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FGF signals are involved in the differentiation of notochord cells and mesenchyme cells of the ascidian *Halocynthia roretzi*

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SUMMARY

Differentiation of notochord cells and mesenchyme cells of the ascidian *Halocynthia roretzi* requires interactions with neighboring endodermal cells and previous experiments suggest that these interactions require fibroblast growth factor (FGF). In the present study, we examined the role of FGF in these interactions by disrupting signaling using the dominant negative form of the FGF receptor. An FGF receptor gene of *H. roretzi* (*HrFGFR*) is expressed both maternally and zygotically. The maternally expressed transcript was ubiquitously distributed in fertilized eggs and in early embryos. Zygotic expression became evident by the neurula stage and transcripts were detected in epidermal cells of the posterior half of embryos. Synthetic mRNA for the dominant negative form of FGFR, in which the intracellular tyrosine kinase domain was deleted, was injected into fertilized eggs to interfere with the possible function of *HrFGFR*. Injected eggs cleaved and gastrulated the same as the control embryos. Analyses of the expression of differentiation markers in the experimental embryos indicated that the differentiation of epidermal cells, muscle cells and endodermal cells was not affected significantly. However, manipulated embryos showed downregulation of notochord-specific *Brachyury* expression and failure of notochord cell differentiation, resulting in the development of tailbud embryos with shorted tails. The expression of an actin gene that is normally expressed in mesenchyme cells was also suppressed. These results suggest that FGF signals are involved in differentiation of notochord cells and mesenchyme cells in *Halocynthia* embryos. Furthermore, the patterning of a neuron-specific tubulin gene expression was disturbed, suggesting that the formation of the nervous system was directly affected by disrupting FGF signals or indirectly affected due to the disruption of normal notochord formation.

Key words: Ascidians, Cell-cell interactions, FGF signals, FGF receptor, Notochord, Mesenchyme, Nervous system, *Halocynthia roretzi*

INTRODUCTION

Patterning of complicated animal body plans requires interactions between constituting cells, and signaling cascades play important roles in the interactions. Examples of some well-characterized signaling cascades include the Wnt family (Moon et al., 1997; Dierick and Bejsovec, 1999), the TGFβ family (Piek et al., 1999), the FGF family (Nugent and Iozzo, 2000; Ornitz, 2000) and the EGF family (Riese II and Stern, 1998; Shen and Schier, 2000). Each of these signaling cascades is established by specific interactions between ligands and receptors followed by a pathway in which these signals eventually activate genes that encode transcription factors. For example, in vertebrate embryos, FGFs function as differentiation factors and play key roles in mesoderm induction (Slack, 1994; Yamaguchi and Rossant, 1995). FGFs can induce muscle, mesenchyme, lateral plate mesoderm and blood islands in *Xenopus*. Inhibition of FGF signaling caused by the truncation of FGF receptors resulted in embryos with reduced mesoderm (Amaya et al., 1991; Yamaguchi et al., 1994; Griffin et al., 1995).

During embryogenesis of ascidians that produce tadpole larvae, a notochord consisting of exactly 40 cells develops within a muscular tail (Satoh, 1994). Developmental processes required for notochord formation have been investigated in detail. First, the entire cell lineage responsible for generating the 40 notochord cells has been completely documented (Conklin, 1905; Nishida, 1987); the anterior 32 cells are derived from a pair of right and left A4.1 (anterior and vegetal) cells of the bilaterally symmetrical 8-cell embryo, while the posterior 8 cells are descendants of the pair of B4.1 (posterior and vegetal) cells. At the 32-cell stage, A-line endodermal cells (A6.1 and A6.3) produce a signal(s) that induces the neighboring presumptive notochord cells (A6.2 and A6.4) to be specified along the notochordal pathway (Nakatani and Nishida, 1994). At the 64-cell stage, the developmental fates of A7.3 and A7.7 are restricted to give rise to notochord cells only. Simultaneously, the *Brachyury* gene is activated (Yasuo

and Satoh, 1993; Corbo et al., 1997), which controls downstream genes that are required for the formation of notochord (Yasuo and Satoh, 1998; Takahashi et al., 1999a; Hotta et al., 2000). The developmental fate of the B-line notochord cell B8.6 is restricted at the 110-cell stage, and then the cell expresses the *Brachyury* gene (Yasuo and Satoh, 1993).

One of the most important steps involved in the notochord formation in ascidian embryos is how the endoderm and the presumptive notochord cells communicate with each other so that the latter becomes specified to give rise to differentiated notochord cells. It has been shown that bFGF, but not activin A, mimics the induction event at the 32-cell stage (Nakatani et al., 1996). A6.2 and A6.4 cells isolated at the early phase of the 32-cell stage were immersed in seawater containing human recombinant bFGF, which resulted in the activation of *Brachyury* and subsequent differentiation of notochord cells as assessed using cell-specific antibody and changes in cell morphology. In vertebrates, it has been shown that the signal cascade triggered by bFGF binding includes the Ras pathway (Satoh et al., 1992; Pawson, 1995). Extending this previous study, Nakatani and Nishida (Nakatani and Nishida, 1997) showed that the injection of a dominant-negative form of Ras, to cause the functional inhibition of endogenous Ras, inhibited the A-line notochord cell differentiation. The mechanism for the B-line notochord cell differentiation, however, remains to be elucidated.

In addition, a recent study by Kim et al. (Kim et al., 2000) demonstrated that the differentiation of mesenchyme cells in *H. roretzi* embryos also requires inducing signals from endodermal cells at the 32-cell stage and that bFGF but not activin A can induce the differentiation of mesenchyme cells. Furthermore, they showed that the differentiation pathway responsible for the development of mesenchyme or notochord cells depends on whether the precursor cells contain the posterior-vegetal cytoplasm (PVC) of the eggs. PVC is present in mesenchyme precursor cells whereas notochord precursor cells do not contain PVC.

Although these experiments strongly suggest an involvement of FGF-like signals in the differentiation of notochord and mesenchyme cells in *Halocynthia* embryos, the reagents used in these studies were from human, not ascidian, sources. The present study was undertaken to overcome this problem and obtain direct evidence of whether or not FGF signals are involved in ascidian cellular interactions. Here, we report the isolation of a cDNA clone for a gene encoding FGF receptor (HrFGFR) from *Halocynthia* embryos, and an examination of the function of HrFGFR by overexpression of dominant-negative form of this receptor.

MATERIALS AND METHODS

Ascidian eggs and embryos

Naturally spawned eggs of *Halocynthia roretzi* were fertilized with a suspension of sperm from another individual. Fertilized eggs were raised at 11-13°C. Embryogenesis proceeded synchronously among different batches of eggs. Embryos at appropriate stages were fixed for in situ hybridization. For northern blotting, specimens were packed by low speed centrifugation and frozen quickly with liquid nitrogen. The frozen samples were kept at –80°C until use.

Isolation and characterization of cDNA clones for an ascidian FGF receptor gene

Polymerase chain reaction (PCR) was used to isolate fragments of *HrFGFR* cDNAs. Degenerate primers were designed to cover the tyrosine kinase domain, which shows the highest conservation among FGFR family members; forward primer, 5′GGNGARGGNTG-YTTYGGNCA3′ and reverse primer, 5′GCYTCNGGNGCCAT-CCAYTT3′.

The targeted template was the reverse transcription product of an *H. roretzi* 110-cell stage embryo (Imai et al., 2000). Several of the cDNA fragments that were obtained were sequenced after subcloning them into pBluescript SK+ (Stratagene). One of them was found to be 592 bp, which encoded a polypeptide corresponding to part of the tyrosine kinase domain. This fragment was used to screen a 3.5×10^5 pfu plate containing an *H. roretzi* gastrula cDNA library (Shimauchi et al., 1997). Nearly 100 positive clones were obtained. Nucleotide sequences for 7 of these clones showed that they encoded an identical protein. The longest clones (about 3.2 kb) was completely sequenced. Nucleotide sequences were determined for both strands with a Big-Dye Terminator Cycle Sequencing Ready Reaction kit and ABI PRISM 377 DNA sequencer (Perkin Elmer).

Genomic Southern blotting

High-molecular mass genomic DNA was extracted from the gonad of a single specimen by the standard procedure (Sambrook et al., 1989). After digestion with *Eco*RI, *Hin*dIII or *Pst*I, the DNA fragments were subjected to 0.7% agarose gel electrophoresis and transferred to a Hybond-N^+ nylon membranes (Amersham). The membranes were hybridized with DNA probes at 42°C for 17 hours and then washed under high-stringency conditions (twice in 2×SSC, 0.1% SDS at 50°C). The DNA probes were excised by *Bam*HI/*Bgl*II (nucleotide position 1314-2076, the C-terminal domain probe) or *Eco*RI/*Hin*dIII (nucleotide position 710-884, the Ig3 domain probe) of *HrFGFR* gene and labeled by [^{32}P]dCTP using a random primed labeling kit (Roche).

RNA extraction and northern blotting

Total RNA was extracted from eggs or embryos using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified using Oligotex beads (Roche Japan). For northern blotting, poly(A)$^+$ RNA was fractionated by agarose gel electrophoresis, and transferred to Hybond-N(+) membranes (Amersham). Blots were hybridized with a ^{32}P-random-labeled DNA probe in 6× SSPE, 0.5% SDS, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA, and 50% formamide at 42°C. The filters were washed twice in 2× SSC/0.1% SDS at 60°C and then exposed to X-ray film (Fuji film).

Deletion constructs and microinjection of eggs

A deletion mutation of HrFGFR without the intracellular tyrosine kinase domain (dnHrFGFR) was made. FGFR that completely lacks the intracellular tyrosine kinase domain effectively abolishes the wild-type receptor function (Amaya et al., 1991). The dnHrFGFR was made by inserting the *Not*I-*Ssp*I fragment into the modified pBluescript-RN3 vector (Lemaire et al., 1995). By digesting at an *Ssp*I site of 1230~1235 bp (58 amino acids downstream from the transmembrane domain), the tyrosine kinase domain was completely deleted (Fig. 1B). As an experimental control, we injected mRNA which encodes full length HrFGFR. Injection of mRNAs into fertilized eggs was performed as described previously (Miya et al., 1997a).

Histochemical staining for alkaline phosphatase and acetylcholinesterase

Differentiation of endodermal cells was monitored by histochemical detection of alkaline phosphatase (AP) activity (Whittaker and Meedel, 1989). Embryos were fixed with 4% paraformaldehyde in

Mops buffer (pH 7.8), 0.2 M NaCl, 0.4 M $MgCl_2$ overnight at 4°C. 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).

Differentiation of muscle cells was examined using a standard histochemical reaction to detect acetylcholinesterase (AchE; Karnovsky and Roots, 1964). Embryos were fixed with 5% formaldehyde in seawater for 10 minutes at room temperature. The specimens were washed in PBT twice, and then the buffer was replaced with AChE staining buffer (65 mM sodium acetate, 3 mM copper sulfate, 0.5 mM potassium ferricyanide, 5 mM sodium citrate, pH 5.5) containing 0.2 mg/ml acetylthiocholine iodide. The reaction was performed at room temperature for 3 hours.

Antibody staining

To monitor the notochord cell differentiation of *H. roretzi*, we used a monoclonal antibody 5F1D5 which recognizes a notochord-specific antigen, Not-1 (Nishikata and Satoh, 1990). Indirect immunochemical staining was carried out using TSA™-DIRECT (NEN™, Life Science Products, Inc. Boston, USA) according to the instructions supplied with the kit, and observed using confocal microscopy.

Whole-mount in situ hybridization

In situ hybridization with whole-mount specimens was carried out using digoxigenin-labeled RNA probes of *HrFGFR*. Specimens were fixed in 4% paraformaldehyde in Mops buffer (pH 7.8), 0.2 M NaCl, 0.4 M $MgCl_2$. After a thorough wash with PBT (phosphate-buffered saline (PBS) containing 0.1% Tween 20), the fixed specimens were treated with 2 μg/ml proteinase K (Merck) in PBT for 30 minutes at 37°C, and then they were post-fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature. After a 1-hour period of prehybridization at 42°C, the specimens were allowed to hybridize with the digoxigenin-labeled antisense or sense probe for at least 16 hours at 42°C. After hybridization, the specimens were washed and treated with RNase A, then washed again extensively with PBT. The samples were then incubated for 1 hour with 1:2000 alkaline-phosphatase-conjugated anti-DIG (Roche) and the colour developed, as indicated in the protocol from Roche. The probes for *HrFGFR* were synthesized according to the instructions supplied with the kit (DIG RNA Labeling Kit; Roche). The gene expression patterns reported here were examined with probes produced from the coding region of the gene.

Probes used to examine *HrFGFR* function in experimental embryos were cDNAs for *H. roretzi Brachyury* gene (*As-T* or *HrBra*; Yasuo and Satoh, 1993), an epidermis-specific gene (*HrEpiC*; Ueki and Satoh., 1995; Ishida et al., 1996), neural tissue-specific gene (*HrTBB2*; Miya et al., 1997b), and cytoplasmic actin gene (*HrCM1*; Araki et al., 1996) which is predominantly expressed in mesenchyme cells. RNA probes were prepared with a DIG RNA Labeling Kit (Roche). Control embryos that were hybridized with sense probes did not show signal above background.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was carried out as described previously (Imai et al., 2000). Twenty embryos at the early gastrula stage were used for cDNA synthesis. PCRs were performed by utilizing primers with the following nucleotide sequence:

HrBra; 5′-CATGCAGCGTCATGCGTTTACGT-3′ and
5′-GTGGTACTCTCTCCAGAACTCGA-3′;
HrEF-1α; 5′-CTACACGCCAGTTTTGGACTG-3′ and
5′-GAAAGTGCCCAGGAAGAAATT-3′.

The reaction was performed for 30 cycles (*HrBra*) or 27 cycles (*HrEF-1α*), each consisting of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. The reactions were resolved by 5% PAGE and subjected to autoradiography.

RESULTS

Characterization of cDNA clones for *H. roretzi* FGF receptor gene

As shown in Fig. 1A, an insert of the longest cDNA that we obtained consisted of 3,199 bp including 18 adenylyl residues at the 3′ end (DDBJ/GenBank/EMBL accession number, AB046873). It contained a long and single open reading frame which predicted a polypeptide of 763 amino acids. BlastX search (Altschul et al., 1997) indicated that the predicted protein resembled FGFR. Referring to a report by Lee et al. (Lee et al., 1989), we identified, from the N terminus to the C terminus of the protein, two immunogloblin (Ig)-like domains (Ig2 and Ig3) bounded by cysteine residues forming disulfide bridges, a transmembrane domain (TM), and two intracellular tyrosine kinase domains split by 14 amino acids (Fig. 1B). Amino acid residues (RDLAARNVL and DFGLAR) characteristic of the tyrosine kinase domains were also seen in the sequence (Fig. 1A). These compositions fit the basis features of a FGF receptor and therefore, the corresponding gene was named *HrFGFR*. However, the cloned *HrFGFR* mRNA lacked Ig1 and acid box sequences that are additional components of FGFR as revealed in, for example, *Xenopus* FGFR (Amaya et al., 1991).

The amino acid sequence of the tyrosine kinase domain of HrFGFR, shown in Fig. 1A, was compared with other FGFR domains and subjected to molecular phylogenetic analysis using neighbor-joining method. The mouse VEGFR2 and PDGFR were used as the outgroup. As shown in Fig. 2, HrFGFR was included within a group of FGFR family members. The tree suggested a routeness of the ascidian FGFR, as well as the amphioxus FGFR (Suga et al., 1999), before the divergence of the ancestral gene into four distinct groups present in vertebrates, FGFR1, FGFR2, FGFR3 and FGFR4.

HrFGFR is present as a single copy in *Halocynthia* genome

Very recently the isolation and characterization of a cDNA clone for *FGFR* gene of *H. roretzi* was reported (Kamei et al., 2000). The cDNA that they isolated is similar to ours, except for two things. First, their cDNA contains Ig1 and an acid box which are absent in our cDNA clone. Second, the sequences of the Ig3 domain are different in these two clones. It is possible that the *HrFGFR* gene may be transcribed into two different types of mRNA by alternative splicing, or that the *Halocynthia* genome contains two *HrFGFR* genes. Therefore, we determined the number of different sequences that correspond to *HrFGFR* in the *Halocynthia* genome by Southern blot hybridization. Two different DNA probes were used: the C-terminal probe common to both *HrFGFR* cDNAs characterized by Kamei et al. (Kamei et al., 2000) and by the present study, and the Ig3 domain probe specific to cDNA characterized by the present study.

When examined with the common probe, only one band was detected in the lanes of *Eco*RI, *Hin*dIII and *Pst*I digestion (Fig. 3), while the specific probe resulted in one band in the lanes of *Eco*RI and *Pst*I, and two bands in the lane of *Hin*dIII digestion (Fig. 3). Our *HrFGFR* cDNA had a restriction site for *Hin*dIII in the Ig3 domain, and a common band between the two probes was seen in the lane of *Eco*RI and *Hin*dIII. In addition, washing the membranes under low-stringency

A

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GGTGTATGAATGTTTCACTGCTTTGCGGTACTCGGTTAAACGTTGGACAGCTTCATATTATTGCGCTTATACCTAATACTACATGGGTTA  90
AGTTAATACAATGAGTTTTATGTAAAGATGAAGGAATTCGAAGTAAAGGTTGCAAGTACGGCCTTCGTTCTTGTACTGTTTTCACTGACT 180
                          M  K  E  F  E  V  K  V  A  S  T  A  F  V  L  V  L  F  S  L  T        21
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E  G  N  E  E  N  V  V  S  A  P  K  Q  D  G  A  S  G  G  Q  K  P  Y  W  T  K  R  E  K  M        81
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M  K  R  L  H  A  E  P  A  G  N  T  V  R  F  R  C  A  V  D  G  N  P  K  P  Q  V  L  W  Y       111
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K  N  D  L  I  V  Q  K  N  D  R  V  G  G  Y  K  Y  R  N  Q  V  L  I  L  E  S  V  V  L  S       141
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D  K  G  N  Y  M  C  V  A  R  N  E  Y  G  S  I  N  H  T  Y  Q  L  D  V  Q  E  R  S  A  S       171
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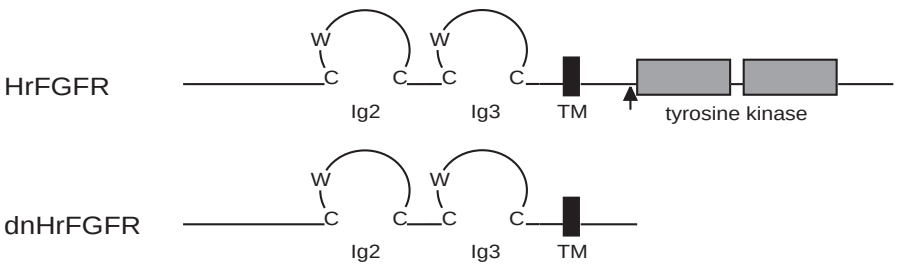

Fig. 1. (A) Nucleotide and predicted amino acid sequences of *HrFGFR* cDNA. The insert of the cDNA encompasses 3,199 bp including 18 adenylyl residues located at the 3′ end. The ATG at positions 118-120 represents the putative start codon for the *HrFGFR* protein. An asterisk indicates the termination codon. The transmembrane domain is indicated by dotted-underline, and the intracellular tyrosine kinase domains are underlined. Bold, italic capitals indicate cysteine residues forming disulfide bridges characteristic of immunogloblin (Ig)-like domains. Bold capitals indicate amino acid residues characteristic of the tyrosine kinase domain. Arrows indicate two regions of amino acid residues that were used in the molecular phylogenetic analysis shown in Fig. 2. (B) Schematic representation of the structural and functional domains of HrFGFR and the mutant dnHrFGFR. The FGF receptor contains two Ig-like domains bounded by cysteine residues that form disulfide bridges (Ig2 and Ig3), a transmembrane domain (TM), and an intracellular tyrosine kinase domain split by 14 amino acids. dnHrFGFR represents a mutant construct in which the entire tyrosine kinase domain starting from amino acid 369 (marked by an arrow on HrFGFR) was deleted.

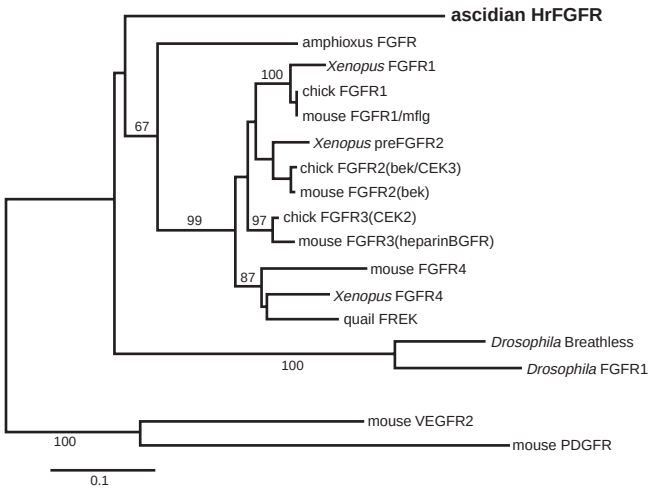

Fig. 2. HrFGFR is a routeness of the chordate FGFR family members. A molecular phylogenetic tree was constructed by means of a neighbor-joining method by comparison of amino acid sequences of the tyrosine kinase domains shown in Fig. 1. The mouse VEGFR2 and PDGFR were used as the outgroup. The numbers indicate the relative robustness of each node as assessed by bootstrap analysis (100 replications).

conditions resulted in a similar result. Therefore, it is likely that *HrFGFR* is present as a single copy per haploid genome of *H. roretzi*, and two different mRNAs are produced by alternative splicing. Nucleotide sequences of the 3′ UTR are identical in the *HrFGFR* cDNAs characterized by Kamei et al. (Kamei et al., 2000) and that of the present study, supporting the idea that the two different mRNAs are produced by alternative splicing.

The *HrFGFR* gene is expressed both maternally and zygotically

HrFGFR is expressed both maternally and zygotically. Northern blot analysis showed that *HrFGFR* transcripts are about 3.2 kb in length and they are present in unfertilized eggs, fertilized eggs and early embryos (Fig. 4). Transcripts are retained through the 64-cell stage, neurula and tailbud stage embryos (Fig. 4). Because in situ hybridizations showed that zygotic transcripts appear at the neurula stage onward, it is likely that maternal transcripts are replaced with zygotic transcripts as development progresses.

In situ hybridization of whole-mount specimens showed that maternal transcripts were distributed evenly in the entire cytoplasm of unfertilized and fertilized eggs (Fig. 5A,B). At the 8-cell stage, hybridization signals were abundant in the animal hemisphere (Fig. 5C). At the 16-cell and 32-cell stages, signals were evident in the animal blastomeres (Fig. 5D,E), whereas the vegetal blastomeres at these stages showed weaker signals (Fig. 5D′,E′,). As development progressed, signals became less and less evident (Fig. 5F) and eventually they were undetectable at the 110-cell and early gastrula stages (Fig. 5G,H).

In ascidians, the first appearance of zygotic transcripts is usually seen in the nucleus of embryonic cells (Yasuo and Satoh, 1993). Zygotic *HrFGFR* transcripts appeared at the neural plate stage. At the neurula stage, hybridization signals were evident within nuclei of epidermal cells situated in the posterior half of the embryo (Fig. 5I). At the early tailbud stage, signals were evident in cells situated in the anterior-most region and in the dorsal and ventral midlines of the tail region (Fig. 5J). This spatial expression pattern of *HrFGFR* expression continued to the mid-tailbud stage (Fig. 5K).

Effects of injection of synthetic mRNA for the dominant negative form of HrFGFR

To deduce a possible role of *HrFGFR*, we constructed a mutated form of HrFGFR (dnHrFGFR) in which the entire tyrosine kinase domain, starting from amino acid position of 369, was deleted from HrFGFR (Fig. 1B). This mutant is thought to act as a dominant negative form to abolish wild-type receptor function (Amaya et al., 1991). We also injected *HrFGFR* mRNA. Eggs injected with 1.0 μg/μl or less of synthetic mRNA cleaved normally and developed into tailbud embryos with normal morphology, and the effect of dnHrFGFR was not evident. Therefore, we examined the effect of dnHrFGFR by injecting mRNA at a concentration of 4.5 μg/μl. The following describes the effects of *dnHrFGFR* mRNA injections on the various tissues of the embryo.

Endoderm

Endodermal cells of the ascidian embryo differentiate

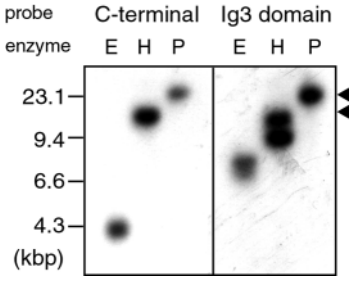

Fig. 3. Genomic Southern analysis of the *HrFGFR* gene. The genomic DNA prepared from a single *H. roretzi* was digested with *Eco*RI (E), *Hin*dIII (H) or *Pst*I (P), resolved by electrophoresis, and transferred to a nylon membrane. Blots were hybridized with two different ^{32}P-labeled DNA probes (common C-terminal probe and specific Ig3 domain probe) and washed under high stringency conditions. Bands that show the same mobility at both probes are marked by arrowheads. Each lane was loaded with 10 μg digested DNA.

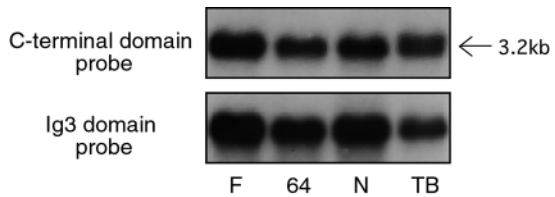

Fig. 4. Temporal expression of *HrFGFR*. Northern blots of poly(A)$^+$ RNA prepared from the fertilized eggs (F), 64-cell stage embryos (64), neurulae (N), and tailbud-stage embryos (TB), hybridized with a ^{32}P-labeled probe prepared from the C-terminal domain (upper) and Ig3 domain (lower). Each lane was loaded with 5 μg poly(A)$^+$ RNA. Exposure time was 1.5 days.

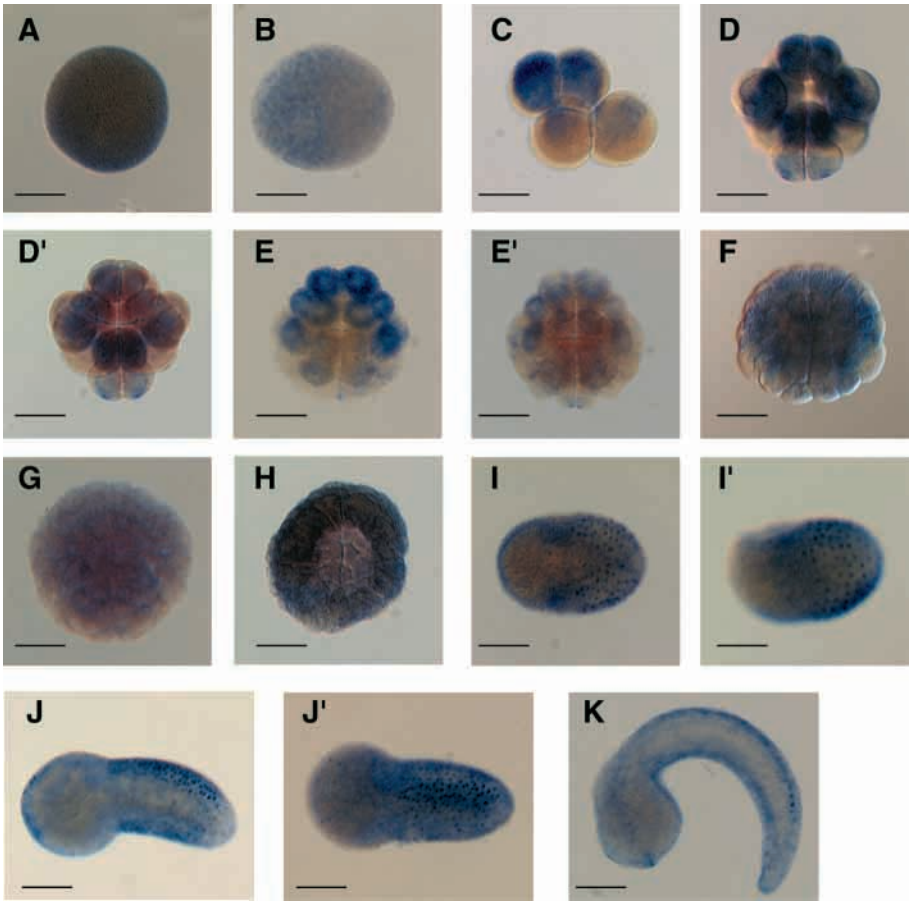

Fig. 5. Spatial expression of *HrFGFR* as revealed by whole-mount in situ hybridization with DIG-labeled antisense probes. (A) An unfertilized egg. (B) A fertilized egg. (C) An 8-cell stage embryo, lateral view. (D,D′) A 16-cell stage embryo viewed from (D) the animal pole (future ventral side) and (D′) vegetal pole (future dorsal side). (E,E′) A 32-cell stage embryo; (E) animal view and (E′) vegetal view. (F) A 64-cell stage embryo, animal view. (G) A 110-cell stage embryo, animal view. (H) A gastrula, vegetal view. (I,I′) A neurula, (I) dorsal view and (I′) lateral view. Signals for zygotic expression are evident in cells of the epidermis in the posterior region. (J,J′) An early tailbud embryo viewed from the lateral (J) and dorsal (J′) side. (K) A mid-tailbud embryo, lateral view. Signal is seen in epidermal cells of the tail. The scale bar is 100 μm for all panels.

autonomously and are dependent on the utilization of prelocalized maternal factors (Satoh, 1994). The gene encoding AP is specifically expressed in endodermal cells (Kumano and Nishida, 1998; Imai et al., 2000), and therefore the histochemical detection of AP is a standard method that is used to assess endoderm differentiation. As shown in Fig. 6A, AP expression was not affected by the injection of *HrFGFR* mRNA (Fig. 6A′) in that 27 of 29 injected embryos (93%) showed distinct AP activity (Table 1). Although the injection of *dnHrFGFR* mRNA resulted in tail shortening of the experimental embryos, AP expression in the trunk region of these embryos did not appear to be affected (Fig. 6A″) in that 18 of 19 injected embryos (95%) showed distinct AP activity (Table 1).

Table 1. Effects of injection of dnHrFGFR mRNA on the appearance of differentiation markers in *H. roretzi* embryos

			No. of embryos with marker expression (%)			
Markers	Injected RNA	Stage examined*	Total	Strong expression	Weak expression	No expression
AP (endoderm)	Not injected	mTB	36	36 (100%)	0	0
	HrFGFR	mTB	29	27 (93%)	2 (7%)	0
	dnHrFGFR	mTB	19	18 (95%)	1 (5%)	0
AChE (muscle)	Not injected	mTB	39	39 (100%)	0	0
	HrFGFR	mTB	22	22 (100%)	0	0
	dnHrFGFR	mTB	29	29 (100%)	0	0
HrEpiC (epidermis)	Not injected	mTB	42	42 (100%)	0	0
	HrFGFR	mTB	22	1 (5%)	20 (90%)	1 (5%)
	dnHrFGFR	mTB	29	23 (79%)	6 (21%)	0
Hr-Bra (notochord)	Not injected	110	31	30 (97%)	0	1 (3%)
	HrFGFR	110	41	41 (100%)	0	0
	dnHrFGFR	110	26	1 (4%)	8 (32%)	16 (64%)
	Not injected	iTB	39	39 (100%)	0	0
	HrFGFR	iTB	44	37 (84%)	1 (2%)	6 (14%)
	dnHrFGFR	iTB	30	16 (53%)	11 (37%)	3 (10%)
Not1	Not injected	mTB	31	30 (97%)	0	1 (3%)
	HrFGFR	mTB	41	36 (88%)	2 (5%)	3 (7%)
	dnHrFGFR	mTB	28	8 (29%)	8 (29%)	12 (42%)

*mTB, mid-tailbud stage; 110, 110-cell stage; and iTB, initial tailbud stage.

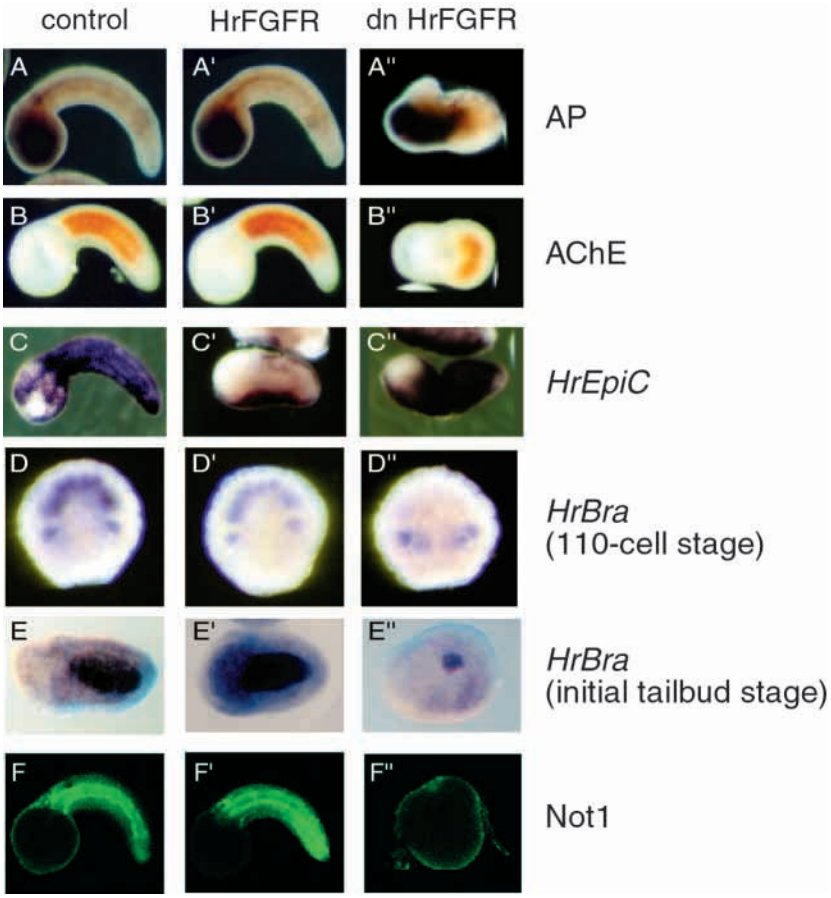

Fig. 6. Effects of injection of mRNAs for *HrFGFR* or *dnHrFGFR* on expression of endoderm-specific AP (A-A″), muscle-specific AChE (B-B"), epidermis-specific *HrEpiC* gene (C-C″), and notochord-specific *HrBra* (D-D″,E-E″) and Not1 antigen (F-F″). Development of the first three markers and the Not1 antigen was examined at the mid-tailbud stage (A-C,F), while that of *HrBra* was examined at the 110-cell (D-D″) and initial tailbud stages (E-E″). (A-E) control, uninjected embryos, (A′-E′) embryos injected with *HrFGFR* mRNA; (A″-E″) embryos injected with *dnHrFGFR* mRNA.

Muscle

Primary lineage muscle cells are also able to differentiate autonomously (Satoh, 1994), and their differentiation can be monitored by the histochemical detection of AChE (Fig. 6B). Because injection of *dnHrFGFR* mRNA resulted in the shortening of tails in the experimental embryos, the posterior region exhibiting AChE expression appeared smaller than that of normal embryos. In some experimental embryos, muscle cells with AChE activity did not separate into the right and left side of the tail, as seen in normal embryos. However, AChE expression was essentially not affected by the injection of *dnHrFGFR* mRNA (Fig. 6B″; Table 1).

Epidermis

Epidermal cell differentiation was examined by in situ hybridization using the epidermis-specific gene *HrEpiC*. Expression of *HrEpiC* begins at the 64-cell stage, and the expression is first seen in the posterior region of the embryo and later in the anterior region too (Ueki and Satoh., 1995; Ishida et al., 1996). The expression of this gene did not appear to be affected by the injection of *dnHrFGFR* mRNA (Fig. 6C″) as 23 of 29 injected embryos (79%) showed distinct *HrEpiC* expression (Table 1). However, injection of *HrFGFR* mRNA sometimes resulted in the slight downregulation of *HrEpiC* (Fig. 6C′), as about 90% of the embryos showed weak in situ signals (Table 1).

Notochord

Brachyury (*As-T* or *HrBra*) is expressed exclusively in the notochord cells of the ascidian embryo, and this gene plays a key role in notochord differentiation (Yasuo and Satoh, 1993; Yasuo and Satoh 1998). Injection of control *HrFGFR* mRNA did not affect *HrBra* expression when it was examined at the 110-cell stage (Fig. 6D′; Table 1) and at the initial tailbud stage (Fig. 6E′; Table 1). In contrast, the injection of *dnHrFGFR* mRNA caused the distinct downregulation of *HrBra* expression. In the embryo shown in Fig. 6D″, the A-line primordial notochord cells of the 110-cell embryo failed to express *HrBra*. As summarized in Table 1, 16 of 26 *dnHrFGFR* mRNA-injected embryos (62%) failed to express *HrBra*, while 8 of the 26 experimental embryos showed only weak signals in fewer notochord cells, usually in the B-line notochord cells, as compared with normal embryos. When examined at the initial tailbud stage, only one-third of the embryos showed the *HrBra* expression in few notochord cells (Fig. 6E″; Table 1). Suppression of the *HrBra* expression by injection of *dnHrFGFR* mRNA was further confirmed by quantitative RT-PCR analysis. As shown in Fig. 7, the band intensity of *HrBra* mRNA in *dnHrFGFR*-mRNA-injected gastrulae was conspicuously reduced when compared with that in normal gastrulae. Negative control (RT–) without reverse transcriptase gave no bands.

Effects of injection of *dnHrFGFR* mRNA upon the notochord differentiation was also examined with a cell-specific monoclonal antibody. Twelve of the 28 injected embryos (43%) failed to express the Not1 antigen (Fig. 6F″;

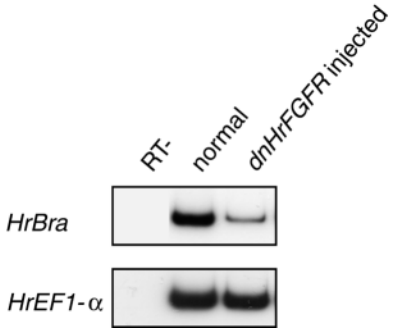

Fig. 7. Semi-quantitative RT-PCR analysis of the amount of *HrBra* mRNA in normal embryos and *dnHrFGFR* mRNA-injected embryos. Negative control (RT–) was without reverse transcriptase in cDNA synthesis.

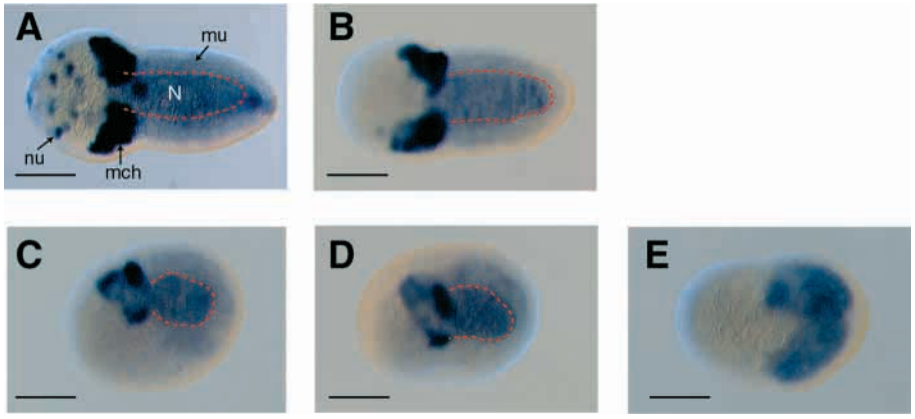

Fig. 8. Effects of injection of *dnHrFGFR* mRNA on expression of *HrCA1*, assessed by whole-mount in situ hybridization. (A) Expression of *HrCA1* in a control tailbud embryo. Strong signals are evident in mesenchyme cells (mch) and in several neuronal cells (nu) in the anterior and dorsal trunk, while weak signal is evident in notochord cells (N, dotted line) and muscle cells (mu). (B) Embryo injected with *HrFGFR* mRNA. (C-E) Three examples of embryos injected with *dnHrFGFR* mRNA. The expression of *HrCA1* was downregulated or suppressed. Scale bar is 100 μm for all panels.

Table 1). Therefore, it was concluded that disturbance of FGF signaling resulted in the downregulation of *HrBra* expression and then the failure of notochord cell differentiation. This caused the tail shortening of the experimental embryos.

Mesenchyme

About 900 mesenchyme cells are formed in the posterior trunk region of the ascidian larva. A recent study by Kim et al. (Kim et al., 2000) showed that mesenchyme cells are specified by at least two factors: vegetal cytoplasm inherited from the egg, and signals emanating from embryonic endodermal cells at the 32-cell stage. They also showed that the formation of mesenchyme cells is induced by treatment with bFGF. *HrCA1* encodes a cytoplasmic actin, however, its zygotic expression is not ubiquitous but is restricted to four types of cells (Araki et al., 1996). Strong in situ hybridization signals are detected in mesenchyme cells using this probe (Fig. 8A). Distinct signals are also evident in about 10 neuronal cells that are situated in a characteristic pattern in the anterior-dorsal trunk region (Fig. 8A). In addition, weak signals are evident in notochord cells and reduced signals are also detected in muscle cells (Fig. 8A).

Injection of *HrFGFR* mRNA resulted in the loss of *HrCA1* expression in the anterior neuronal cells, although *HrCA1* expression in the three other cell-types was not affected (Fig. 8B), and tail lengths were similar to those of normal embryos (Fig. 8A,B). However, when *dnHrFGFR* mRNA was injected into eggs, the resulting tails did not elongate. The number of cells showing an intense *HrCA1* signal decreased in number (Fig. 8C,D) and in some experimental embryos signals were absent (Fig. 8E). These results strongly suggest that disrupting FGF signaling interferes with normal mesenchyme cell differentiation.

The number of notochord cells capable of undergoing intercalation and showing weak *HrCA1* expression also decreased (Fig. 8B-D; red dotted line). In the embryo injected with *dnHrFGFR* mRNA shown in Fig. 8E, notochord cells are absent, and, instead, there was an increase in *HrCA1* expression in muscle cells. These results, together with the *HrBra* and Not1 experiments, suggest that notochord differentiation was disrupted by *dnHrFGFR* mRNA injection.

Nervous system

The effects of microinjection of *dnHrFGFR* mRNA on development of the nervous system was examined by in situ hybridization using the *HrTBB2* probe, which encodes for β-tubulin and is expressed in cells of the nervous system (Miya et al., 1997b). *HrTBB2* expression is seen in the anterior-most cells, in some cells within the dorsal brain region, in pairs of several cells located in the posterior trunk region, and in pairs of cells situated in the tail region (Fig. 9A).

Injection of *HrFGFR* mRNA did not affect the pattern of *HrTBB2* expression (Fig. 9B). However, the injection of *dnHrFGFR* mRNA did affect *HrTBB2* expression. In some of the experimental embryos shown in Fig. 9C, the patterning of *HrTBB2*-positive cells was disturbed and there are two patches of *HrTBB2*-expressing cells in the anterior region. However, the anterior-most domain of *HrTBB2* expression is absent (Fig. 9C).

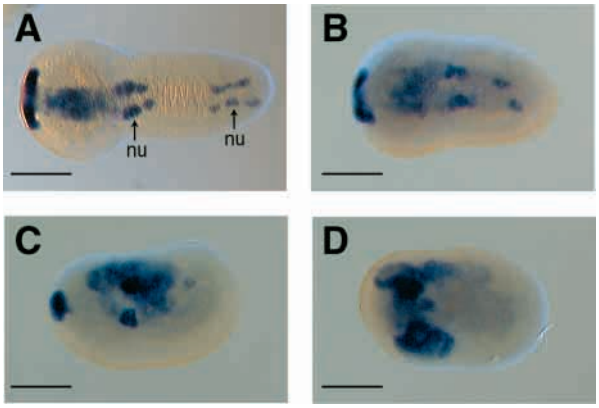

Fig. 9. Effects of injection of *dnHrFGFR* mRNA on expression of *HrTBB2*, assessed by whole-mount in situ hybridization. (A) Expression of *HrTBB2* in a control tailbud embryo. Signals are evident in neuronal cells (nu) in the anterior and dorsal trunk and dorsal tail regions. Embryo injected with (B) *HrFGFR* mRNA and (C) *dnHrFGFR* mRNA. The expression pattern of *HrTBB2* is disturbed. Scale bar, 100 μm for all panels.

DISCUSSION

The roles of FGF signals in the differentiation of notochord and mesenchyme cells in *Halocynthia* embryos

We examined the role of FGF signals in the differentiation of notochord and mesenchyme cells in *Halocynthia* embryos by microinjection of *dnHrFGFR* mRNA. First, we isolated a

cDNA clone for *HrFGFR*. Northern blot analyses and in situ hybridization revealed that the *HrFGFR* gene is expressed both maternally and zygotically (Figs 4 and 5); the latter method indicated the zygotic *HrFGFR* expression begins after gastrulation and that the gene is expressed mainly in epidermal cells. Previous blastomere isolation and recombination experiments demonstrated that the cell-cell interactions that are required for notochord and mesenchyme differentiation occur during the period when the 32-cell embryo divides to produce the 64-cell embryo (Nakatani and Nishida, 1994; Nakatani et al., 1996; Kim et al., 2000). We investigated the distribution of HrFGFR protein in the early embryos by immunostaining with commercially available antibodies with no avail, and this should be investigated in future studies. However, it is likely that the effects of *dnHrFGFR* mRNA injection on the differentiation of notochord cells and mesenchyme cells can be attributed to a disturbance of HrFGFR function derived from maternally transcribed mRNA. However, the effects on the nervous system formation assessed by *HrTBB2* expression may be explained by the following two observations. First, the zygotic expression of *HrFGFR* is seen in the midline cells as well as the anterior most cells. Therefore, disturbance in the patterning of *HrTBB2*-positive cells is attributable to the failure of *HrFGFR* function derived from zygotically transcribed mRNA. Second, it is possible that the patterning of the nervous system requires interactions with underlying notochord directly or indirectly through nerve cord cells, and therefore the failure of notochord differentiation would disturb the patterning of *HrTBB2*-positive cells. Although we cannot yet exclude the possibility that zygotically derived HrFGFR plays a role, it is more likely that the observed changes in *HrTBB2*-positive cell patterning was caused by the disruption of the notochord.

The degree of the disturbance of *HrFGFR* function caused by the microinjection of *dnHrFGFR* mRNA was not always absolute, and the results were not always the same in different batches of eggs (Table 1). This is mainly due to degradation of injected mRNA. In addition, as reported by Kamei et al. (Kamei et al., 2000) and shown by the present study, it is likely that the *HrFGFR* gene may be transcribed into two different types of mRNA by alternative splicing. If the HrFGFR that was characterized by Kamei et al. (Kamei et al., 2000) functions as a receptor of bFGF signals, it is possible that the injection of *dnHrFGFR* mRNA does not disrupt the FGF signaling cascade completely. This may be another reason for that the downregulation of *HrBra* expression by microinjection of *dnHrFGFR* mRNA was not always absolute.

Relationship between endoderm differentiation and notochord specification in the *Halocynthia* embryos

The requirement of endodermal cells for specification of notochord cells has been shown in two different ascidian species *H. roretzi* and *Ciona intestinalis* (as well as *C. savignyi*). In *H. roretzi*, it was shown that cell contact between presumptive notochord cells and endodermal cells during the early phase of the 32-cell stage is necessary and sufficient for the subsequent differentiation of notochord cells (Nakatani and Nishida, 1994). However, one intriguing result that these authors showed is that contact of two presumptive notochord cells during this stage will also result in the subsequent differentiation of notochord cells (Nakatani and Nishida, 1994). That is, in their experiments, endodermal cells are not always required for notochord differentiation. Therefore, this inductive process cannot always be explained by a scenario in which endodermal cells release FGF that functions as the ligand which binds to receptors on the presumptive notochord cells, even if exogenous bFGF can mimic normal cellular interactions (Nakatani et al., 1996). However, there is evidence that FGF regulates *Brachyury* in the process of mesoderm formation of vertebrate embryos (Issacs et al., 1994; Schulte-Merker and Smith, 1995; Griffin et al., 1995).

In *Ciona* embryos, endodermal cells are first specified by maternally derived cytoplasmic determinants, and β-catenin is involved in the endodermal cell specification of *Ciona* embryos (Yoshida et al., 1998; Imai et al., 2000). During early cleavages of *Ciona* embryos, β-catenin was shown to accumulate in the nuclei of vegetal blastomeres, suggesting that it plays a role in the specification of endoderm. Mis- and/or overexpression of β-catenin induced the development of ectopic endodermal cells from presumptive notochord cells and epidermal cells. Downregulation of β-catenin induced by the overexpression of cadherin resulted in the suppression of endodermal cell differentiation. This suppression was accompanied by the differentiation of extra epidermal cells. Both the overexpression of β-catenin in presumptive notochord cells and the downregulation of β-catenin in presumptive endodermal cells led to the suppression of *Brachyury* gene expression, resulting in the failure of notochord specification, indicating that specification of notochord cells does not take place in the absence of endoderm differentiation.

Brachyury (both *HrBra* and *Ci-Bra*) is a key regulator gene for notochord differentiation in ascidian embryos. This gene begins to be expressed at the 64-cell stage, immediately after interaction with endodermal cells at the 32-cell stage. The minimal promoter required for notochord-specific expression of this gene has been characterized both for *Ci-Bra* (Corbo et al., 1997) and *HrBra* (Takahashi et al., 1999b). Within about 500 bp upstream of the transcription start site of *Ci-Bra*, there are three distinct regions which regulate the notochord-specific expression of the gene: first, there is a distal region responsible for repression of expression in non-notochord mesoderm (mesenchyme and muscle) cells; second, there is an intermediate region for the activation of expression in notochord cells; and third, there is a proximal region for the activation of non-notochord mesoderm cells (Corbo et al., 1997). The notochord-specific activation domain of the *Ci-Bra* promoter contains the Suppressor of Hairless [Su(H)] binding site, and thereforeit has been suggested that the Notch signal cascade is involved in the interaction with endodermal cells (Corbo et al., 1998). Furthermore, the non-notochord mesoderm suppression domain of the promoter contains Snail binding sites. Fujiwara et al. (Fujiwara et al., 1998) showed that the *snail* gene of *C. intestinalis* (*Ci-sna*) is expressed in mesenchyme and muscle to prevent *Ci-Bra* expression in inappropriate lineages. Together with a scenario first proposed by Kim et al. (2000) in which endodermal cells emanate signals to induce both notochord and mesenchyme cells, the above mentioned results may explain how the *Brachyury* gene is expressed solely in notochord cells.

However, interaction between endoderm and notochord cells and the initiation of *Brachyury* gene expression in ascidian embryos seems to be much more complex than we presently

can appreciate. For example, the *snail* gene of *H. roretzi* (*HrSna*) shows a slightly different pattern of expression when compared with *Ci-sna* (Wada and Saiga, 1997). In addition to its expression in muscle and mesenchyme lineages, *HrSna* is also expressed in an overlapping pattern with *HrBra* at the 64-cell and 110-cell stages, suggesting that the mechanism in which *HrSna* expression defines the boundary between the notochord and non-notochord mesoderm is not applicable to *Halocynthia* embryos despite the fact that the minimal promoter of *HrBra* does not contain HrSna binding sites (Takahashi et al., 1999b). Analysis of the minimal promoter of *HrBra* that is required for its notochord-specific expression revealed the importance of two critical sequences and gel-shift assays suggest that specific types of nuclear proteins bind to these sequences (Takahashi et al., 1999b). Therefore, it looks promising that we will be able to characterize these DNA-binding proteins that regulate the expression of *HrBra*.

In conclusion, the present study provides evidence for the involvement of FGF signals in the differentiation of notochord and mesenchyme cells in *Halocynthia* embryos. However, the results also raise many unresolved questions that need to be answered by future studies so that our understanding of the cellular and molecular mechanisms responsible for notochord differentiation of ascidian embryos can be applied to the fundamental question how the notochord arose during evolution of chordates.

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