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An essential role of a *FoxD* gene in notochord induction in *Ciona* embryos

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SUMMARY

A key issue for understanding the early development of the chordate body plan is how the endoderm induces notochord formation. In the ascidian *Ciona*, nuclear accumulation of β-catenin is the first step in the process of endoderm specification. We show that nuclear accumulation of β-catenin directly activates the gene (*Cs-FoxD*) for a winged helix/forkhead transcription factor and that this gene is expressed transiently at the 16- and 32-cell stages in endodermal cells. The function of *Cs-FoxD*, however, is not associated with differentiation of the endoderm itself but is essential for notochord differentiation or induction. In addition, it is likely that the inductive signal that appears to act downstream of *Cs-FoxD* does not act over a long range. It has been suggested that FGF or Notch signal transduction pathway mediates ascidian notochord induction. Our previous study suggests that *Cs-FGF4/6/9* is partially involved in the notochord induction. The present experimental results suggest that the expression and function of *Cs-FGF4/6/9* and *Cs-FoxD* are not interdependent, and that the Notch pathway is involved in B-line notochord induction downstream of *Cs-FoxD*.

Key words: *FoxD*, *Ciona* embryos, Transient expression, Endoderm, Notochord specification

INTRODUCTION

A key issue for understanding the early development of the chordate body plan is how the endoderm induces notochord formation (reviewed by Kimelman and Griffin, 2000; Rodaway and Patient, 2001). One possible scenario for notochord induction is that differentiation of the endoderm is tightly associated with the potential of the endoderm to induce the notochord, so that these two phenomena cannot be separated. Alternatively, the endoderm expresses a gene or produces a molecule that is not involved in the differentiation of the endoderm itself but is essential for notochord induction. We provide evidence for the latter scenario.

The endoderm of the ascidian tadpole larva is a simple tissue comprising about 500 cells, while the notochord is composed of exactly 40 cells (reviewed by Satoh, 1994; Satoh, 1999; Satoh, 2001). Cell lineage studies have documented that both the endodermal cells and notochord cells are derived from the vegetal A4.1 and B4.1 blastomeres of the eight-cell stage embryo (see Fig. 1). As early as the 32-cell stage, a pair of vegetal blastomeres (A6.1) becomes restricted to the generation of endoderm only (Fig. 1C), 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 (Fig. 1D). Reflecting such an early fate restriction, presumptive endodermal blastomeres show strong potential for autonomous differentiation when they are isolated from early embryos (Whittaker, 1990; Nishida, 1993). It has been shown in *Halocynthia roretzi* embryos and may also be the case in *Ciona* embryos that, at the 32-cell stage, the endodermal cells (A6.1 and A6.3) induce the neighboring cells (A6.2 and A6.4) to differentiate into notochord cells (Fig. 1C), and at the 64-cell stage, A7.3 and A7.7 are destined to give rise to notochord cells (Fig. 1D) (Nakatani and Nishida, 1994). Later, B7.3 also receives induction signal(s) from B6.1, A7.5 or A7.6 that induce B7.3 to give rise to notochord cells (B8.6) (Darras and Nishida, 2001). Therefore, the 40 notochord cells are constituted from two different lineages: 32 cells are of A-line (primary), while eight cells are of B-line (secondary) lineage.

Recently, convincing evidence has been accumulated showing the 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). β-catenin, together with TCF/LEF1, enters the nucleus and activates downstream genes, which include *siamois* (Brannon et al., 1997; Fan and Sokol, 1997), *twins* (Laurent et al., 1997), *Nodal-related3* (McKendry et al., 1997) and *fibronectin* (Gradl et al., 1999) in *Xenopus* embryos; *boz/dharma* in zebrafish embryos (Fekany et al., 1999); and *Brachyury* (Yamaguchi et al., 1999; Arnold et al., 2000) in mouse embryos. In early *Ciona* embryos, β-catenin accumulates in the nuclei of endoderm precursor cells by the 32-cell stage, and this nuclear accumulation of β-catenin is the first step of endodermal cell specification (Imai et al., 2000). If β-catenin is mis- and/or overexpressed, the fate of presumptive notochord cells and epidermal cells changes so that they become endodermal cells. If β-catenin nuclear localization is downregulated by the overexpression of cadherin, which binds to cytoplasmic β-catenin, endodermal cell differentiation is suppressed and

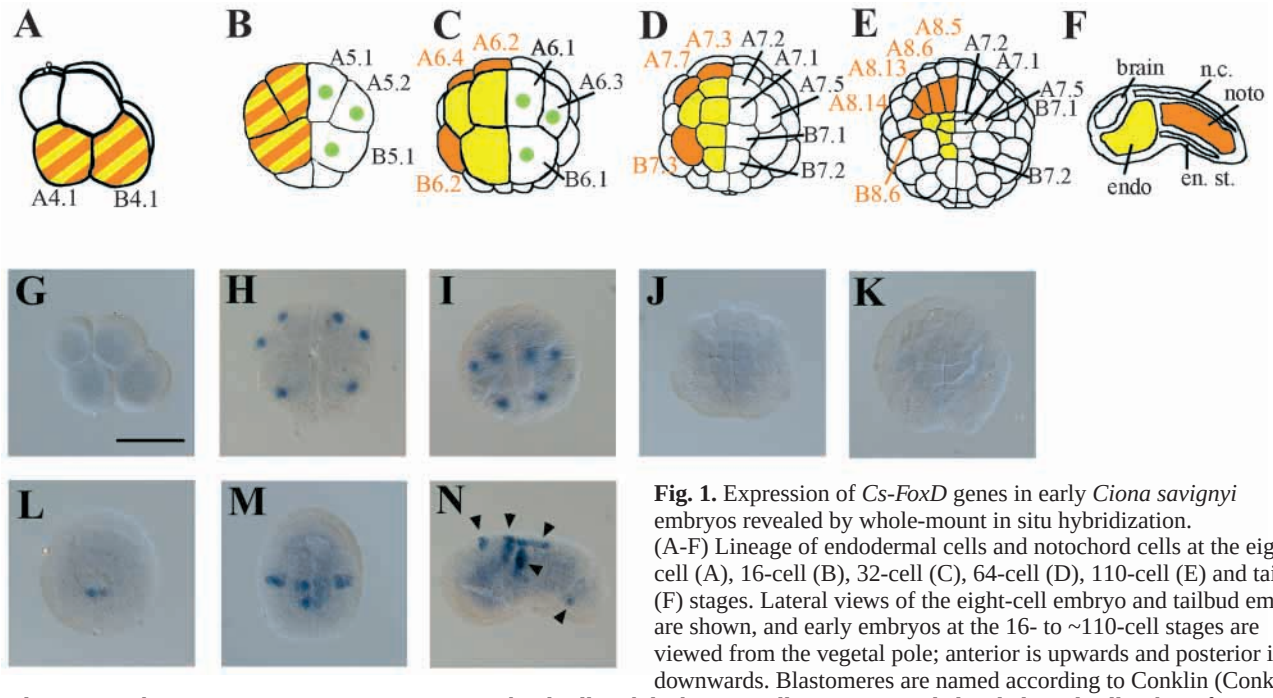

Fig. 1. Expression of *Cs-FoxD* genes in early *Ciona savignyi* embryos revealed by whole-mount in situ hybridization. (A-F) Lineage of endodermal cells and notochord cells at the eight-cell (A), 16-cell (B), 32-cell (C), 64-cell (D), 110-cell (E) and tailbud (F) stages. Lateral views of the eight-cell embryo and tailbud embryo are shown, and early embryos at the 16- to ~110-cell stages are viewed from the vegetal pole; anterior is upwards and posterior is downwards. Blastomeres are named according to Conklin (Conklin, 1905). Blastomeres shown in orange are presumptive notochord cells, while those in yellow are primordial endodermal cells whose fates are restricted to the endoderm. Light green dots in B and C show the expression of *Cs-FoxD* in endodermal cells. (G-N) Expression of *Cs-FoxD* at the eight-cell (G), 16-cell (H), 32-cell (I), 64-cell (J), 110-cell (K), gastrula (L), neurula (M) and tailbud (N) stages. Arrowheads in N indicate the five expression domains. In ascidians, in situ signals for zygotic gene expression are first detected in the nuclei of embryonic cells. Scale bar: 100 μm.

increased differentiation of epidermal cells occurs (Imai et al., 2000). We conducted subtractive hybridization screens of mRNAs between β-catenin-overexpressing embryos and cadherin-overexpressing embryos to identify potential β-catenin target genes in *Ciona savignyi* embryos (Satou et al., 2001a). We describe one β-catenin target gene belonging to the winged-helix/forkhead class of transcription factors (this class of transcription factors was recently renamed the Fox proteins, for Forkhead box) (Kaufmann and Knöchel, 1996; Kaestner et al., 2000). The transient expression of this gene is essential for notochord specification but not endodermal cell differentiation itself in the ascidian embryo.

MATERIALS AND METHODS

Ascidian embryos

Adults of *Ciona savignyi* were collected near the Otsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo, Iwate and the Maizuru Fisheries Research Station of Kyoto University, Maizuru, Japan. Adults were 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 of cDNA clones for *Ciona FoxD* gene

The procedures for isolation of cDNA clones for strongly induced β-catenin target genes are described elsewhere (Satou et al., 2001a). Briefly, β-catenin-overexpressing embryos and cadherin-overexpressing embryos were prepared by microinjection of synthetic mRNAs as described previously (Imai et al., 2000). Total RNA was isolated from 120 of the former type of embryos and 129 of the latter type of embryos, both at the 110-cell stage, and cDNAs were synthesized from 0.3 μg of each type 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 (Satou et al., 2001a). The cDNA fragments amplified by PCR after three subtraction cycles were inserted into pGEM-T vector (Promega). The average length of the cDNA fragments obtained by PCR was about 300 to 400 bp. First, 300-400 bp of either end of the cDNAs were completely sequenced, and the nucleotide sequence information was used to check the independence of clones and sequence similarity to reported genes.

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

Microinjection of fusion gene constructs and histochemical detection of β-galactosidase (β-gal) activity

Genomic DNA was isolated from the gonad of *Ciona savignyi* and was completely digested with *Eco*RI or *Mun*I, and then cloned into lambdaZAPII (Stratagene). By screening the libraries with *Cs-FoxD* cDNA probe, we isolated a genomic clone that covers the 3.6 kb upstream region of *Cs-FoxD*.

The entire upstream region was amplified with KOD DNA polymerase (TOYOBO) and ligated to the *lacZ* gene cloned into pSP1.72 (Promega) in frame to produce the –3.6 kb construct. The amplified nucleotide sequence was confirmed by sequencing. The –2.3 kb, –2.0 kb, –1.6 kb and –0.66 kb constructs were made by cleavage of the –3.6 kb construct at internal restriction sites (*Sal*I, *Pst*I, *Xba*I and *Hin*dIII, respectively). The constructs harboring the 1138, 1119, 1051 and 983 bp upstream regions were made from the –1.6 kb

construct by the standard deletion method using exonuclease. The mutation constructs were made from the –1138 bp construct using a Quick Change site-directed mutagenesis kit (Stratagene). The mutations introduced consisted of transversions (A↔C, T↔G). The nucleotide sequences of the resultant constructs were confirmed by sequencing.

Before injection, the constructs were linearized by digestion with *Sca*I. The constructs were microinjected into fertilized *Ciona savignyi* eggs, and the eggs were allowed to develop to early gastrula stage and then fixed for β-gal staining. The details of the methods for injection and histochemical detection of β-gal activity have been described before (Satou and Satoh, 1996)

The relative β-gal activity was calculated as follows. First, the number of blastomeres with *lacZ* expression was counted in control or experimental embryos (the number of embryos examined is shown in parentheses in Fig. 4), and the average number was calculated. Second, the average number in the control embryos was represented as 1.0, and the ratio of blastomeres with expression in experimental embryos/blastomeres with expression in control embryos was calculated.

Isolation of cDNA clones for *Ciona Notch* gene and construction of dominant-active form of Cs-Notch

We isolated a PCR fragment of *Cs-Notch* using the following degenerate primers: 5′-TGGGCIGCIGCIGTIAAYAA-3′ and 5′-TCC-ATRTGRTCIGTIAYITC-3′. Using the obtained fragment, a cDNA clone with a 2.7 kb insert was isolated from the fertilized egg cDNA library. The cDNA (5.4 kb), containing four EGF repeats, three DSL domains, a transmembrane domain and six ankyrin repeats, was amplified by two successive 5′ RACE reactions using a SMART RACE cDNA Amplification Kit (Clontech).

To make a synthetic mRNA for a constitutively active form of Cs-Notch protein, the region encoding the transmembrane domain and intracellular domain was cloned into pBS-RN3 vector. Because the cloned cDNA did not have the sequence encoding the N terminus of Cs-Notch, a fragment encoding the initiator methionine and the signal peptide of Ci-cadherin was inserted into 5′-end of the constitutively active Cs-Notch construct.

Whole-mount in situ hybridization and histochemical staining for alkaline phosphatase (AP)

In situ hybridization was carried out using standard protocols (Satou et al., 1995). Differentiation of endodermal cells was monitored by two methods: histochemical detection of AP activity (Whittaker and Meedel, 1989) and in situ hybridization with a probe for an endoderm-specific thyroid hormone receptor gene, *Cs-THR*, of *C. savignyi*. The *Cs-THR* gene (GenBank Accession Number, AB057767) is expressed in endodermal cells of embryos at the neurula and later stages. Differentiation of epidermal cells was monitored with a probe for an epidermis-specific gene, *Cs-Epi1* (Chiba et al., 1998), and that of muscle cells with a probe for a muscle actin gene, *Cs-MA* (Chiba et al., 1998). Differentiation of mesenchyme cells was monitored with a probe for a mesenchyme-specific gene, *Cs-mech1*, whose expression begins at the tailbud stage (Imai et al., 2002a; Imai et al., 2002b), and that of neuronal cells with a probe for a nervous system-specific gene, *Cs-ETR* (Imai et al., 2002a). Notochord differentiation was assessed with probes for *C. savignyi Brachyury* gene (*Cs-Bra*) (Imai et al., 2000; Imai et al., 2002b) and a notochord-specific fibrinogen-like gene, *Cs-fibrn* (Imai et al., 2002a). Control embryos that were hybridized with sense probes did not show signals above background.

Morpholino oligo and synthetic capped mRNAs

In the present study, we used a 25 mer morpholino oligo (Gene Tools LLC) for *Cs-FoxD*. The nucleotide sequence of the *Cs-FoxD* morpholino is shown in Fig. 2A. For rescue experiments, synthetic capped mRNA for *Cs-FoxD* was synthesized from *Cs-FoxD* cDNA cloned into pBluescript RN3 vector (Lemaire et al., 1995) using a Megascript T3 kit (Ambion, Austin, TX, USA). To obtain 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. The synthetic *Cs-FoxD* mRNA was designed to lack the morpholino sequence, and therefore the *Cs-FoxD* morpholino does not bind the synthetic mRNA.

The isolation and characterization of *Cs-FGF4/6/9* (GenBank Accession Number, AB057767) were reported in Imai et al. (Imai et al., 2002a). Synthetic capped mRNA for *Cs-FGF4/6/9* was synthesized as described above.

After insemination, fertilized eggs, usually with intact chorion, were microinjected with 15 fmole of morpholino and/or synthetic capped mRNA (Satou et al., 2001b). For each microinjection, 30 pl of solution was injected using a micromanipulator (Narishige Science Instruments Laboratories, Tokyo, Japan) as described (Imai et al., 2000). Injected eggs were reared at about 18°C in MFSW containing 50 μg/ml streptomycin sulfate. Cleavage of some embryos was arrested at the 110-cell stage with cytochalasin B and the embryos were then cultured for about 12 hours as control embryos that reached the early tailbud stage.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was carried out as described (Imai et al., 2000; Imai et al., 2002b). RNA extracted from 20 normal tailbud embryos and 20 embryos at the same stage developed from eggs injected with *Cs-FoxD* morpholino was used for cDNA synthesis. PCR was performed by using the following primers:

Cs-THR, 5′-GATGGACGTGAACCAATTG-3′ and 5′-GCGTATG-TCGTGTATTCATA-3′; and

Cs-ETR, 5′-TTCAATGCACCGTGTACATA-3′ and 5′-GGACCTC-GGGTTCTAGC-3′.

Each reaction (for *Cs-THR* or *Cs-ETR*) was performed for 23 cycles, with each cycle consisting of incubation using [α-^{32}P]dCTP at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The reaction products were resolved by 5% PAGE and subjected to autoradiography.

RESULTS

Isolation and characterization of β-catenin-downstream *Fox* genes

Subtractive hybridization screening of mRNAs from β-catenin-overexpressing embryos versus cadherin-overexpressing embryos in *C. savignyi* yielded two β-catenin-downstream genes belonging to the winged-helix/forkhead class of transcription factors or Fox proteins (Fig. 2). The first cDNA clone consisted of 1949 nucleotides, which encoded a predicted polypeptide of 579 amino acids, and represented a gene identical to the previously characterized *forkhead/HNF-3β* (*Cs-FoxA5*) (Shimauchi et al., 2001a) (Fig. 2B,C). The second cDNA was 1915 base pairs in length and encoded a novel Fox protein of 506 amino acid residues (Fig. 2A) (DDBJ/GenBank/EMBL database Accession Number, AB057738). Comparative analyses of the amino acid sequences of the forkhead domain suggested that the second gene is a FoxD class member (Fig. 2B,C), and we therefore named it *Cs-FoxD*.

Cs-FoxD is transiently expressed in endodermal cells at the 16- and 32-cell stages

Expression of *Cs-FoxD* began at the 16-cell stage, and the in situ hybridization signal was evident in A5.1, A5.2 and B5.1,

which have the developmental potential to give rise to endoderm, notochord and other tissues (Fig. 1H, also see 1B). During the next cleavage to form the 32-cell stage embryo, the expression was inherited by only one of each of the daughter cells, namely A6.1, A6.3 and B6.1 (Fig. 1I, also see 1C). A6.1 and B6.1 are destined to give rise to endoderm only, while A6.3 has the potential to form endoderm and trunk lateral cells (a type of mesenchyme cell). This early embryonic expression of *Cs-FoxD* was transient: the in situ signal was not detected by the 64-cell stage (Fig. 1J,K).

Later during embryogenesis, *Cs-FoxD* was expressed again in cells that included cells in the nervous system (Fig. 1L-N). This second phase of expression was detected in a pair of cells in the posterior neural plate (Fig. 1L). In the neurula, the expression was seen in several pairs of cells, and in the tailbud embryo, the transcript was detected in five domains (arrowheads in Fig. 1N), including anterior nerve cord cells (Fig. 1N).

The early embryonic expression of *Cs-FoxD* is directly controlled by nuclear accumulation of β-catenin

If the early transient expression of *Cs-FoxD* is triggered by β-catenin nuclear localization, the expression of *Cs-FoxD* should be upregulated in embryos injected with in vitro synthesized β-catenin mRNA and downregulated in embryos injected with in vitro synthesized cadherin mRNA. As shown in Fig. 3A, injection of β-catenin mRNA into fertilized eggs resulted in an ectopic expression of *Cs-FoxD* in all of the blastomeres in 32-cell stage embryos (eight out of nine, the number of embryos with ectopic *Cs-FoxD* expression/the number of embryos examined). By contrast, injection of cadherin mRNA into fertilized eggs resulted in no expression of *Cs-FoxD* in 32-cell stage embryos (0/9; Fig. 3B). The embryos in which the expression of *Cs-FoxD* was completely suppressed (Fig. 3B) did not form notochords, presumably

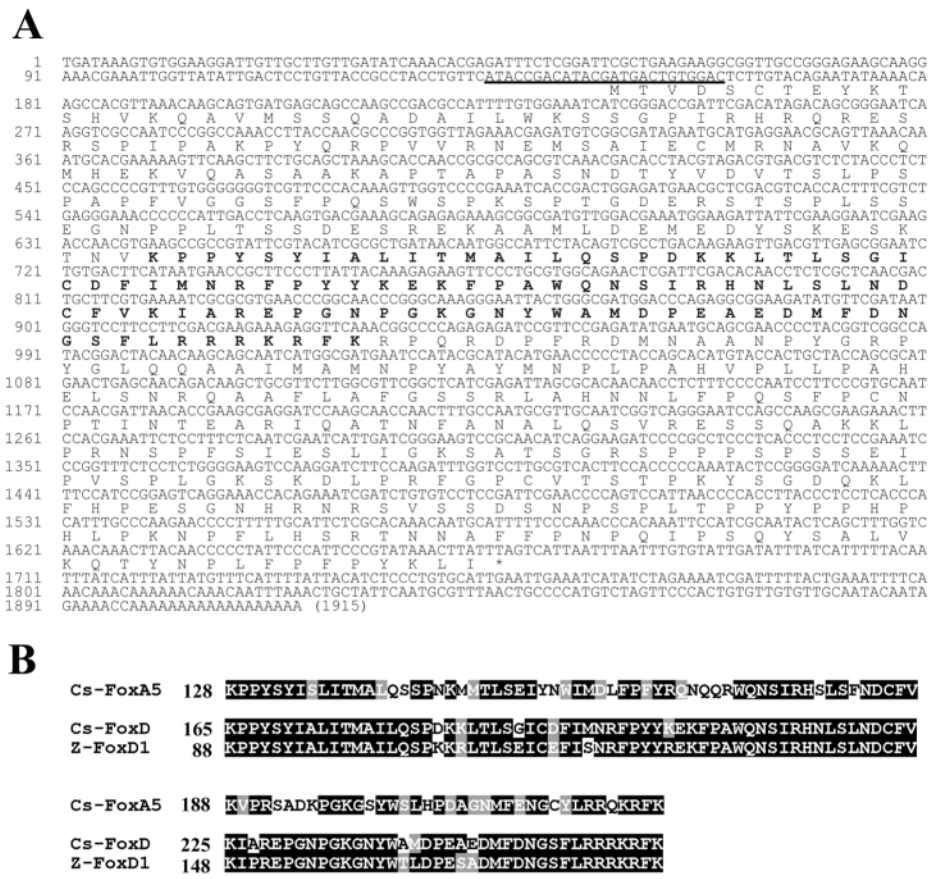

Fig. 2. Gene product of *Cs-FoxD*. (A) Nucleotide and deduced amino acid sequence of *Cs-FoxD* clone. The 1915 bp insert includes a single open reading frame that encodes a polypeptide of 506 amino acids. The termination codon is shown by an asterisk. The predicted forkhead domain is shown by bold letters. The nucleotide sequence of the 5′ region used to prepare morpholino oligonucleotides is underlined. (B) Comparison of amino acid sequences of the forkhead domains of Cs-FoxA5, Cs-FoxD and zebrafish z-FoxD1. (C) Molecular phylogenetic analysis of relationships of *Cs-FoxD* with Fox gene members. The tree was constructed by comparison of the amino acid sequences of the forkhead domains using the neighbor-joining method. Human FoxC1 and FoxC2 were used as the outgroup. Branch lengths are proportional to the evolutionary distance corrected for multiple substitutions with the scale denoting 0.01 amino acid substitutions per site. The numbers indicate the relative robustness of each node as assessed by bootstrap analysis (100 replications). This analysis strongly suggests that Cs-FoxD is a member of the FoxD subclass.

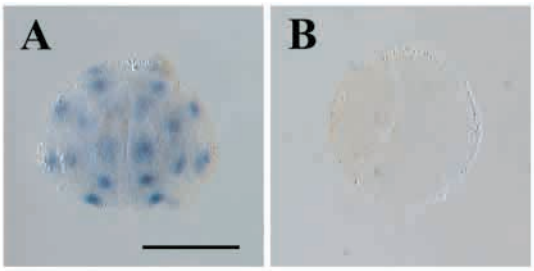

Fig. 3. Expression of *Cs-FoxD* gene in β-catenin-overexpressing embryos (A) and in cadherin-overexpressing embryos (B) at the 110-cell stage. Vegetal pole view; anterior is upwards and posterior is downwards. Scale bar: 100 μm.

owing to the lack of endodermal cell differentiation (data not shown) (Imai et al., 2000).

β-catenin acts together with Tcf/LEF as a transcription activator (reviewed by Miller and Moon, 1996; Willert and Nusse, 1998). To determine whether nuclear localization of β-catenin directly controls the initiation of *Cs-FoxD* expression, we carried out the four types of experiments, described below, and all of the findings strongly supported that the initiation of *Cs-FoxD* expression is directly controlled by nuclear accumulation of β-catenin via Tcf-binding elements. First, we examined the sequence of about 3.6 kb of the 5′ flanking region of *Cs-FoxD*, and found that there are three Tcf-binding elements, TBE1, TBE2 and TBE3 [(C/G)TTTG(A/T)(A/T)], between nucleotide positions –1202 and –963 upstream from the transcription start site (Fig. 4A).

Second, we injected various deletion constructs of p(–3.6kb)CsFoxD/*lacZ* into fertilized eggs and examined the activity of the reporter gene expression or *lacZ* protein expression at the early gastrula stage. Various deletion constructs between –3600 and –1138 showed the same level of *lacZ* expression (data not shown). Therefore, we used p(–1138)Cs-FoxD/*lacZ*, which contained all three TBEs, as a control, and compared its expression level with those of three further deleted and mutated constructs. The first deletion construct, p(–1119)Cs-FoxD/*lacZ*, which lacked TBE1, and the second deletion construct, p(–1051)Cs-FoxD/*lacZ*, which lacked TBE1 and TBE2, showed about 60% and 80% reduction in the number of cells with the reporter gene expression as compared with the control (Fig. 4B). In addition, the third deletion construct, p(–983)Cs-FoxD/*lacZ*, which lacked all three TBEs, did not show detectable reporter gene expression (Fig. 4B).

Third, when p(–1138)Cs-FoxD/*lacZ* was co-injected with β-catenin mRNA, the number of cells with *lacZ* expression increased more than nine times compared with that in embryos injected with p(–1138)Cs-FoxD/*lacZ* only (second lane in Fig. 4C). However, co-injection of p(–1138)Cs-FoxD/*lacZ* with cadherin mRNA abolished *lacZ* expression completely (third lane in Fig. 4C).

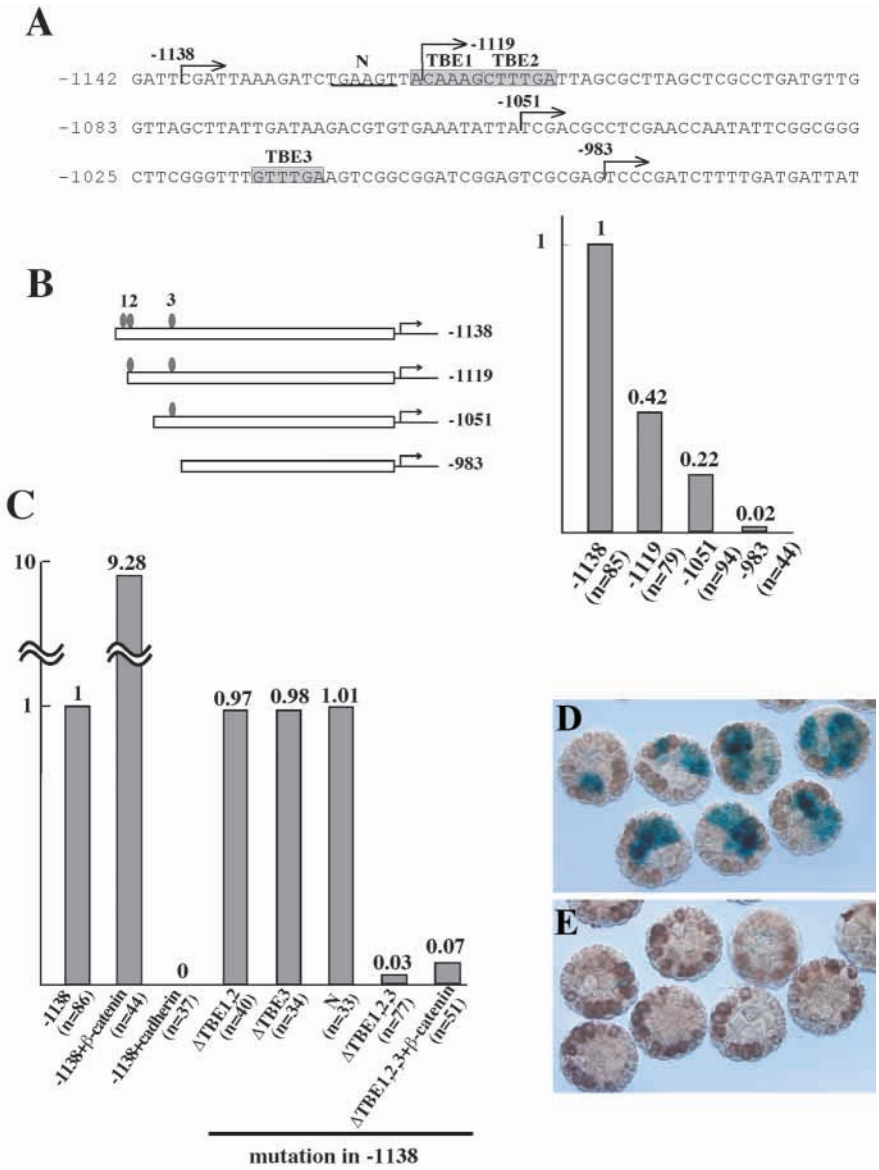

Fig. 4. (A) Sequence of the 5′ flanking region between nucleotide positions –1142 and –963 of *Cs-FoxD*, which contains three Tcf-binding motifs [TBE1-3: CTTTG(A/T)(A/T)]. (B) Relative levels of *lacZ* expression when various deletion constructs were injected. Deletion constructs are shown on the left and relative ratios of *lacZ* expression on the right. The relative expression level was calculated as described in the Materials and Methods. (C) Relative levels of *lacZ* expression in embryos injected with β-catenin mRNA, cadherin mRNA or various mutation constructs derived from p(–1138)Cs-FoxD/*lacZ*. Mutation N is shown in A. (D) Expression of *lacZ* when control p(–1138)Cs-FoxD/*lacZ* was injected. *lacZ* was detected in progeny cells of A5.1, A5.2 and B5.2 (endoderm, notochord and nerve cord cells, respectively) presumably due to the stability of β-gal protein. (E) Expression of *lacZ* was not detected when p(–1138/del TBE1/2/3)Cs-FoxD/*lacZ* was injected.

Fourth, mutation of control sequences (N region of Fig. 4A) resulted in the same level of reporter gene expression as intact p(–1138)Cs-FoxD/*lacZ* (Fig. 4C,D). Mutation of either TBE1, TBE2 or TBE3 also resulted in comparable reporter gene expression. However, mutations in all three TBEs resulted in a lack of detectable reporter gene expression (Fig. 4C,E). Co-injection of β-catenin mRNA along with the construct with mutations in all three TBEs did not rescue the reporter gene expression (Fig. 4C).

Cs-FoxD is not involved in the differentiation of the endoderm itself

The transient expression of *Cs-FoxD* occurs primarily in endoderm precursor cells, suggesting a pivotal role of this gene in endodermal cell differentiation. We examined the role of *Cs-FoxD* in the differentiation of endoderm and of other tissues and organs by suppressing *Cs-FoxD* gene function with a specific morpholino oligo. Recently, we found that morpholinos specifically inhibit *Ciona* gene function and that their functional specificity can be ascertained by rescue experiments with in vitro synthesized mRNAs that lack the target sequences of the morpholinos (Satou et al., 2001a; Satou et al., 2001b). As a control, we injected morpholino against *lacZ*, and this injection had no effect on *C. savignyi* embryogenesis (data not shown) (Satou et al., 2001b).

Fertilized eggs injected with *Cs-FoxD* morpholino cleaved normally, and gastrulation took place as in normal embryos, but formation of tailbud embryos was usually affected, resulting in disordered tailbud embryos (Figs 5, 6). Unexpectedly, however, suppression of *Cs-FoxD* function did not affect endodermal cell differentiation (Table 1; Fig. 5). *Cs-FoxD* morpholino-injected embryos showed expression of endodermal markers, i.e. alkaline phosphatase (Cs-AP) (Fig. 5A′,B′) and a gene for thyroid hormone receptor (*Cs-THR*) (Fig. 5C′,D′), comparable with that of normal control embryos (Fig. 5A,B for Cs-AP; Fig. 5C,D for *Cs-THR*).

We also examined the differentiation of epidermis, muscle and mesenchyme in embryos developed from eggs injected with *Cs-FoxD* morpholino. Differentiation of these cell-types was assessed by monitoring the expression of an epidermis-specific gene, *Cs-Epi1* (Fig. 6A), a muscle-specific actin gene, *Cs-MA1* (Fig. 6B), and a mesenchyme-specific gene, *Cs-mech1* (Fig. 6C). As shown in Fig. 6 and Table 1, the expression of *Cs-Epi1* (Fig. 6A′), *Cs-MA1* (Fig. 6B′) and *Cs-mech1* (Fig. 6C′) was not affected by injection of *Cs-FoxD* morpholino. Therefore, *Cs-FoxD* is not likely to be involved in the differentiation of epidermis, muscle or mesenchyme.

Table 1. Effects of *Cs-FoxD* morpholino oligo on differentiation of embryonic cells

Tissue or organ	Marker	Rescue experiment	Number of embryos expressing marker/ number of embryos examined	Fig.
Endoderm	Cs-AP		14/14 (100%)	5B′
	Cs-THR		18/18 (100%)	5D′
Epidermis	*Cs-Epi1*		18/18 (100%)	6A′
Muscle	*Cs-MA1*		17/17 (100%)	6B′
Mesenchyme	*Cs-mech1*		20/20 (100%)	6C′
Nervous system	*Cs-ETR*		11/12 (92%)	6D′
Notochord	*Cs-Bra*		0/19 (0%)	7B
		+*Cs-FoxD* mRNA	20/23 (87%)	7C
	Cs-fibrn		2/41 (5%)	7E
		+*Cs-FoxD* mRNA	19/19 (100%)	7F

An essential role of *Cs-FoxD* in differentiation of the notochord

In the ascidian embryo, the induction of notochord cells by signal(s) from endodermal cells takes place at the 32-cell stage (Nakatani and Nishida, 1994), and immediately after the induction or at the 64-cell stage, the primordial notochord cells begin to express the *Brachyury* gene (Yasuo and Satoh, 1993;

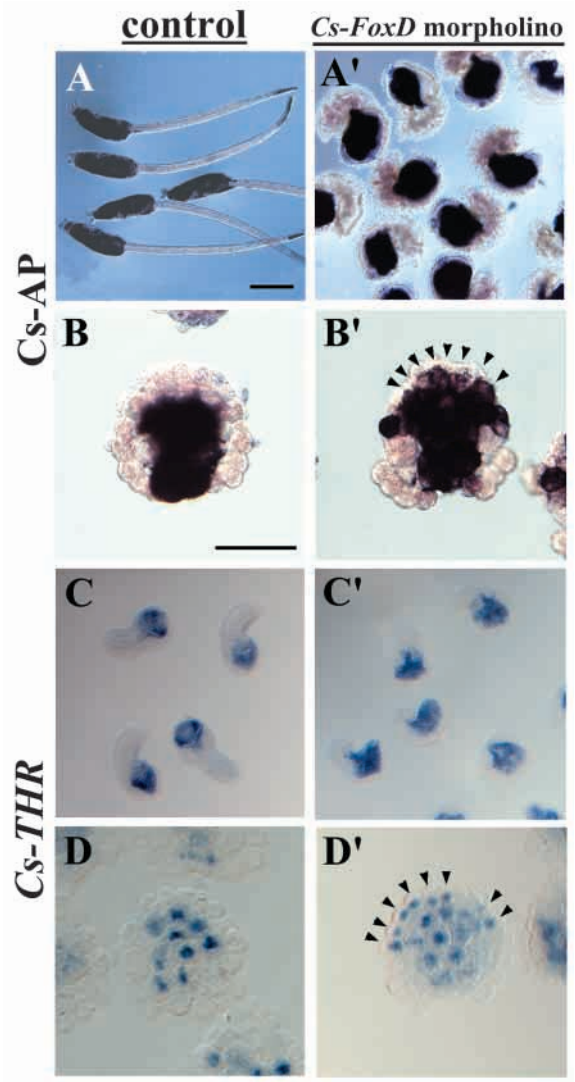

Fig. 5. Effects of suppression of *Cs-FoxD* function with specific morpholino on the differentiation of endoderm. (A,B) Histochemical detection of endoderm-specific alkaline phosphatase (Cs-AP) activity. (C,D) In situ hybridization examining expression of an endoderm-specific gene for thyroid hormone-receptor (*Cs-THR*). (A-D) Normal embryos; (B,D) control embryos arrested at the 110-cell stage. (A′,B′,C′,D′) Injection of *Cs-FoxD* morpholino into fertilized eggs: (A′,C′) injected embryos at the tailbud stage and (B′,D′) injected embryos arrested at the 110-cell stage. Arrowheads in D′ indicate the expression of Cs-THR in presumptive notochord cells in addition to endoderm cells. Scale bars: in A, 100 μm for A′,C,C′; in B, 100 μm for B′,D,D′.

Corbo et al., 1997), which encodes a T-box transcription factor. As mentioned above, *Cs-FoxD* is expressed transiently in endodermal cells, but suppression of its function does not block differentiation of endodermal cells themselves. However, it is possible that this gene functions in the induction and subsequent differentiation of notochord cells. The effects of *Cs-FoxD* morpholino on notochord induction were examined using two markers, expression of *Brachyury* (*Cs-Bra*) (Fig. 7A,B) and expression of a notochord-specific fibrinogen-like gene (*Cs-fibrn*) (Fig. 7G,H).

As shown in Fig. 7B and Table 1, injection of *Cs-FoxD* morpholino into fertilized eggs resulted in failure of the expression of *Cs-Bra*. In addition, suppression of the function of *Cs-FoxD* in the right AB2 cell of the two-cell stage embryo resulted in the failure of *Cs-Bra* expression in the right A7.3, A7.4, A7.7, A7.8 (white arrowheads in Fig. 7D) and B7.6 (white arrow in Fig. 7D). The suppression of the function of *Cs-FoxD* in one of the blasomeres of the four-cell stage embryo resulted in the failure of *Cs-Bra* expression in notochord precursors derived from the *Cs-FoxD*-suppressed blastomere (white arrow in Fig. 7E and white arrowheads in Fig. 7F). Expression of *Cs-fibrn* was also suppressed in embryos developed from eggs injected with *Cs-FoxD* morpholino (Fig. 7H; Table 1).

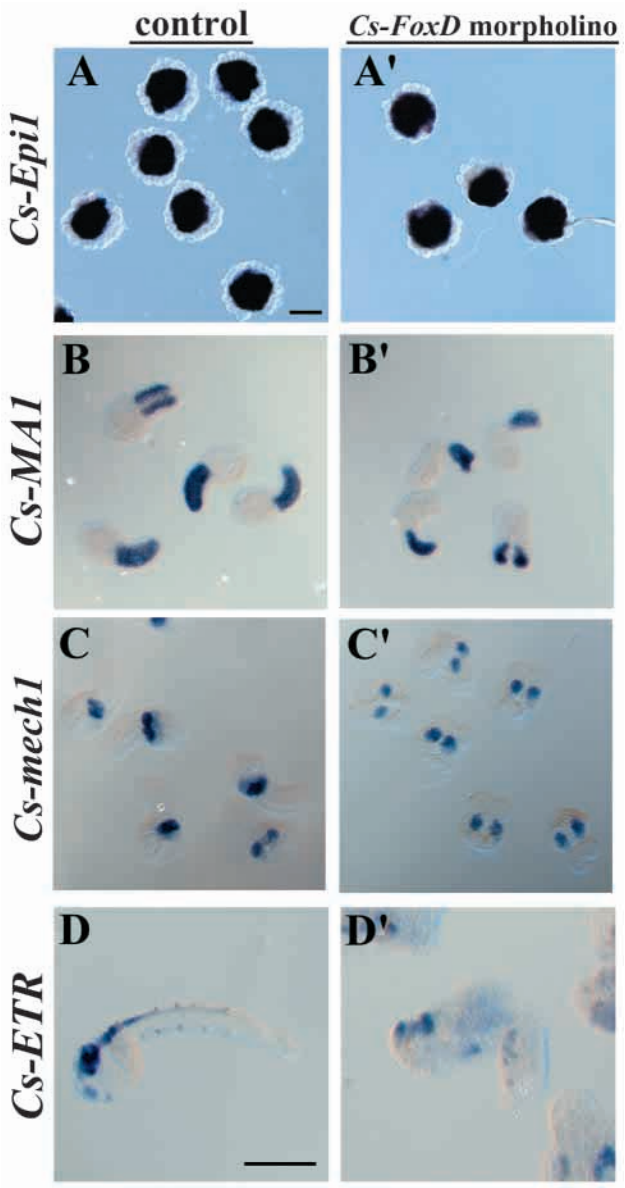

Fig. 6. Effects of suppression of *Cs-FoxD* function with morpholino on differentiation of (A,A′) epidermal cells, (B,B′) muscle cells, (C,C′) mesenchyme cells and (D,D′) the nervous system. In situ hybridization with probes for (A) epidermis-specific gene *Cs-Epi1*, (B) muscle-specific actin gene *Cs-MA*, (C) mesenchyme-specific gene *Cs-mech1* or (D) nervous system-specific gene *Cs-ETR*. (A-D) Control embryos and (A′-D′) embryos injected with *Cs-FoxD* morpholino. Scale bars: in A, 100 μm for A,A′; in D, 100 μm for B-D′.

To examine whether the suppression is specifically caused by downregulation of *Cs-FoxD* function, we attempted rescue experiments. mRNA for *Cs-FoxD* lacking the *Cs-FoxD* morpholino recognition sequences was synthesized in vitro and injected into fertilized eggs together with *Cs-FoxD* morpholino. This co-injection rescued the expression of *Cs-Bra* (Fig. 7C; Table 1) and *Cs-fibrn* (Fig. 7I; Table 1) in the resultant embryos, although the morphology of the embryos were not recovered possibly because the effect of *Cs-FoxD* mRNA overexpression. All of these experimental results strongly suggest that *Cs-FoxD* is essential for the differentiation of notochord cells. It is also likely that the *Cs-FoxD*-downstream inductive signal does not act over a long range.

The presumptive notochord cells in *Cs-FoxD*-suppressed embryos were likely to have changed their fate into that of endodermal cells. As shown in Fig. 5B′,D′, in *Cs-FoxD*-suppressed embryos, the expression of endodermal markers Cs-AP and *Cs-THR* was detected not only in the endodermal cells but also in presumptive notochord cells (arrowheads); this was confirmed in 22 out of 30 embryos examined for Cs-AP and in all 22 embryos examined for *Cs-THR*. To confirm this result further, we performed semi-quantitative RT-PCR analysis. As is evident in Fig. 8, the quantity of *Cs-THR* transcripts in *Cs-FoxD* morpholino-injected embryos was higher than that in control embryos.

We also examined the effects of overexpression of *Cs-FoxD* by injection of its synthetic *Cs-FoxD* mRNA into fertilized eggs, and found that this injection did not affect notochord formation; that is, notochord cells were formed in the experimental embryos as in normal embryos (data not shown). However, we noticed that the epidermis failed to differentiate in *Cs-FoxD* mRNA-injected embryos (data not shown).

Notochord cells of the ascidian embryo are involved, either directly or indirectly, in pattern formation in the nervous system, and therefore inhibition of notochord formation by suppression of *Cs-FoxD* function may affect the nervous system formation. We examined this question with a marker gene, *Cs-ETR* (Fig. 6D). Injection of *Cs-FoxD* morpholino did not diminish the expression of *Cs-ETR* (Fig. 6D'), but the quantity of *Cs-ETR* transcripts in *Cs-FoxD* morpholino-injected embryos decreased when compared with that in the control (Fig. 8).

Notch as a possible *Cs-FoxD*-downstream signal transduction pathway

Two signal transduction pathways have been suggested for notochord induction in ascidian embryos: a Notch-related

pathway in *Ciona intestinalis* embryos (Corbo et al., 1998) and a bFGF-related pathway in *Halocynthia roretzi* embryos (Nakatani et al., 1996; Shimauchi et al., 2001b). We examined the possible involvement of these signal pathways downstream of *Cs-FoxD*.

To examine the Notch signal pathway, we characterized a cDNA for *Cs-Notch*. As shown in Fig. 9A, although the cDNA lacks the 5′ region encoding the N-terminal half of Cs-Notch, it contains sequences encoding four EGF repeats, three DSL domains, the transmembrane domain and six ankyrin repeats. As in the case of *Hr-Notch* (*Halocynthia* homolog of *Notch*) (Hori et al., 1997), *Cs-Notch* is expressed maternally and zygotically. The maternal expression of *Cs-Notch* is evident from the fact that we isolated the *Cs-Notch* clone from a cDNA library of *C. savignyi* fertilized eggs. Whole-mount in situ hybridization with a *Cs-Notch* probe showed that the maternal transcript is distributed evenly in fertilized eggs and early embryos (Fig. 9B-D). Zygotic *Cs-Notch* expression begins at the neurula stage, and the *Cs-Notch* transcript is evident in the cells of the central nervous system (Fig. 9E,F).

A construct that lacks the extracellular domain of Notch acts as constitutively active form (Kopan et al., 1996). We made a constitutively active form (ΔE-Notch) of Cs-Notch (Fig. 9A), and examined whether the overexpression of ΔE-Notch was able to rescue the notochord development in *Cs-FoxD*-suppressed embryos. As shown in Fig. 10C, injection of ΔE-Notch mRNA into fertilized eggs resulted in the recovery of *Cs-fibrn* gene expression in B-line notochord cells of *Cs-FoxD*-suppressed embryos, but none of the A-line notochord cells expressed *Cs-fibrn*. In addition, the experimental embryos showed ectopic expression of *Cs-fibrn* in B-line endodermal cells and mesenchyme cells (Fig. 10C).

We also examined the effects of injection of the ΔE-Notch construct into intact fertilized eggs, and found that all 40 experimental embryos showed ectopic expression of *Cs-fibrn* in B-line endodermal cells and mesenchyme cells (Fig. 10B). In addition, eleven out of the 40 experimental embryos showed ectopic *Cs-fibrn* expression in A-line endodermal cells as well (data not shown). Therefore, it is likely that there is a difference in the genetic cascade for the induction of notochord between the A- and B-lines, even though *Cs-FoxD* functions in the notochord induction in both lines.

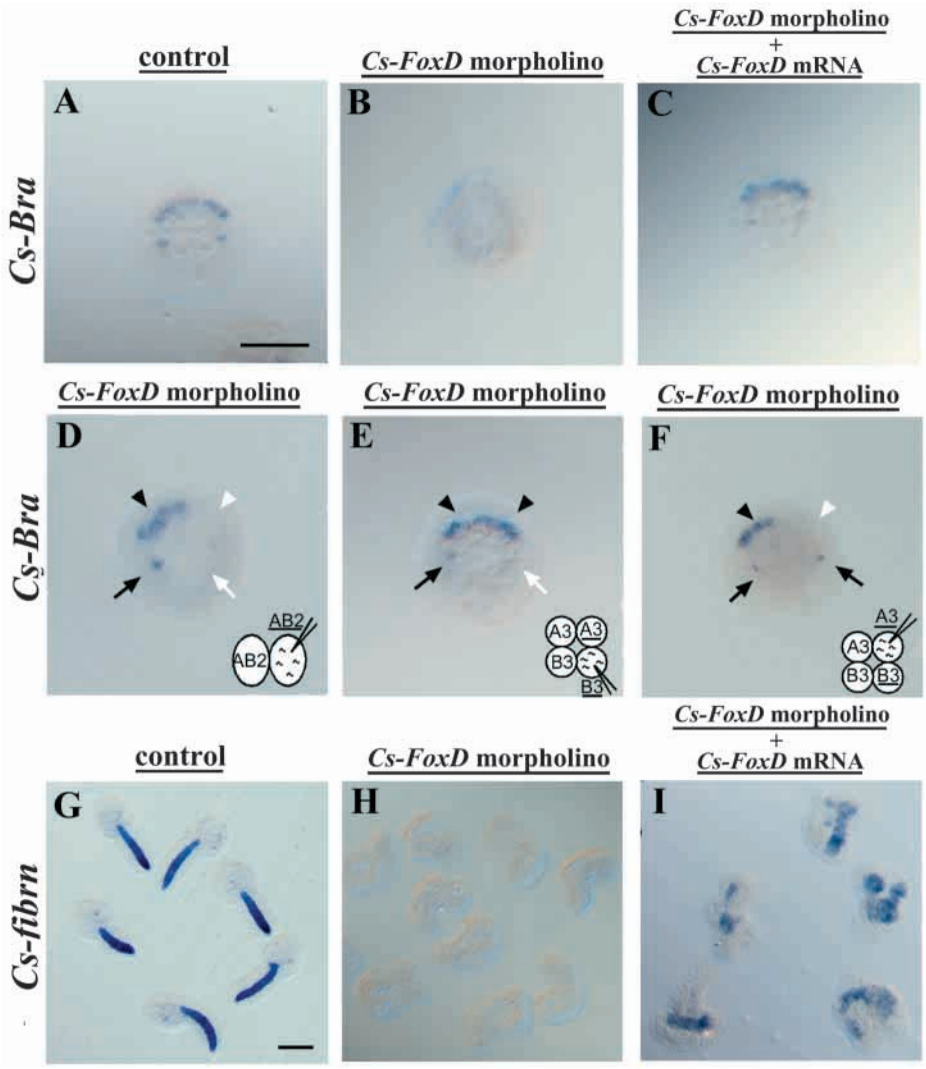

Fig. 7. Effects of suppression of *Cs-FoxD* function with morpholino on induction of notochord cells. (A-F) In situ hybridization to examine *Cs-Bra* expression. (A) Normal 110-cell embryo, (B) *Cs-FoxD* morpholino-injected embryo and (C) an embryo injected with both *Cs-FoxD* morpholino and synthetic *Cs-FoxD* mRNA. (D) A 110-cell stage embryo developed from two-cell embryo whose right AB2 was injected with *Cs-FoxD* morpholino; (E) 110-cell stage embryo developed from four-cell embryo whose right B3 was injected with *Cs-FoxD* morpholino, and (F) 110-cell stage embryo developed from four-cell embryo whose right A3 was injected with *Cs-FoxD* morpholino. Black arrowheads indicate Cs-Bra expression in A-line notochord cells. Black arrows indicate Cs-Bra expression in B-line notochord cells. White arrowheads and white arrows indicate the failure of Cs-Bra expression. (G-I) Expression of a notochord-specific gene, *Cs-fibrn*. (G) Normal tailbud embryo; (H) *Cs-FoxD* morpholino-injected embryo, and (I) an embryo injected with both *Cs-FoxD* morpholino and synthetic *Cs-FoxD* mRNA. Scale bars: in A, 100 μm for B,C,D,E,F; in G, 100 μm for H,I.

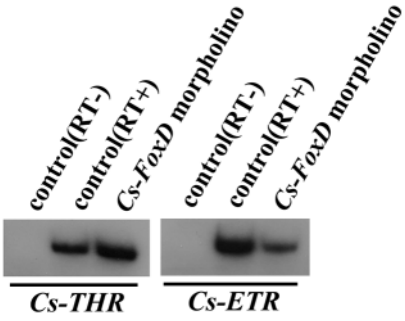

Fig. 8. Semi-quantitative RT-PCR analysis of the amounts of *Cs-THR* and *Cs-ETR* mRNAs in control embryos and embryos injected with *Cs-FoxD* morpholino.

FGF as a possible *Cs-FoxD*-downstream signal transduction pathway

Recently we found that *Ciona* contains at least four members of the FGF family, FGF3/7/10, FGF4/6/9/20, FGF8/17/18 and FGF11/12/13/14 (K. S. I., N. S. and Y. S., unpublished). Among these FGF family genes, *Cs-FGF4/6/9* encodes an FGF protein with features of both FGF4/6 and FGF9/20 of vertebrates, and the gene is expressed in endodermal cells and notochord-lineage cells around the notochord induction stage (Imai et al., 2002a). When *Cs-FGF4/6/9* function was suppressed with morpholino, the differentiation of mesenchyme cells was completely blocked (Imai et al., 2002a). However, the role of *Cs-FGF4/6/9* in the induction of notochord cells is only partial; the initiation of notochord-specific gene expression was inhibited by *Cs-FGF4/6/9* morpholino, but notochord-specific genes were expressed later, resulting in formation of a partial notochord.

The previous study showed that injection of *Cs-FGF4/6/9* morpholino into fertilized eggs did not affect *Cs-FoxD* expression (Imai et al., 2002a). Vice versa, injection of *Cs-FoxD* morpholino did not affect *Cs-FGF4/6/9* expression, suggesting that the expression and function of *Cs-FGF4/6/9* and *Cs-FoxD* are not interdependent (Imai et al., 2002a). In the present study, we first confirmed these experimental results (data not shown). Then, we examined whether overexpression of *Cs-FGF4/6/9* is able to rescue the notochord development in *Cs-FoxD*-suppressed embryos. As shown in Fig. 10D, injection of *Cs-FGF4/6/9* mRNA into fertilized eggs failed to rescue the *Cs-fibrn* gene expression in *Cs-FoxD*-suppressed embryos (0/22, the number of embryos with *Cs-fibrn* expression/the number of embryos examined). Therefore, it is unlikely that *Cs-FGF4/6/9* plays a role as a downstream signal of *Cs-FoxD* in notochord specification.

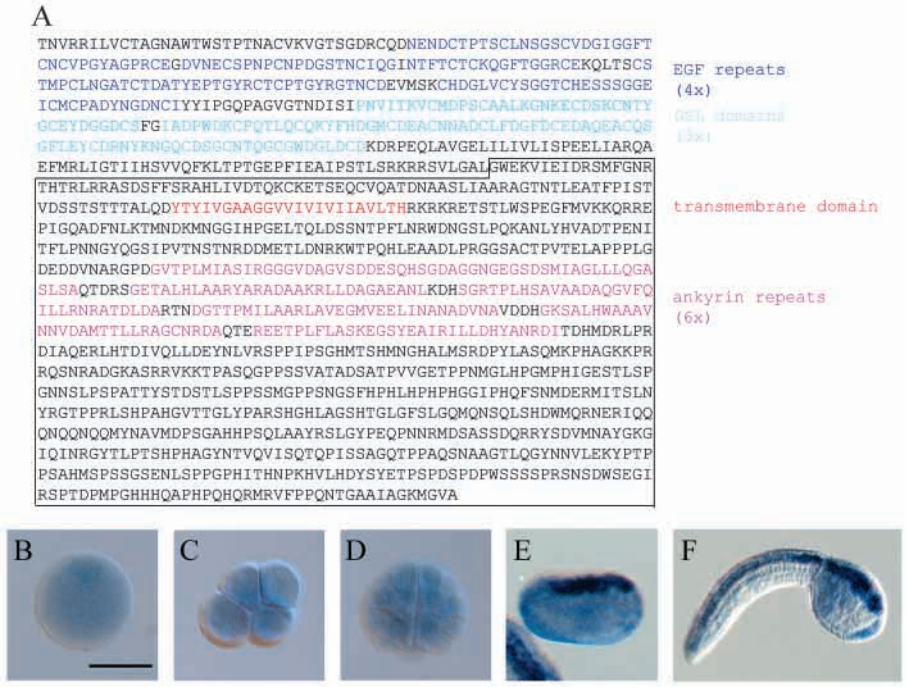

Fig. 9. Expression of *Cs-Notch* gene. (A) The partial amino acid sequence of Cs-Notch encoded by the cDNA used. Although the sequence lacks the N-terminal half, it contains four EGF repeats, three DSL domains, the transmembrane domain and six ankyrin repeats. (B-F) Expression of *Cs-Notch* revealed by whole-mount in situ hybridization. (B) Fertilized egg, (C) eight-cell stage embryo, (D) 32-cell stage embryo, (E) neurula and (F) tailbud embryo. Scale bar: 100 μm.

DISCUSSION

The results of the present study indicate that (1) during early *Ciona* embryogenesis, nuclear localization of β-catenin triggers the transient expression of *Cs-FoxD* in endodermal cells at the 16- and 32-cell stages, (2) the function of *Cs-FoxD* is not associated with the differentiation of the endoderm itself but is essential for the induction and subsequent differentiation of notochord cells, and the putative *Cs-FoxD*-downstream inductive signal does not act long-range, (3) the expression and function of *Cs-FoxD* and *Cs-FGF4/6/9* are not interdependent, and (4) there is a difference between the induction processes in the A-line and B-line notochord cells, and the Notch pathway is likely to be involved in the B-line notochord induction downstream of *Cs-FoxD*.

Genetic cascade underlying *Cs-FoxD* expression and function: upstream

Unfertilized eggs of various kinds of animals are provided maternally with a considerable amount of β-catenin mRNA and protein in the cytoplasm. During early cleavages after fertilization, β-catenin, together with TCF/LEF1, enters the nucleus and activates downstream genes which include *siamois* (Brannon et al., 1997; Fan and Sokol, 1997), *twins* (Laurent et al., 1997), *Nodal-related3* (McKendry et al., 1997) and *fibronectin* (Gradl et al., 1999) in *Xenopus* embryos, and *boz/dharma* in zebrafish embryos (Fekany et al., 1999). Via the function of these induced genes, β-catenin plays pivotal roles in early developmental processes including axis determination and embryonic cell specification (reviewed by Cadigan and Nusse, 1997; Moon and Kimelman, 1998; Sokol, 1999). As in the case of vertebrate embryos, β-catenin accumulates in the nuclei of blastomeres in early *Ciona* embryos (Imai et al., 2000). However, *Ciona* β-catenin enters into the nuclei of endoderm precursor cells and triggers the activation of, for example, a LIM-homeodomain gene, *Cs-lhx3*, which is required for endodermal cell differentiation (Satou et al., 2001a).

As shown in Fig. 4A, about 1.2 kb of the 5′ flanking region of *Cs-FoxD*, which contains three Tcf-binding elements between nucleotide positions –1202 and –963 upstream from the transcription start site, is sufficient to drive specific expression of a reporter gene. Deletion of or mutation in the three Tcf-binding elements resulted in dramatic reduction of the reporter gene expression (Fig. 4B,C). Co-injection of p(–1138)Cs-FoxD/*lacZ* with β-catenin mRNA increased the

number of cells with *lacZ* expression, while co-injection of cadherin mRNA with this construct abolished *lacZ* expression completely (Fig. 4C). In addition, co-injection of p(–1138)Cs-FoxD/*lacZ* without the three Tcf-binding elements along with β-catenin mRNA resulted in a failure to express *lacZ*. All of these data strongly suggest that the initiation of *Cs-FoxD* expression is directly controlled by nuclear accumulation of β-catenin, via Tcf-binding elements, although it remains possible that other binding element(s) are also involved in the control of *Cs-FoxD* expression.

Genetic cascade underlying *Cs-FoxD* expression and function: downstream

Brachyury

In ascidians, *Brachyury* is essential for notochord cell differentiation. *Hr-Bra*, *Ci-Bra* and *Cs-Bra* are expressed exclusively in notochord cells (Yasuo and Satoh, 1993; Corbo et al., 1997; Imai et al., 2000). Ectopic expression of *Hr-Bra* (Yasuo and Satoh, 1998) or *Ci-Bra* (Takahashi et al., 1999a) promotes notochord cell differentiation of non-notochord lineage cells. Inhibition of expression of *Hr-Bra* (H. Takahashi, K. Hotta. and N. S., unpublished) or *Cs-Bra* (Satou et al., 2001b) results in failure of notochord cell differentiation. In addition, *Ci-Bra* activates more than 20 specific genes that may be associated with the structural construction and subsequent function of the notochord in *Ciona* embryos (Takahashi et al., 1999a; Di Gregorio and Levine, 1999; Hotta et al., 2000). Therefore, a key issue to explore in order to understand notochord induction is the pathway that lies between *Cs-FoxD* and *Brachyury*: what molecules are activated or repressed by transcription factor Cs-FoxD, and how do they trigger the activation of *Brachyury*?

Cs-Notch

In the above-mentioned context, the 5′ flanking sequences required for notochord-specific expression of *Ci-Bra* have been studied in detail (Corbo et al., 1997). The basal promoter of *Ci-Bra* is present in about a 600 bp 5′ flanking region of *Ci-Bra*, and consists of at least three regions: i.e. from distal to proximal to the transcription start site; a region responsible for suppression of the gene in mesodermal regions (mesenchyme and muscle) other than the notochord, a region responsible for activation of the gene in the notochord, and a region responsible for activation in other mesodermal regions (Corbo et al., 1997). The region responsible for the notochord-specific activation contains binding sites for suppressor of hairless [Su(H)], and these sites are essential for the notochord-specific expression of *Ci-Bra*, leading to the suggestion that Notch is a signal molecule (Corbo et al., 1998).

As shown in the present study, the overexpression of ΔE-Notch in normal embryos changes the fate of B-line endodermal cells into that of notochord cells (Fig. 10B). In addition, overexpression of ΔE-Notch in *Cs-FoxD* morpholino-injected embryos could rescue the B-line notochord differentiation (Fig. 10C). Therefore, it is highly likely that Notch protein is produced by translation of its maternal message, and its signal transduction pathway via binding sites for Su(H) is involved in B-line notochord induction. These results also suggest that both B-line presumptive notochord cells and endodermal cells are competent to respond to Notch signal transduction molecules, and some mechanisms may block the competence of the endodermal cells.

However, overexpression of ΔE-Notch in *Cs-FoxD* morpholino-injected embryos could not rescue the A-line notochord differentiation. This suggests that there are different induction mechanisms in the A-line and B-line notochord cells. The identity of the molecules that act downstream of *Cs-FoxD* and trigger the activation of *Cs-Bra* in A-line notochord cells will be discussed below in relation to *Cs-ZicL*.

Cs-FGF4/6/9

Regarding the role of *Cs-FGF4/6/9* in *Ciona* notochord induction, the previous study showed that its role in the notochord induction is partial, because the suppression of *Cs-FGF4/6/9* function cannot completely inhibit the notochord formation (Imai et al., 2002a). As shown in the previous and present studies, the expression of *Cs-FGF4/6/9* and *Cs-FoxD* are independent. Injection of *Cs-FGF4/6/9* mRNA into fertilized eggs did not rescue the *Cs-fibrn* gene expression in *Cs-FoxD*-suppressed embryos. Therefore, some signals other than Cs-FGF4/6/9 may be involved in the notochord induction in *Ciona* embryos. However, it remains possible that *Cs-FoxD* is required for the competence for Cs-FGF4/6/9 or other signals, because *Cs-FoxD* is expressed in notochord/endoderm precursors at 16-cell stage.

Cs-ZicL

Recently, we isolated cDNA clone for a Zic-like zinc finger

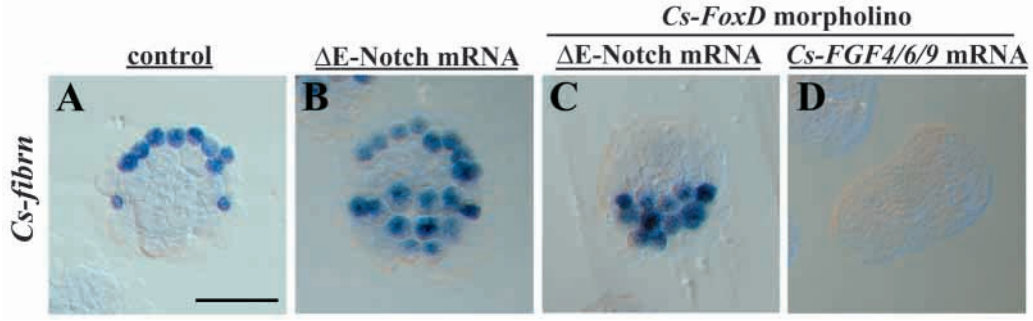

Fig. 10. Relationships of *Cs-FoxD* with *Cs-FGF4/6/9* and *Cs-Notch*. Expression of a notochord-specific *Cs-fibrn* gene in (A) control embryos, (B) embryos developed from eggs injected with the constitutively active form of Notch mRNA, (C) embryos developed from eggs co-injected with *Cs-FoxD* morpholino and constitutively active form of Notch mRNA, and (D) embryos developed from eggs co-injected with *Cs-FoxD* morpholino and *Cs-FGF4/6/9* mRNA.

transcription factor gene *Cs-ZicL*, as one of β-catenin downstream genes (Imai et al., 2002b). *Cs-ZicL* is transiently expressed in the A-line notochord/nerve cord lineage and in B-line muscle lineage from the 32-cell stage, and later in a-line CNS lineage from the 110-cell stage. Suppression of *Cs-ZicL* function with morpholino indicated that *Cs-ZicL* is essential for the formation of A-line notochord cells but not of B-line notochord cells (Imai et al., 2002b). The expression of *Cs-ZicL* in the A-line cells is downstream of *Cs-FoxD*. Therefore, it is highly likely that in the A-line notochord cells acts *Cs-ZicL* as downstream of *Cs-FoxD*, while in the B-line notochord cells acts *Cs-Notch* as downstream of *Cs-FoxD*.

Cs-FoxA5

Besides the genes discussed above, some other genes may be involved in this induction process. For example, *FoxA5* (*forkhead/HNF3β*) gene is expressed in the blastomeres of both endoderm and notochord lineages (Shimauchi et al., 1997). Suppression of the function of *Cs-FoxA5* results in a deficiency of endoderm development, which in turn leads to the failure of notochord induction (K. S. I., unpublished). However, it has been shown that *FoxA* and *Brachyury* act synergistically in the notochord formation in *Halocynthia* embryos (Shimauchi et al., 2001b), as previously demonstrated in *Xenopus* (O'Reilly et al., 1995). In addition, other genes such as *BMP2/4* are involved in notochord formation (Darras and Nishida, 2001). The genetic cascade of notochord induction in ascidian embryos appears to be not so simple.

Notochord induction in the two ascidian species *Halocynthia* and *Ciona*

Ascidians are subdivided into two major orders: the Enterogona and the Pleurogona (see Satoh, 1994). *Ciona* species belong to the former order, while *Halocynthia roretzi* belong to the latter. Recent molecular phylogenetic studies suggest that the two orders diverged comparatively early in the history of evolution of urochordates (Wada et al., 1992; Cameron et al., 2000). Therefore, it is possible that the two ascidian species adopt different molecular mechanisms in notochord induction. Indeed, previous analyses of notochord-specific enhancer of the 5′ flanking region of ascidian *Brachyury* indicated that the enhancer sequences of *Ciona Ci-Bra* (Corbo et al., 1997) appear to be different from those of *Halocynthia Hr-Bra* (Takahashi et al., 1999b).

Using *H. roretzi* embryos, Nishida and colleagues conducted a series of blastomere isolation and recombination experiments, and studied the effects of the treatment of the blastomeres with exogenous human bFGF (Nakatani and Nishida, 1994; Nakatani and Nishida, 1997; Nakatani et al., 1996; Kim et al., 2000; Kim and Nishida, 2001; Darras and Nishida, 2001). They suggested that FGF signal transduction pathways are involved in notochord induction in this species. Because a dominant-negative form of FGF receptor (HrFGFR) of *H. roretzi* causes suppression of *Hr-Bra* (*As-T*) expression, it is likely that FGF-like molecules are involved in notochord formation (Shimauchi et al., 2001b). Therefore, it is important to isolate FGF-related genes from *H. roretzi* to determine their roles in the notochord induction.

In *Ciona* embryos, *Cs-FGF4/6/9* is partially involved in the notochord induction (Imai et al., 2002a), and therefore molecules other than FGFs appear to be responsible for this process. As shown in the present study, *Cs-FoxD* play a pivotal role in the specification of notochord cells in *Ciona* embryos. In addition, Cs-Notch acts downstream of *Cs-FoxD* in the B-line notochord induction while *Cs-ZicL* acts downstream of *Cs-FoxD* in the A-line notochord induction. Therefore, it should be determined whether these genes are involved in the notochord formation in *Halocynthia* embryos. Such efforts may disclose evolutionary changes in the mechanism involved in the formation of ascidian larval notochord, the most prominent characteristic feature of chordates (Satoh and Jeffery, 1995).

Cs-FoxD as a member of the FoxD gene family

Recent studies have shown that vertebrate FoxD genes are expressed in the central nervous system and are involved in neural crest formation and function (Pohl and Knöchel, 2001; Sasai et al., 2001; Kos et al., 2001). One important question to be answered is whether vertebrate FoxD genes are also expressed in early embryos and are involved in the specification of notochord cells. As shown in the present study, *Cs-FoxD* expression is very transient, as it is seen at just the 16-cell and 32-cell stages. Therefore, it is possible that vertebrate FoxD genes are also expressed at early stages of embryogenesis, as is *Cs-FoxD*. Although the functions reported for vertebrate FoxD genes are mainly associated with the neural lineage, some FoxD genes are also expressed in the mesoderm at early embryonic stages. For example, zebrafish *fkh6*, *fkh8* and *fkh9* are expressed in paraxial mesoderm (Odenthal and Nüsslein-Volhard, 1998). *Xenopus foxD5a* is expressed in paraxial mesoderm during primary gastrulation, and ventral injection of *Xenopus foxD5a* mRNA induces partial secondary axes composed of expanded mesoderm and ectoderm (Sullivan et al., 2001). *Xenopus foxD5a* is strongly induced by *siamois* (Sullivan et al., 2001). Mouse *Fkh2* is expressed in notochord and anterior endoderm at the headfold stage (Kaestner et al., 1995). These results suggest that vertebrate FoxD family genes might have functions in the patterning of the mesoderm.

Conversely, it is also interesting that there is later expression of *Cs-FoxD* in epidermal cells and nerve cord cells in the early tailbud embryo. Is there any evolutionary link between neural crest cells and this later expression in ascidian embryos, which do not develop neural crests?

In conclusion, an ascidian *FoxD* gene is expressed transiently and primarily in endodermal cells, and its expression is essential for notochord differentiation. Further characterization of *Cs-FoxD* target genes is important for understanding the molecular mechanisms involved in the notochord induction of ascidian embryos.

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