DDBJ/GenBank/EMBL Accession Number AB057734). The *C. intestinalis* homologue of *ttf1* (*Ci-titf1*) has been characterized and reported to play a significant role in endodermal cell differentiation (Ristoratore et al., 1999). The homeodomain of Cs-TTF1 showed a significant identity to that of *C. intestinalis* and of mammals (Fig. 5B).

As in the case of *C. intestinalis Ci-titf1*, the expression of *Cs-ttf1* was first detected in A7.1, A7.2, A7.5, B7.1 and B7.2 blastomeres at the 64-cell stage (Fig. 1R). *Cs-ttf1* expression in the endoderm lineage persisted during early gastrulation (Fig. 1S), and was then downregulated. It has been reported that *Ci-titf1* is expressed once again in the endostyle primordium during metamorphosis of the larva to the adult (Ogasawara et al., 1999).

As shown in Fig. 3K, *Cs-ttf1* expression was upregulated in the β-catenin mRNA-injected 110-cell embryos. However, *Cs-ttf1* expression was not detected in β-catenin overexpressed 32-cell embryos (Fig. 3I), suggesting that β-catenin overexpression did not accelerate the timing of initiation of *Cs-ttf1* expression. The gene expression was suppressed in the *Ci-cadherin* mRNA injected embryos at the 110-cell stage (Fig. 3L).

A

```
  1   MSYLKSPHYA MNGLGLGHDM NLLHPTVTYP GDSAACYFQR IQGMHQGTKN MQFAAPPVYS
 61   SSRKQRRERT TFTRAQLDIL EALFGKTRYP DIFMREEVAL KINLPESRVQ VWFKNRRAKC
121   RQQVQQQQQK QKSGSGSGSS SSANNSSGSS SGSTNSNNNS SSSASKKKSP PTTPTPTATG
181   PAASTSPFQQ LTAPPAHQPL AQSSSPPLPS SPVSSNGQIG PSVTPSELMG SSGAPLNHQG
241   SHGAHSGMVH TGSSSNIWSP ASVSPGASSD GASNVPGIGY SGALVASNNS PYMAAAAAVA
301   HAAHSNYSAT SNAQVTSGGY PQNYHTSHSY FGTMEASYLP SVPFPGAAPG CGMGDMSMSG
361   QQSASQLGHH GVGQAHSAPH HQFMPQSYPY HHATSGVTPT YPTVSECLDY KDQTQAWKFQ
421   VL  (422)
```

B

```
Cs-OTX   KQRRERTTFTRAQLDILEALFGKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQ
Ci-OTX   KQRRERTTFTRAQLDILEALFGKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQ
Hr-OTX   KQRRERTTFTRAQLDILEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQ
         *********************.**************************************
```

Fig. 4. The *Cs-otx* gene product. (A) The predicted amino acid sequence. The homeodomain is underlined. (B) Alignment of amino acid sequence of the homeodomain of Cs-OTX protein with those of Ci-OTX (Hudson and Lemaire, 2001) and Hr-OTX (Wada et al., 1996). Identities of the amino acid residues are shown by asterisks and similarities by dots.

A

```
  1   MSVSPKHSTT TPFSVTDILS PLEESYGNGS VDGIVSGRRE FSELYCGMDS GTTSSTGIHV
 61   PNLGGGGSTG SISSPMPSYR QPVMGSPPHH SIHHQQMPVT NPYQSATGVN GAYNLHPMPT
121   PPQTTFHTMS GTGGGPMYGN GGVSDLPPYN NVQSHWYGAP SNPDPRFVPS YPRLQIGYGS
181   MMPGTGMDGM HKSLLPPSQR RKRRVLFSQA QVFELERRFK QQKYLSAPER EHLAQMIHLT
241   PTQVKIWFQN HRYKNKRSLK DKQTQDMGVQ QQQPQNSVNP QQQHTNEMTH SNPAQLQPIP
301   SNIAPGGSRT PQETYSPVPS GIPPQQGMEE VSDAAHRMVH ITNQNGEQSS PRQIAMSDGQ
361   ACHETTIIES PNLQMSMPSV VHSTDSESGL VPFSSVIKLE EVNVESNETA AGNHGINVPQ
421   THYLGSTHHH DLMNVNVAAL NSVDAYQNHH HHNHIPIGNS VLHDPSILYS VYR (473)
```

B

```
Cs-TTF1      RRKRRVLFSQAQVFELERRFKQQKYLSAPEREHLAQMIHLTPTQVKIWFQNHRYKNKRSL
Ci-TTF1      RRKRRVLFSQAQVFELERRFKQQKYLSAPEREHLAQMIHLTPTQVKIWFQNHRYKNKRSL
mouse TTF1   RRKRRVLFSQAQVYELERRFKQQKYLSAPEREHLASMIHLTPTQVKIWFQNHRYKMKRQA
             *************.********************.*******************.**..
```

Fig. 5. The *Cs-ttf1* gene product. (A) The predicted amino acid sequence. The homeodomain is underlined. (B) Alignment of amino acid sequence of the homeodomain of Cs-TTF1 protein with those of Ci-TTF1 (Ristoratore et al., 1999) and mouse TTF1 (Lazzaro et al., 1991). Identities of the amino acid residues are shown by asterisks and similarities by dots.

Functional assay of *Cs-lhx3*, *Cs-otx* and *Cs-ttf1*

In *Ciona* embryos, the nuclear localization of β-catenin in vegetal blastomeres was assessed using a specific antibody that becomes evident by the 32-cell stage, and this localization is most probably the first step in endodermal cell specification (Imai et al., 2000). As shown above, all three transcription factor genes are upregulated in β-catenin overexpressed embryos and downregulated in cadherin overexpressed embryos. In addition, all three genes are expressed in embryonic endodermal cells around the time of their fate restriction, suggesting an involvement of the three genes in endodermal cell differentiation. This issue was investigated by inhibition of possible gene function by morpholino oligonucleotides or by over and/or ectopic expression of the gene by injection of synthetic mRNAs into eggs. Nucleotide sequences of the morpholino oligonucleotides are listed in Table 1, and it has been shown that morpholino oligos specifically suppress certain gene function in *Ciona* embryos (Satou et al., 2001a).

Fig. 6A-C shows larvae that developed from eggs injected with *lacZ* morpholino oligo as a control. Although the *lacZ* morpholino completely inhibits *lacZ* development in larvae developed from eggs injected with *lacZ* mRNA (Satou et al., 2001a), this morpholino injection does not affect morphogenesis of *Ciona* embryos (Fig. 6B,C) and development endoderm-specific alkaline phosphatase (AP) (Fig. 6A).

Cs-otx

Fig. 6D-F shows larvae developed from eggs injected with *Cs-otx* morpholino oligo. Development of AP was evident by histochemical staining (Fig. 6D). This was confirmed in two series of 41 experimental larvae (Table 2). However, *Cs-otx*-morpholino injected larvae showed a failure of central nervous system formation (Fig. 6E,F). They did not develop the two sensory organs, otolith and ocellus, that are able to be assessed by observing pigment cell differentiation (Fig. 6E,F). The development of the anterior head was also suppressed by *Cs-otx* morpholino, so that this region looks smooth (Fig. 6E,F). These results suggest that *Cs-otx* is not directly involved in the larval endodermal cell differentiation but is essential for the neuronal cell differentiation.

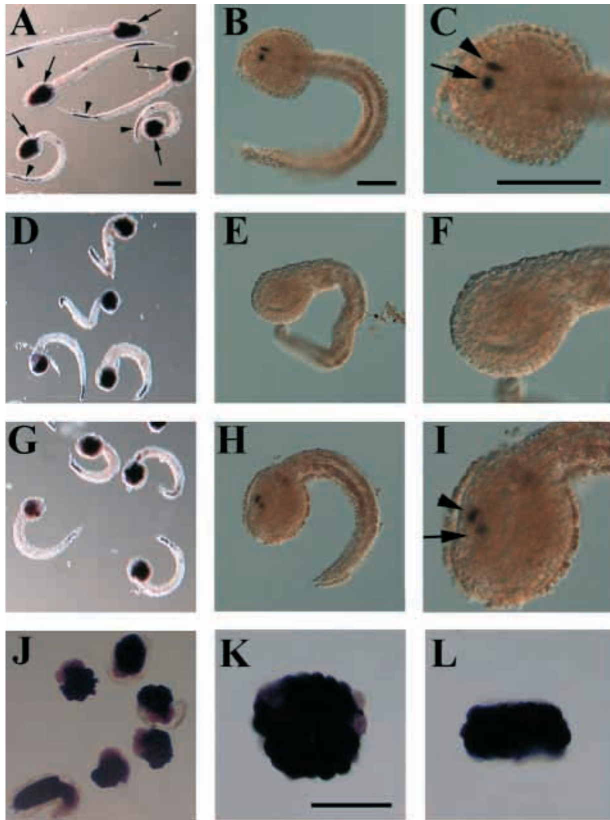

Fig. 6. Effects of functional suppression with morpholino oligos of *Cs-otx* (D-F) and *Cs-ttf1* (G-I) or overexpression of *Cs-ttf1* (J-L) on the endodermal cell differentiation. (A-C) Larvae developed from eggs injected with *lacZ* morpholino (control). (A) Histochemical staining of AP in the endodermal cells (arrows) of the larval trunk. In *Ciona* larvae, AP is also expressed B-line notochord cells (arrowhead). (B,C) Larvae that show normal development of two sensory organs, otolith (arrow) and ocellus (arrowhead), which is evident by pigment cell development. (D-F) Larvae developed from eggs injected with *Cs-otx* morpholino. (D) Histochemical staining of AP. (E,F) Larvae that show a failure of development of two sensory organs. The development of the anterior head is also suppressed by *Cs-otx* morpholino, causing this region to appear smooth. (G-I) Larvae that developed from eggs injected with *Cs-ttf1* morpholino. (G) Histochemical staining of AP activity. (H,I) Larvae that show normal development of two sensory organs, otolith (arrow) and ocellus (arrowhead). (J-L) Larvae developed from eggs injected with *Cs-ttf1* mRNA. Histochemical staining of AP activity in tailbud embryos (J) and in 110-cell arrested embryo. Ventral view (K) and anterior view (L). Scale bars: in A, 100 μm for A,D,G,J; in B, 100 μm for B,E,H; in C, 100 μm for C,F,I; and in K, 100 μm for K,L.

Cs-ttf1

Injection of *Cs-ttf1* morpholino oligo also did not affect the development of AP activity (Fig. 6G). This was confirmed by two series of 17 manipulated larvae (Table 2). The morphology of resultant larvae looked normal (Fig. 6H,I).

Ristoratore et al. (Ristoratore et al., 1999) showed that injection of *Ci-titf1* mRNA resulted in an ectopic expression of AP in *C. intestinalis* embryos. We confirmed their result: injection of 1.5 pg of *Cs-ttf1* mRNA caused an ectopic expression of AP so that many embryonic cells showed AP activity (Fig. 6J). To examine cells with ectopic AP activity, we took advantage of cleavage-arrested embryos. When early ascidian embryos are immersed in seawater containing cytochalasin B, cytokinesis but not nuclear division of blastomeres is blocked. Blastomeres of these cleavage-arrested embryos express differentiation markers depending on their lineage (Whittaker, 1973). Embryos injected with *Cs-ttf1* mRNA were arrested at the 110-cell stage, because the developmental fate of almost all of the blastomeres is restricted to one tissue by this stage. AP activity in the cleavage-arrested embryos was examined when untreated control embryos hatched. In uninjected embryos, AP activity was detected in 10 vegetal cells, as expected based on the endoderm lineage (data not shown; cf. Fig. 7E). It became evident that in embryos injected with *Cs-ttf1* mRNA, AP activity was evident in almost all of the blastomeres including the primary lineage muscle cells (Fig. 6K,L).

Table 2. Effects of morpholino oligonucleotides on alkaline phosphatase expression

	Number of experiments	Total number of embryos	AP expression		
			Normal	Reduced	Not detected
Cs-otx	2	41	41	0	0
Cs-ttf1	2	17	17	0	0
Cs-lhx3	4	54	2	20	32

Cs-lhx3

In contrast to *Cs-otx* and *Cs-ttf1*, morpholino oligonucleotides against *Cs-lhx3* markedly inhibited AP development (Fig. 7A). The *Cs-lhx3* morpholino oligo was designed to match 24 nucleotides between −39 and −16 in the 5′ UTR of the mRNA (Table 1) as A of the first ATG was regarded position 0. Fertilized eggs injected with *Cs-lhx3* morpholino underwent cleavages that were slightly delayed and failed to undergo normal morphogenesis to form the larvae (Fig. 7A). These experimental larvae showed the expression of an epidermis-specific gene *Cs-Epi1* (data not shown). However, as shown in Table 2, among four series of 54 experimental larvae, 32 did not develop AP activity, while 20 showed only reduced activity. Suppression of endodermal cell differentiation by *Cs-lhx3* morpholino was examined by another differentiation marker of endodermal cells. We have isolated cDNA clone for a thyroid hormone receptor gene of *C. savignyi* and have characterized its endoderm-specific expression (Fig. 7B, insert; DDBJ/GenBank/EMBL Accession Number AB057767). Injection of *Cs-lhx3* morpholino also inhibited development of this endoderm-specific gene expression (Fig. 7B).

The suppression of endodermal cell differentiation by *Cs-lhx3* morpholino was rescued by injection of 9 pg of synthetic mRNA for *Cs-lhx3*. The synthetic *Cs-lhx3* mRNA was designed to be initiated from position −4 and therefore the *Cs-lhx3* morpholino does not recognize the synthetic mRNA. As a control, injection of 9 pg of synthetic mRNA for *lacZ* did not rescue the AP expression (Fig. 7C); AP expression was not detected in 20 out of the 25 experimental embryos injected together with *Cs-lhx3* morpholino and *lacZ* mRNA (Table 3). By contrast, as shown in Fig. 7D and Table 3, more than a half of 23 experimental embryos injected together with *Cs-lhx3* morpholino and *Cs-lhx3* mRNA rescued the AP activity.

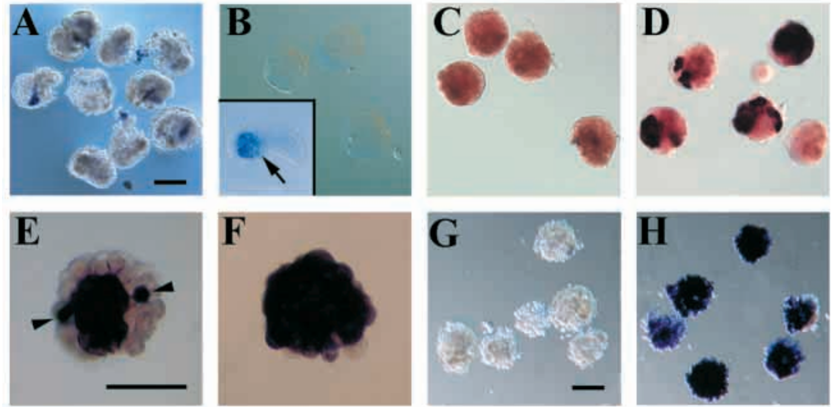

Fig. 7. Effects of functional suppression of *Cs-lhx3* with morpholino oligos, rescue of the suppression by injection of synthetic *Cs-lhx3* mRNA or overexpression of *Ci-lhx3* on endodermal cell differentiation. (A,B) The embryos developed from eggs injected with morpholino against *Cs-lhx3. Cs-lhx3* morpholino inhibits development of AP activity (A) and endoderm-specific thyroid hormone receptor gene expression (B). Insert in B shows the endoderm-specific expression of thyroid hormone receptor gene in control embryo (arrow). (C,D) Co-injection of *Cs-lhx3* morpholino together with *lacZ* mRNA (C) or *Cs-lhx3* mRNA (D). In control, AP activity did not develop (C), but embryos developed from eggs injected with *Cs-lhx3* morpholino and synthetic mRNA showed AP activity (D). (E,F) Effects of *Cs-lhx3* overexpression. AP expression in embryos developed from eggs injected with *Cs-lhx3* mRNA or *lacZ* mRNA (control) and arrested at the 110-cell stage, vegetal view. (E) Control embryo showing AP activity in blastomeres of the endoderm lineage, while (F) *Cs-lhx3* overexpressed embryos showing AP activity in non-endodermal blastomeres as well. Arrowheads indicate pigment cell development in the cleavage-arrested embryo. (G) AP expression in embryos developed from eggs injected with *delE-Ci-cadherin* mRNA and *lacZ* mRNA. (H) AP expression in embryos developed from eggs injected with *delE-Ci-cadherin* mRNA and *Cs-lhx3* mRNA. Scale bars: in A, 100 μm for A-D; in E, 100 μm for E,F; in G, 100 μm for G,H.

As an ectopic expression experiment, *Cs-lhx3* mRNA was injected into fertilized eggs, and this injection promoted ectopic AP development (Fig. 7E,F). When this promotion was examined using cleavage-arrested embryos, AP activity was evident in almost all of the blastomeres, except in the primary lineage muscle cells (Fig. 7F).

As mentioned before, injection of 60 pg of *delE-Ci-cadherin* into fertilized eggs downregulates the β-catenin nuclear localization, which in turn results in the failure of AP development in resultant larvae (Imai et al., 2000). As shown in Fig. 7G and Table 4, embryos developed from eggs injected with 60 pg of *delE-Ci-cadherin* mRNA and 9 pg of *lacZ* mRNA showed a suppression of AP activity. However, when eggs were injected with 60 pg of *delE-Ci-cadherin* mRNA together with 9 pg of *Cs-lhx3* mRNA, resultant embryos recovered the AP activity (Fig. 7H). This was confirmed in all of the 31 experimental embryos (Table 4).

All of these results strongly suggest that the early embryonic expression of *Cs-lhx3* is involved in the endodermal cell differentiation in *C. savignyi* embryos.

DISCUSSION

Subtraction screening of β-catenin downstream genes in *C. savignyi* embryos

In the present study, we conducted subtractive hybridization screens of mRNAs between β-catenin overexpressed embryos and cadherin overexpressed embryos to identify potential β-catenin target genes in *C. savignyi* embryos. In early *Ciona* embryos, when β-catenin is mis- and/or overexpressed, presumptive notochord cells and epidermal cells change their fates into endodermal cells, whereas when β-catenin nuclear localization is downregulated by the overexpression of cadherin, endodermal cell differentiation is suppressed, which is accompanied by the differentiation of extra-epidermal cells (Imai et al., 2000). The enrichment of an endoderm-related gene *Cs-HNF3* (Shimauchi et al., 2001) in β-catenin overexpressed library and that of an epidermis-specific gene *Cs-Epi1* in cadherin overexpressed library were confirmed by Southern blot hybridization. In the previous study, we demonstrated that the development of primary muscle cells is

Table 3. Alkaline phosphatase expression suppressed by *Cs-lhx3* morpholino oligonucleotide was restored by injection of *Cs-lhx3* mRNA but not by *lacZ* mRNA

			Alkaline phosphatase expression		
Morpholino oligos	Synthetic mRNA	Total number of embryos	Detected in more than a half region of embryo	Detected in small region of embryo	Not detected
Cs-lhx3	*Cs-lhx3*	23	8	5	10
Cs-lhx3	*lacZ*	25	1	4	20

Table 4. Alkaline phosphatase expression which is diminished by nuclear β-catenin depletion is rescued by microinjection of *Cs-lhx3* mRNA

			Alkaline phosphatase expression		
	Number of experiments	Total number of embryos	Detected in entire region of embryo	Detected in partial region of embryo	Not detected
delE-Ci-cadherin + lacZ	2	22	0	6	16
delE-Ci-cadherin + Cs-lhx3	2	31	31	0	0

not affected by overexpression of β-catenin nor by depletion of nuclear β-catenin, whereas cells with neural fates are transformed into endoderm by overexpression of β-catenin (Imai et al., 2000). *Cs-ZICR1* (a ZIC-related gene 1 of *C. savignyi*) is expressed in both presumptive muscle cells (B6.2 and B6.4) and presumptive nerve cord cells (A6.2 and A6.4) at the 32-cell stage (K. S. Imai, Y. S. and N. S., unpublished). Using this gene probe, we determined whether the transcription activity of genes expressed in the vegetal cells is non-specifically upregulated by β-catenin overexpression and downregulated by depletion of nuclear β-catenin. As expected, the *Cs-ZICR1* expression in muscle lineage cells was not altered in experimental embryos, whereas the gene expression in neuronal lineage cells was downregulated by β-catenin injection (K. S. Imai, Y. S. and N. S., unpublished). The present screening thus yielded cDNAs for twelve different genes, including a LIM-homeobox gene (*Cs-lhx3*), an *otx* gene (*Cs-otx*), a *ttf1* gene (*Cs-ttf1*), two *forkhead* genes, a type II *cadherin* gene, a *protocadherin* gene, a *netrin* gene, a *frizzled* gene, a *lefty/antivin* homolog, an *EPH* gene and a gene encoding a protein with no known sequence similarity; the first three transcription genes were studied in detail.

An essential role for *Cs-lhx3* in the endoderm differentiation of ascidian embryos

As shown in the present study, *Cs-lhx3* is a possible target of nuclear localization of β-catenin in *Ciona* embryos, because *Cs-lhx3* expression was upregulated in the β-catenin overexpressed embryos and downregulated in the cadherin overexpressed embryos (Fig. 3). The first embryonic expression of *Cs-lhx3* is seen in A6.1, A6.2 and B6.1 blastomeres of 32-cell stage embryos (Fig. 1G), all three pairs being presumptive endodermal cells, and A6.1 and B6.1 are restricted at this stage to give rise to the endoderm only (Fig. 1B). This early and transient expression of *Cs-lhx3* is found mainly in endodermal cells. *Cs-lhx3* misexpression by injection of its synthetic mRNA causes development of extra cells with AP activity (Fig. 7F). In addition, its morpholino oligonucleotide blocks development of cells with AP activity (Fig. 7A). The *Cs-lhx3* morpholino also completely suppresses expression of an endoderm-specific thyroid hormone receptor gene (Fig. 7B). This suppression was rescued by injection of *Cs-lhx3* mRNA (Fig. 7D) but not by injection of *lacZ* mRNA (Fig. 7C). Furthermore, injection of *Cs-lhx3* mRNA rescues development of cells with AP activity in cadherin-overexpressed embryos that would not express AP. These results strongly suggest that a LIM-homeobox gene *Cs-lhx3* is one of the β-catenin downstream genes and its early expression in embryonic endodermal cells is responsible for their differentiation.

Future studies therefore should determine whether the early expression of *Cs-lhx3* is directly controlled by the complex of β-catenin and Tcf/LEF-1. A preliminary investigation suggests that the 5′ flanking region of the *Cs-lhx3* gene contains consensus sequence motifs for Tcf-binding site. One of these motifs is likely to be required for the *Cs-lhx3* expression, although other factors are also required for proper *Cs-lhx3* expression (K. S. Imai, Y. S. and N. S., unpublished). In addition, future studies should explore the mechanisms of *Cs-lhx3* function, whether or not *Cs-lhx3* directly regulates the transcription of *Cs-AP* gene or thyroid hormone receptor gene or other genes required for endodermal cell differentiation. A recent study of gene expression profile of *C. intestinalis* tailbud embryos has revealed that more than 30 genes are specifically expressed in the endodermal cells (Satou et al., 2001b). Counterparts of these *C. intestinalis* endoderm-specific genes may be expressed in *C. savignyi* embryonic endodermal cells. Therefore, it should also be determined how *Cs-lhx3* controls the expression of these endoderm-specific genes.

During later embryogenesis, *Cs-lhx3* is again expressed in one pair of cells in the nerve cord and a subset of brain cells, as in the case of *Hrlim* (Wada et al., 1995). Because most of the LIM-homeobox genes are involved in neuronal development and function (reviewed by Hobert and Westphal, 2000), it is conceivable that the ascidian LIM-homeobox genes are also involved in the differentiation of neuronal cells. Injection of *Cs-lhx3* morpholino disrupts morphogenesis; therefore, we could not investigate the role of *Cs-lhx3* in the differentiation of neuronal cells. This should be examined in future studies by experiments in which this gene is temporally controlled suppressed and/or misexpressed at different times in development. In addition, because the endoderm cells emanate signals required for the specification of notochord cells (Nakatani and Nishida, 1994; Nakatani et al., 1996) and mesenchyme cells (Kim et al., 2000) in *Halocynthia* embryos, whether *Cs-lhx3* regulates genes associated with these cellular interactions is also an intriguing issue.

Roles for *Cs-otx* and *Cs-ttf1* in the endoderm differentiation of ascidian embryos

Cs-otx

Wada et al. (Wada et al., 1996) have shown that *Hroth* of *H. roretzi* begins to be expressed as early as the 32-cell stage and exhibits a rather complex spatial expression pattern in early embryos, namely, at the 32-cell stage *Hroth* is expressed in both involuting mesoendoderm and anterior ectoderm during gastrulation, and later expression is restricted to the sensory vesicle and anterior epidermis. *C. intestinalis Ci-otx* shows a similar but slight different expression pattern (Hudson and Lemaire, 2001). In addition, Wada and Saiga (Wada and Saiga, 1999) have shown that microinjection of *Hroth* mRNA into fertilized eggs leads to embryos with an expanded trunk and a reduced tail, in which development of muscle and notochord is effected. *Hroth* overexpression also causes ectopic formation of the anterior neuroectoderm, together with the suppression of epidermis development. They suggested that *Hroth* plays roles in both specification of mesoendodermal cells and anterior neuroectoderm formation (Wada and Saiga, 1999).

As in the case of *Hroth* and *Ci-otx*, *Cs-otx* is first expressed at the 32-cell stage in vegetal blastomeres of B6.1 (endoderm lineage), B6.2 (mesoderm lineage) and B6.4 (muscle and mesenchyme lineages), and at the 64-cell stage A-line endodermal cells begin to express *Cs-otx*. However, *Cs-otx* morpholino did not block AP expression in endodermal cells, and differentiation of mesenchyme, muscle and notochord cells appeared normal (Fig. 6D). Instead, *Cs-otx* morpholino oligo severely inhibited the formation of the central nervous system in that the two sensory pigment cells did not develop and the anterior head structure was affected. These results suggest that the primary function of *otx* during ascidian embryogenesis is associated with the patterning of the CNS, as in *Drosophila*

and vertebrate embryos (Finkelstein and Boncinelli, 1994; Matsuo et al., 1995; Simone, 1998).

Nevertheless, expression of *Cs-otx* in mesoendoderm cells of early *Ciona* embryos suggests its role in the differentiation of the mesoendoderm cells. This possibility is also suggested in sea urchin embryos (reviewed by Angerer and Angerer, 2000). In sea urchins, the nuclear localization of β-catenin is seen in vegetal blastomeres and is essential for vegetal fate determination of embryonic cells (Wikramanayake et al., 1998; Logan et al., 1999). This nuclear localization of β-catenin, in complex with Tcf (Vonica et al., 2000), may activate several transcription factor genes, including Otx (*SpOtx*) (Li et al., 1999). Because the Engrailed-SpOtx fusion protein reduced the expression of endoderm- and aboral ectoderm-specific genes, the *SpOtx* gene is likely to be involved in the endoderm differentiation (Li et al., 1999). The sea urchin *Otx* gene also controls the aboral-specific gene *Ars* (Kiyama et al., 2000). In sea urchin embryos, a *lim1*-related homeobox gene, *Hplim1* is expressed transiently in the vegetal plate cells of hatching blastulae (Kawasaki et al., 1999). Overexpression experiments suggest that *Hplim1* is involved in endoderm development. Therefore, whether *Hplim1* is a downstream gene of β-catenin remains an intriguing issue.

Furthermore, an ascidian *otx* gene is highly likely to play an essential role in the pharynx formation after metamorphosis (Hinman and Degnan, 2000). Ascidian tadpole larvae are nonfeeding larvae and the gut does not fully differentiate until after metamorphosis of the larvae. One of the primary tissues that the endoderm differentiates into after metamorphosis is the pharynx. Hinman and Degnan (Hinman and Degnan, 2000) have shown that *otx* (*Hec-Otx*) of another ascidian *Herdmania curvata* is expressed in the pharynx. In addition, treatment of juveniles with retinoic acid suppresses expression of otx after metamorphosis, which results in development of adult ascidians lacking the pharynx (Hinman and Degnan, 1998; Hinman and Degnan, 2000). These results strongly suggest that the ascidian *otx* gene functions during very early embryogenesis in endomesoderm specification, then in neuroectoderm differentiation during larval formation, and later in the pharynx formation of juveniles after metamorphosis.

Cs-ttf1

Ci-titf1 of *C. intestinalis* (Ristoratore et al., 1999; Ogasawara et al., 1999), *Cs-ttf1* of *C. savignyi* (the present study) and *Hrtitf1* of *H. roretzi* (Ogasawara et al., 1999) are ascidian members of the *NK-2* gene family. These genes are expressed in the endodermal cells of the 64-cell to early gastrula stages, and later in the endostyle of young adults (Ristoratore et al., 1999; Ogasawara et al., 1999). Ristoratore et al. (Ristoratore et al., 1999) have shown that misexpression of these genes causes the development of extra cells with AP activity. As shown in the present study, the expansion of AP activity in extra cells by *Cs-ttf1* is as strong as that of β-catenin or *Cs-lhx3*, suggesting a role of the ascidian *NK-2* family gene in the embryonic endoderm differentiation, as suggested by Ristoratore et al. (Ristoratore et al., 1999). However, the expression of this gene is initiated at the 64-cell stage, and its morpholino oligonucleotide does not block the appearance of AP activity in the endodermal cells (Fig. 6G). These results suggest that the ascidian *ttf1* gene is not involved in the initial phase of endodermal cell specification and/or differentiation but in the process of the maintenance of differentiated state. Because ascidian *ttf1* seems to upregulate the AP gene activity, it is interesting to ask whether the Cs-TTF1 binds to *cis*-elements of endoderm-specific genes of *Ciona* embryos.

β-Catenin target downstream genes

In *Xenopus* and zebrafish, a key step in the establishment of the dorsoventral axis is nuclear localization of β-catenin, which is supplied by maternal transcript (Heasman et al., 1994; Funayama et al., 1995; Guger and Gumbiner, 1995; Kelly et al., 1995; Schneider et al., 1996; Larabell et al., 1997). Dorsally localized β-catenin forms a complex with Tcf-like transcription factor, and this complex is thought to trigger the zygotic activation by transcription factor genes: *siamois* (Brannon et al., 1997; Fan and Sokol, 1997) and *twins* (Laurent et al., 1997) in *Xenopus* embryos, and *boz/dharma* in zebrafish embryos (Fekany et al., 1999). These transcription factor genes are likely to act upstream of transforming growth factor β, which plays a pivotal role in the development of both axial mesoderm and anteroposterior neural patterning (Cadigan and Nusse, 1997; Moon and Kimelman, 1998; Sokol, 1999). Therefore, it is expected that the nuclear localization of β-catenin activates several other transcription factor genes that in turn may regulate other genes required for the differentiation of specific cell types in sea urchin and ascidian embryos or for cell-cell signaling cascades involved in axis formation in frog and fish embryos.

In conclusion, the present attempt to identify β-catenin target downstream genes in *C. savignyi* embryos demonstrates that a LIM-homeobox gene *Cs-lhx3* is probably a direct target of β-catenin and that the expression of this gene is required for endodermal cell differentiation.

We thank Dr Yasuaki Takagi, Mr Kouichi Morita and all of the members in the Otsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo, Iwate, Japan for collecting *Ciona savignyi*. This research was supported by Grant-in-Aids from the Ministry of Education, Science, Sports and Culture, Japan to Y. S., K. S. I. and N. S.

REFERENCES

Angerer, L. M. and Angerer, R. C. (2000). Animal-vegetal axis patterning mechanisms in the early sea urchin embryo. *Dev. Biol.* **218**, 1-12.

Arnold, S. J., Stappert, J., Bauer, A., Kispert, A., Herrmann, B. G. and Kemler, R. (2000). *Brachyury* is a target gene of the Wnt/β-catenin signaling pathway. *Mech. Dev.* **91**, 249-258.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T. and Kimelman, D. (1997). A β-catenin/XTcf-3 complex binds to the *siamois* promoter to regulate dorsal axis specification in *Xenopus*. *Genes Dev.* **11**, 2359-2370.

Cadigan, K. M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.* **11**, 3286-3305.

Chiba, S., Satou, Y., Nishikata, T. and Satoh, N. (1998). Isolation and characterization of cDNA clones for epidermis-specific and muscle-specific genes in *Ciona savignyi* embryos. *Zool. Sci.* **15**, 239-246.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

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

Fan, M. J. and Sokol, S. Y. (1997). A role for Siamois in Spemann organizer formation. *Development* **124**, 2581-2589.

Fekany, K., Yamanaka, Y., Leung, T. C., Sirotkin, H. I., Topczewski, J., Gates, M. A., Hibi, M., Renucci, A., Stemple, D., Radbill, A., Schier, A. F., Driever, W., Hirano, T., Talbot, W. S. and Solnica-Krezel, L. (1999).

The zebrafish *bozozok* locus encodes Dharma, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures. *Development* **126**, 1427-1438.
Finkelstein, R. and Boncinelli, E. (1994). From fly head to mammalian forebrain: the story of *otd* and *Otx*. *Trends Genet*. 310-315.
Funayama, N., Fagotto, F., McCrea, P., Gumbiner, B. M. (1995). Embryonic axis induction by the armadillo repeat domain of β-catenin: evidence for intracellular signaling. *J. Cell Biol.* **128**, 959-968.
Gradl, D., Kühl, M. and Wedlich, D. (1999). The Wnt/Wg signal transducer β-catenin controls fibronectin expression. *Mol. Cell. Biol*. **19**, 5576-5587.
Guger, K. A. and Gumbiner, B. M. (1995). β-Catenin has Wnt-like activity and mimics the Nieuwkoop signaling center in *Xenopus* dorsal-ventral patterning. *Dev. Biol.* **172**, 115-125.
Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C,. Noro, C. Y. and Wylie, C. (1994). Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell* **79**, 791-803.
Hinman, V. F. and Degnan, B. M. (1998). Retinoic acid disrupts antrior ectodermal and endodermal development in ascidian larvae and postlarvae. *Dev. Genes Evol*. **208**, 336-345.
Hinman, V. F. and Degnan, B. M. (2000). Retinoic acid perturbs *Otx* gene expression in the ascidian pharynx. *Dev. Genes Evol*. **210**, 129-139.
Hobert, O. and Westphal, H. (2000). Functions of LIM-homeobox genes. *Trends Genet*. **16**, 75-83.
Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C. M., Cramer von Laue, C., Snyder, P., Rothbacher, U. and Holstein, T. W. (2000). WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra. *Nature* **407**, 186-189.
Hudson, C. and Lemaire, P. (2001). Induction of anterior neural fates in the ascidian *Ciona intestinalis*. *Mech. Dev*. **100**, 189-203.
Imai, K., Takada, N., Satoh, N. and Satou, Y. (2000). β-catenin mediates the specification of endoderm cells in ascidian embryos. *Development* **127**, 3009-3020.
Kawasaki, T., Mitsunaga-Nakatsubo, K., Takeda, K., Akasaka, K. and Shimada, H. (1999). Lim1 related homeobox gene (*HpLim1*) expressed in sea urchin embryos. *Dev. Growth Differ*. **41**, 273-282.
Kelly, G. M., Erezyilmaz, D. F. and Moon, R. T. (1995). Induction of a secondary embryonic axis in zebrafish occurs following the overexpression of β-catenin. *Mech. Dev*. **53**, 261-273.
Kelly, C., Chin, A. J., Leatherman, J. L., Kozlowski, D. J. and Weinberg, E. S. (2000). Maternally controlled β-catenin-mediated signaling is required for organizer formation in the zebrafish. *Development* **127**, 3899-3911.
Kim, G. J., Yamada, A. and Nishida, H. (2000). An FGF signal from endoderm and localized factors in the posterior-vegetal egg cytoplasm pattern the mesodermal tissues in the ascidian embryo. *Development* **127**, 2853-2862.
Kiyama, T., Sasai, K., Takata, K., Mitsunaga-Nakatsubo, K., Shimada, H. and Akasaka, K. (2000). CAAT sites are required for the activation of the *H. pulcherrimus Ars* gene by Otx. *Dev. Genes Evol*. **210**, 583-590.
Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D. and Moon, R. T. (1997). Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in β-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol*. **136**, 1123-1136.
Laurent, M. N., Blitz, I. L., Hashimoto, C., Rothbacher, U. and Cho, K. W. (1997). The *Xenopus* homeobox gene *twin* mediates Wnt induction of goosecoid in establishment of Spemann's organizer. *Development* **124**, 4905-4916.
Lazzaro, D., Price, M., De Felice, M. and Di Lauro, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113**, 1093-1104.
Lemaire, P., Garrett, N. and Gurdon, J. B. (1995). Expression cloning of *Siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94.
Li, X., Wikramanayake, A. H. and Klein, W. H. (1999). Requirement of SpOtx in cell fate decisions in the sea urchin embryo and possible role as a mediator of β-catenin signaling. *Dev. Biol.* **212**, 425-439.
Logan, C. Y., Miller, J. R., Ferkowicz, M. J. and McClay, D. R. (1999). Nuclear β-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* **126**, 345-357.
Matsuo, I., Kuratani, S., Kimura, C., Takeda, N. and Aizawa, S. (1995). Mouse Otx2 functions in the formation and patterning of rostral head. *Genes Dev*. **9**, 2646-2658.
McKendry, R., Hsu, S. C., Harland, R. M. and Grosschedl, R. (1997). LEF-1/TCF proteins mediate Wnt-inducible transcription from the *Xenopus nodal-related 3* promoter. *Dev. Biol*. **192**, 420-431.
Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L. and Moon, R. T. (1999). Establishment of the dorsal-ventral axis in *Xenopus* embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *J. Cell Biol*. **146**, 427-437.
Moon, R. T. and Kimelman, D. (1998). From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in *Xenopus*. *BioEssays* **20**, 536-545.
Nakatani, Y. and Nishida, H. (1994). Induction of notochord during ascidian embryogenesis. *Dev. Biol*. **166**, 289-299.
Nakatani, Y., Yasuo, H., Satoh, N. and Nishida, H. (1996) Basic fibroblast growth factor induces notochord formation and the expression of *As-T,* a *Brachyury* homolog, during ascidian embryogenesis. *Development* **122**, 2023-2031.
Nishida, H. (1987). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. *Dev. Biol.* **121**, 526-541.
Nishida, H. (1992). Developmental potential for tissue differentiation of fully dissociated cells of the ascidian embryo. *Roux's Arch. Dev. Biol*. **201**, 81-87.
Nishida, H. (1993). Localized regions egg cytoplasm that promote expression of endoderm-specific alkaline phosphatase in embryos of the ascidian *Halocynthia roretzi*. *Development* **118**, 1-7.
Ogasawara, M., Di Lauro, R. and Satoh, N. (1999). Ascidian homologs of mammalian thyroid transcription factor-1 gene are expressed in the endostyle. *Zool. Sci*. **16**, 559-565.
Ristoratore, F., Spagnuolo, A., Aniello, F., Branno, M., Fabbrini, F. and Di Lauro, R. (1999). Expression and functional analysis of *Cititf1*, an ascidian *NK-2* class gene, suggest its role in endoderm development. *Development* **126**, 5149-5159.
Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y., H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**, 707-716.
Satoh, N. (1994). *Developmental Biology of Ascidians*. New York: Cambridge University Press.
Satou, Y. and Satoh, N. (1997). *posterior end mark 2* (*pem-2*), *pem-4*, *pem-5* and *pem-6*: Maternal genes with localized mRNA in the ascidian embryo. *Dev. Biol*. **192**, 467-481.
Satou, Y., Imai, K. S. and Satoh, N. (2001a). Action of morpholinos in *Ciona* embryos. *genesis* (in press).
Satou, Y., Takatori, N., Yamada, R., Mochizuki, Y., Hamaguchi, M., Ishikawa, H., Chiba, S., Imai, K., S., Kano, S., Murakami, S. D. et al. (2001b). Gene expression profile in *Ciona intestinalis* tailbud embryos. *Development* **128**, 2893-2904
Schneider, S., Steinbeisser, H., Warga, R. M. and Hausen, P. (1996). β-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mech. Dev*. **57**, 191-198.
Shimauchi, Y., Chiba, S. and Satoh, N. (2001). Synergistic action of *HNF-3* and *Brachyury* in the notochord differentiation of ascidian embryos. *Int. J. Dev. Biol*. (in press).
Simeone, A. (1998). Otx1 and Otx2 in the development and evolution of the mammalian brain. *EMBO J*. **17**, 6790-6798.
Sokol, S. Y. (1999). Wnt signaling and dorso-ventral axis specification in vertebrates. *Curr. Opin. Genet. Dev*. **9**, 405-410.
Thorpe, C. J., Schlesinger, A., Carter, J. C. and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. *Cell* **90**, 695-705.
Vonica, A., Weng, W., Gumbiner, B. M. and Venuti, J. M. (2000). TCF is the nuclear effector of the β-catenin signal that patterns the sea urchin animal-vegetal axis. *Dev. Biol*. **217**, 230-243.
Wada, S., Katsuyama, Y., Yasugi, S. and Saiga, H. (1995). Spatially and temporally regulated expression of the LIM class homeobox gene *Hrlim* suggests multiple distinct functions in development of the ascidian, *Halocynthia roretzi*. *Mech. Dev*. **51**, 115-126.
Wada, S., Katsuyama, Y., Sato, Y., Itoh, C. and Saiga, H. (1996). *Hroth*, an *orthodenticle*-related homeobox gene of the ascidian, *Halocynthia roretzi*: its expression and putative roles in the axis formation during embryogenesis. *Mech. Dev*. **60**, 59-71.
Wada, S. and Saiga, H. (1999). Vegetal cell fate specification and anterior

neuroectoderm formation by *Hroth*, the ascidian homologue of *orthodenticle*/*otx*. *Mech. Dev*. **82**, 67-77.

Wang, Z. and Brown, D. D. (1991). A gene expression screen. *Proc. Natl. Acad. Sci. USA* **88**, 11505-11509.

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

Whittaker, J. R. (1990). Determination of alkaline phosphatase expression in endodermal cell lineages of an ascidian embryo. *Biol. Bull*. **178**, 222-230.

Whittaker, J. R. and Meedel, T. H. (1989) Two histospecific enzyme expression in the same cleavage-arrested one-celled ascidian embryos. *J. Exp. Zool*. **250**, 168-175.

Wikramanayake, A. H., Huang, L. and Klein, W. H. (1998). β-Catenin is essential for patterning the maternally specified animal-vegetal axis in the sea urchin embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9343-9348.

Willert, K. and Nusse, R. (1998). β-catenin: a key mediator of Wnt signaling. *Curr. Opin. Genet. Dev*. **8**, 95-102.

Yamaguchi, T. P., Takada, S., Yoshikawa, Y., Wu, N. and McMahon, A. P. (1999). *T* (*Brachyury*) is a direct target of Wnt3a during paraxial mesoderm specification. *Genes Dev*. **13**, 3185-3190.