Limited functions of Hox genes in the larval development of the ascidian *Ciona intestinalis*

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SUMMARY

In animals, region specific morphological characters along the anteroposterior axis are controlled by a number of developmental genes, including Hox genes encoding homeodomain transcription factors. Although Hox genes have been regarded to play a key role in the evolution of morphological diversity, as well as in the establishment of the body plan, little is known about the function of Hox genes in invertebrates, except for in insects and nematodes. The present study addresses the role of Hox genes in body patterning during the larval development of the ascidian *Ciona intestinalis* conducting knockdown experiments of the seven Hox genes expressed during embryogenesis. Experimental results have demonstrated that Ci-Hox12 plays an important role in tail development through the maintenance of expression of Ci-Fgf8/17/18 and Ci-Wnt5 in the tail tip epidermis. Additionally, it has been shown that Ci-Hox10 is involved in the development of GABAergic neurons in the dorsal visceral ganglion. Surprisingly, knockdown of Ci-Hox1, Ci-Hox2, Ci-Hox3, Ci-Hox4 and Ci-Hox5 did not give rise to any consistent morphological defects in the larvae. Furthermore, expression of neuronal marker genes was not affected in larvae injected with MOs against Ci-Hox1, Ci-Hox3 or Ci-Hox5. In conclusion, we suggest that the contribution of Hox genes to the larval development of the ascidian *C. intestinalis* might be limited, despite the fact that Ci-Hox10 and Ci-Hox12 play important roles in neuronal and tail development.

KEY WORDS: Hox, Ascidian, Larval development, *Ciona intestinalis*

INTRODUCTION

Morphological diversification of body regions depends on the formation of characteristic structures along the various body axes. It has long been noted that the system specifying positional identity along the anteroposterior (AP) axis is based on an evolutionary conserved set of regulators, the Hox genes (Carroll, 1995; McGinnis and Krumlauf, 1992). Hox genes are known for the notable coordination, referred to as ‘colinearity’, between their expression patterns along the AP axis and the position within their clustered arrangement on a chromosome. This leads to the unique combination of Hox genes expressed at the different AP axial levels during development, which is also referred to as the ‘Hox code’ (Kessel and Gruss, 1991). Despite the fact that Hox genes have been regarded to play a key role in the evolution of morphological diversity, as well as in the establishment of the body plan, little is known about the function of Hox genes in invertebrates, except for in insects and nematodes. Therefore, it is considered necessary to understand the developmental role of Hox genes in a wide variety of invertebrates, especially in animal species occupying key positions in the phylogeny.

Ascidians belong to the subphylum Urochordata, a branch within the phylum Chordata. Their eggs develop into tadpole larvae, which share a prototypical morphogenesis and chordate body plan, characterized by the presence of a hollow dorsal neural tube, a notochord, paraxial mesoderm and a postanal tail (Satoh, 1994; Satoh, 2008). However, there are some crucial differences in the development between ascidians and vertebrates. In vertebrates, body patterning along the AP axis proceeds from anterior to posterior, generating elaborate segmental vertebra, spinal cord and gut, for example. In this process, Hox genes play a crucial role; loss of the function of one Hox gene frequently leads to a drastic morphological defect, the homeotic transformation, in which the character of one body segment transforms into that of the flanking segment (Favier and Dolle, 1997). By contrast, ascidians exhibit extremely rapid and predominantly mosaic development, generating a simple tadpole-like body consisting of approximately 2600 cells without conspicuous segmental structures. Furthermore, they subsequently metamorphose into sessile adults with a unique body structure.

In the ascidian species *Ciona intestinalis*, nine Hox genes have been identified (Dehal et al., 2002). Previously we demonstrated that *Ciona* Hox genes are dispersed on two chromosomes and that eight Hox genes, *Ci-Hox1, 2, 3, 4, 5, 10, 12* and *13* are expressed up to the juvenile stage. *Ci-Hox1, 3, 5, 10* and *12* exhibit limited spatially coordinated expression in the larval neural tube, as do *Ci-Hox10, 12* and *13* in post-larval gut development (Ikuta et al., 2004). Based on these observations, a scenario for the evolution of the ascidian body plan has been proposed, in which ascidians must have acquired their simple body plan and rapid embryogenesis together with extensive genomic rearrangement and gene loss, including disintegration of the Hox gene cluster and loss of some Hox gene members (Ikuta and Saiga, 2005). To fully evaluate the scenario, an in-depth functional analysis of the ascidian Hox genes needs to be performed.

To investigate the biological functions of the seven Hox genes of *C. intestinalis, Ci-Hox1, 2, 3, 4, 5, 10* and *12*, of which expression has been detected by whole-mount in situ hybridization (WISH) during embryogenesis, translational inhibition experiments using antisense morpholino oligonucleotides (MOs) directed against them were conducted. We have found that *Ci-Hox12* plays an important role in tail development by maintaining the expression of *Ci-Fgf8/17/18* and *Ci-Wnt5* after the early tailbud stage. Additionally, *Ci-Hox10* is involved in the development of GABAergic neurons in...
the dorsal visceral ganglion. Conversely, for Ci-Hox1, 2, 3, 4 and 5, no morphological defects were detected in the knockdown experiments. Furthermore, the expression of GABAergic and cholinergic neuronal marker genes was unaffected in larvae injected with MOs against Ci-Hox1, 3 and 5, despite the fact that Ci-Hox1, 3, 5, 10 and 12 are coordinately expressed in the larval CNS (Ikuta and Saiga, 2005; Ikuta and Saiga, 2007; Ikuta et al., 2004). These results suggest that the roles of Hox genes are limited in ascidian larval development.

MATERIALS AND METHODS

Ascidians

C. intestinalis were cultivated at the Maizuru Fisheries Research Station of Kyoto University or at the International Coastal Research Centre, Ocean Research Institute, the University of Tokyo. Eggs and sperm were obtained surgically from the gonoducts.

Antisense morpholino oligonucleotides (MOs) and synthetic mRNAs

MOs (Gene Tools) used in the present study were as follows:

Hox1 MO: equimolar mixture of 5'-AATTCATCTCTACACCTTTCTATATAA-3' and 5'-CAGATCTTCTGTTGTCGCTGACATC-3' (see below);

Hox2 MO: 5'-TCTGGCTACACATTTCTCCAATTCT-3';

Hox3 MO: 5'-AGTACATGTCGATCTCTGTA-3';

Hox4 MO: 5'-TTTGACAGAGAATATGAATACC-3';

Hox5 MO: 5'-AGATTCGGTTAATCATCATCG-3';

Hox10 MO: 5'-TCGAAAATGGAATGGATGATAACATC-3';

Hox16 MO: 5'-TGGATGGAATGATCCATGTTTAA-3';

Hox12 MO: 5'-CATAGGGATTCAACAGACTAAATAT-3';

Hox12 MO2: 5'-TGAGGTTGAATATCCATTGTTTTAA-3';

Hox12 MO3: 5'-CGCCGCTAAAAACCTTTGCTGTCAT-3';

Hox5 MO1: 5'-AAATTCATCTTACCTCCTTTGTT -3';

Hox1 MO1: 5'-CATCGGGATACAACAC -3';

Hox1 MO2: 5'-CATATTCGCGCAACGATCCTGATACACTT-3';

Fg8/17/18 MO: TACTGCGATGCTWACATTAAATC-3'.

Because both Hox1/2MO1 and Hox1/2MO2 produced the same morphological defects, only Hox1/2MO1 was used in the following experiments. In Ci-Hox10 and Ci-Hox12 knockdown experiments, mutated Hox10/1MO1 and Hox12/1MO1 were used as controls, respectively, with five base-pair mismatches: Hox10/1 muMO, 5'-TGGATGGAATGATCCATGTTTAA-3'; and Hox12/1 muMO, 5'-TCGAAAATGGAATGGATGATAACATC-3'. Additionally, a fluorescein-labelled standard control MO, FeMO (5'-CCTCTTACCTCAGTTACATTTGATA-3'), was used as the cell-line tracer.

The ability of Hox1 MO, Hox3 MO, Hox4 MO, Hox5 MO, Hox10 MO1 and Hox12 MO1 to inhibit translation was checked by examining the effect of the MOs on the expression of a lacZ reporter gene prepared by inserting a lacZ reporter construct (data not shown).

Microinjection and sample preparation

For preparation of samples at the tailbud stage, unfertilized eggs were dechorionated and microinjected with 10-20 fmoles of MO and/or 200 fg of reporter construct DNA, which were dissolved in 0.1 × TE containing 0.5 mg/ml of Fast Green (Wako). For the knockdown of Ci-Wnt5, 2.5 fmoles of Wnt5MO was co-injected with 1.25 fmoles of FeMO into both b/b4/2 cells at the eight-cell stage. In rescue experiments, 10 fmoles of MO and 20 pg of synthetic mRNA were introduced into an egg. Injected eggs or embryos were reared at 16-20°C in Millipore-filtered sea water (MFSW), or artificial sea water (Rohtomarine; Rohto, Japan). For preparation of samples of larvae, fertilized eggs with intact chorion were microinjected.

Histochemical staining and WISH

lacZ reporter gene expression was visualized by histochemical staining for β-galactosidase as described previously (Hikosaka et al., 1994). Phalloidin staining was carried out as described previously (Christiaen et al., 2005). Images were captured using a CCD camera on a Keyence BZ-8000 microscope, a Nikon E600 microscope equipped with Nikon EZ C1 confocal scanning system, or a Zeiss LSM 5 Exciter confocal scanning system. To measure the length of the notochord, AP dimensions of all the notochord cells were taken sequentially in a confocal sagittal section through the midline with ZEN LSM micro-imaging software (Zeiss) and added up for each specimen. For each measurement, two individuals were used.

WISH was carried out as described previously (Ikuta and Saiga, 2007; Ikuta et al., 2004; Katsuyama et al., 1995). Antisense RNA probes for Ci-MRLC2, Ci-Wnt5, Ci-En, Ci-SoxB1, Ci-Eph3, Ci-EphC, CiGC25h02 and CiGc16g22 were synthesized using EST clones as templates, obtained from Ciona intestinalis Gene Collection release 1 (http://ghost.zool.kyoto-u.ac.jp/index1.html). Templates for RNA probe synthesis of Ci-Hox1, 3, 5, 10 and 12, Ci-En, Ci-Eph3, Ci-vatP, Ci-vatGAT, Ci-Pax2/5/8 and Ci-Fg8/17/18 were as described previously (Ikuta and Saiga, 2007; Ikuta et al., 2004). For the simultaneous detection of three-marker gene expression, hybridization was performed in the presence of fluorescein-, digoxigenin-, and DNP-labelled probes. DNP was detected by anti-DNP-HRP and stained with cyanine-5 tyramide (Perkin Elmer). Imaging was as described previously (Ikuta and Saiga, 2007).

Treatment with Fgf signalling pathway inhibitor

U0126 (Promega), a MAPK kinase (MEK) inhibitor, or SU5402 (Calbiochem), an Fgf receptor inhibitor, was dissolved in dimethylsulfoxide (DMSO) to 10 mM and stored as stock solution at −80°C. Embryos at the neurula or early tailbud stages were transferred to freshly prepared 2, 10 or 20 μM U0126 or SU5402 in sea water, or to 0.02, 0.1 or 0.2% DMSO in sea water, reared until the mid-tailbud stage and fixed for phalloidin staining or WISH.

RESULTS

Translational inhibition of Ci-Hox12 causes a morphological defect in the tailbud embryos

To address the role of the seven Hox genes expressed during the larval development of Ciona intestinalis, translational inhibition experiments using MOs were carried out. In tailbud embryos and larvae injected with MOs against Ci-Hox1, 2, 3, 4, 5 or 10, no morphological defects were consistently detected (data not shown; see Fig. S1 in the supplementary material), despite the positive results of confirmatory experiments for the effectiveness of the MOs used (see Materials and methods). Furthermore, expression of the genes that share the expression domain with the Hox genes at tailbud or larval stage was unaffected in the tailbud embryos or larvae injected with MOs against Ci-Hox1, 2, 3, 4 and 5 (see Fig. S1 in the supplementary material).

By contrast, embryos injected with a MO against Ci-Hox12 had a round-ended tail, which was slightly shorter in length (9.9% on average, n = 12, P < 0.001) than that of control embryos (Fig. 1A,B;
Fig. 4I,J). Eggs injected with the control mutated MO (Hox12muMO) developed into normal tailbud embryos with a tapered tail end (Fig. 1C).

In normal larval development, Ci-Hox12 is expressed from neurula stage onwards in posterior ectodermal cells, and at the tailbud stage in the end of the posterior nerve cord and epidermis (Ikuta et al., 2004). To see the effect of Ci-Hox12 knockdown, the morphology of Hox12MO-injected embryos was examined in detail by confocal microscopy. First, in embryos injected with Hox12MO, the epidermal cells at the tail tip were cuboidal, whereas they are columnar in control embryos (Fig. 1D-G). Second, the nerve cord did not reach the tail end and, in the region posterior to the nerve cord end, epidermal cells, or even more frequently one or two large cells, were located on the dorsal side of the notochord (Fig. 1F,G). The number of notochord cells was unchanged between Hox12MO-injected and control embryos (data not shown). Confocal transverse sections close to the tail end revealed that the muscle cells met on the dorsal side of the notochord in Hox12MO-injected embryos, whereas the nerve cord on the dorsal side of the notochord separated muscle cells laterally in control embryos (Fig. 1K,O). This was confirmed by visualizing the expression of a muscle marker gene Ci-MRLC2, the Ciona homologue of the myosin regulatory light chain gene (Fig. 1H-J,L,M). Furthermore, the number of muscle cells in Hox12MO-injected embryos was eighteen on each side of the tail, similar to the control embryos (Fig. 1P,Q); therefore, the abnormal localization of muscle cells is most likely to be indirectly produced by the defect of nerve cord elongation.

These observations indicate that Ci-Hox12 plays an important role in tail development by controlling elongation of both the tail nerve cord and the tail itself, and by controlling the morphology of the tail end and of the epidermal cells at the tail tip.

Translational inhibition of Ci-Hox12 affects signal transduction gene expression

To further understand the function of Ci-Hox12 in the tail development, the effects of Ci-Hox12 knockdown on gene expression at the tail end of tailbud stage embryos were examined. We identified Ci-Fgf8/17/18 and Ci-Wnt5 as the genes expressed in the tail tip epidermis in normal mid-tailbud embryos (our unpublished results; Fig. 2A,C; see also http://hoya.zool.kyoto...
Expression of Ci-Fgf8/17/18 and Ci-Wnt5 in the tail tip epidermis was extensively reduced in Hox12MO-injected embryos, whereas expression outside of the tail tip was unaffected (Fig. 2A-D). Closer examination of the expression of these genes in embryos injected with Hox12muMO revealed that their expression was normal (data not shown).

In an attempt to demonstrate specificity of the action of Hox12MO, a rescue experiment was carried out in which embryos were co-injected with Hox12MO and Ci-Hox12 synthetic mRNA. The positioning of the muscle cells was normal and the expression of Ci-Fgf8/17/18 and Ci-Wnt5 at the tail tip was recovered (Fig. 2E,H). Furthermore, the nerve cord reached the tail end (Fig. 2I) and in some embryos epidermal cells at the tail tip recovered, exhibiting columnar morphology (Fig. 2J). Thus, the phenotypes observed in embryos injected with Hox12MO originated from the specific knockdown of Ci-Hox12 gene function.

**Ci-Hox12 controls tail length through the maintenance of Ci-Fgf8/17/18 expression**

To explore further the role of Ci-Hox12 in controlling tail morphology, the roles of the signalling genes regulated by Ci-Hox12 at the tail tip, Ci-Fgf8/17/18 and Ci-Wnt5, were examined. At first, the effect of blocking Fgf signalling on tail morphogenesis was evaluated. Embryos were treated with SU5402, an Fgf receptor inhibitor, and U0126, a MEK inhibitor, from neurula stage, when the expression of Ci-Fgf8/17/18 at the posterior end becomes detectable by WISH, and morphology was examined at the mid-tailbud stage. Embryos treated with 10 μM SU5402 exhibited morphological defects over the whole body, tail elongation was severely inhibited and was accompanied by a slight intercalation defect of notochord cells; embryos treated with 2 μM SU5402 did not show any defects (Fig. 3G,H). In embryos treated with U0126, a similar tail elongation inhibition was observed and was dose dependent (Fig. 3D-F). In this case, however, some columnar cells were observed on the dorsal side close to the tail tip (Fig. 3D,F, insets). This was in contrast to what was observed in embryos treated with SU5402, in which the columnar cells were located at the tail tip, similar to in normal embryos (Fig. 3H, inset). This difference might reflect a wider inhibitory effect of U0126 on activities downstream of various signalling molecules, including Fgfs. Furthermore, embryos injected with Fgf8/17/18MO had a shortened tail with columnar epidermal cells at the tip (Fig. 3I).

In normal development, Ci-Fgf8/17/18 transcripts are detectable by WISH at the posterior end of the embryo from the neurula stage onwards, which is the same timing as the onset of Ci-Hox12 expression. Therefore, it is likely that Ci-Hox12 is involved in the maintenance but not the onset of Ci-Fgf8/17/18 transcription. This is supported by the experiment examining Ci-Fgf8/17/18 expression in Hox12MO-injected embryos at neurula and early tailbud stages. As shown in Fig. 4A-D, the frequency of Ci-Fgf8/17/18-positive embryos, which were indistinguishable from normal embryos, was 100% at the neurula stage and about 50% at the early tailbud stage. This is in contrast with the mid-tailbud stage, at which downregulation of Ci-Fgf8/17/18, as well as shortening of the tail, was observed in over 90% of Hox12MO-injected embryos. These observations suggest that the function of Ci-Hox12 in maintaining Ci-Fgf8/17/18 expression at the tail tip occurs from around the early tailbud stage onwards. Next, embryos were treated with SU5402 from the early tailbud but not neurula stage onwards and the length of the notochord was measured to see whether the treatment reproduces the tail shortening effect. The extent of tail shortening was 14.6% (n=12, P<0.001) in SU5402-treated embryos (Fig. 4K,L), which was comparable to the 9.9% (n=12, P<0.001) observed in Hox12MO-treated embryos (Fig. 4L,J). It should be noted that the shortening of the tail was the only conspicuous morphological defect observed in the Fgf receptor inhibitor-treated embryos.

These observations suggest that Ci-Hox12 plays an important role in maintaining the expression of Ci-Fgf8/17/18 at the tail tip, which in turn controls the length of the tail, but not the cell morphology, after the early tailbud stage.

**Ci-Hox12 controls epidermal cell shape at the tail end through the maintenance of Ci-Wnt5 expression**

Next, the involvement of Ci-Wnt5 in tail morphogenesis was examined. For this, the b4.2 cells of the eight-cell stage embryo, the precursors of the posterior epidermal cells (Nishida, 1987), were co-injected with the MO against Ci-Wnt5 and the tracer fluorescein-labelled standard control MO (FcMO). The morphology at the mid-tailbud stage was examined after phalloidin staining. Control embryos injected with FcMO did not exhibit any defects. By contrast, embryos co-injected with Wnt5MO had cuboidal but not columnar cells at the tail tip, and a rounded rather than a tapered tail end (Fig. 5A,B). The length of the tail did not seem to be affected (data not shown). This observation suggested that Ci-Wnt5 might be involved in the formation of columnar cells at the tail tip under the control of Ci-Hox12. As it was noted in the previous section that columnar cells were present in the embryos in which Fgf signalling was inhibited, the embryos treated with Fgf signalling inhibitors
were examined for Ci-Wnt5 expression. As shown in Fig. 6, expression of Ci-Wnt5 was detected in the tail end where the columnar epidermal cells were located (Fig. 6D-H). Furthermore, expression of Ci-Wnt5 was also detected in the tapered tail end of the embryos injected with the Fgf8/17/18MO (Fig. 6I-L). Conversely Ci-Fgf8/17/18 expression in the tail tip epidermis was unaffected in embryos injected into the epidermal lineage with Wnt5MO (Fig. 5C), which suggests that the function of Ci-Wnt5 in the tail epidermis is independent of Ci-Fgf8/17/18.

Because the expression of Ci-Wnt5 in the posterior epidermis becomes detectable by WISH at the gastrula stage, before the onset of Ci-Hox12 expression, Ci-Hox12 does not seem to be involved in the onset of Ci-Wnt5 expression. Thus, the expression of Ci-Wnt5 in Hox12MO-injected embryos at neurula and early tailbud stage was investigated. Expression of Ci-Wnt5 was detected in the tail tip epidermis in 100% of the Hox12MO-injected embryos at both stages (Fig. 4E-H). This is in strong contrast to the mid-tailbud stage, in which over 90% of the Hox12MO-injected embryos showed a downregulation of Ci-Wnt5. These observations suggest that Ci-Wnt5, the expression of which at the tail tip is maintained by Ci-Hox12 after early tailbud stage, plays a role in the formation of columnar cells at the tail tip and in the formation of tapered tail end morphology.

**Functional analysis of Ci-Hox1, 3, 5 and 10 in larval CNS development**

As described above, morphological defects were not detected in the tailbud embryos and larvae injected with MOs against Ci-Hox1, 2, 3, 4, 5 and 10. However, because they are coordinately expressed in the neural tube during C. intestinalis embryogenesis, it has been envisaged that Ci-Hox1, 3, 5 and 10 might be involved in regionalization of the larval CNS (Ikuta and Saiga, 2007; Ikuta et al., 2004). Therefore, we examined whether a function of Ci-Hox1, 3, 5 or 10 in CNS development was detectable.

Ci-Hox1, 3, 5 and 10 have been reported to be expressed in a region corresponding to the prospective visceral ganglion and the anterior nerve cord (Ikuta and Saiga, 2007). Within the region, it has been shown that GABAergic neurons are present in the visceral ganglion and the anterior nerve cord, and that cholinergic neurons are located in the visceral ganglion. Both types of neuron are involved in controlling the locomotor activity of Ciona larvae (Brown et al., 2005; Takamura et al., 2002; Yoshida et al., 2004). To investigate the function of Ciona Hox genes in neuronal development, the expression of Ci-vAChTP, a cholinergic neuron marker, and of Ci-vGAT, a GABAergic neuron marker, was examined in larvae injected with MOs against Ci-Hox1, 3, 5 or 10.

In un.injected control larvae, expression of Ci-vAChTP was detected in the sensory vesicle and the ventral visceral ganglion, and expression of Ci-vGAT was detected in the sensory vesicle, the dorsal visceral ganglion and the anterior nerve cord (Fig. 7A). Injection with MOs against Ci-Hox1, 3 and 5 did not affect the expression of either marker gene (Fig. 7B-D). Next, double knockdown experiments were conducted, as Hox genes have been described to function redundantly in a variety of animals (Akin and Nazarali, 2005; Favier and Dolle, 1997; Maconochie et al., 1996; Schilling and Knight, 2001). At the tailbud stage, Ci-Hox1 and Ci-Hox3 are co-expressed in a part of the presumptive visceral ganglion, and Ci-Hox1 and Ci-Hox5 are co-expressed in the anterior nerve cord (Ikuta and Saiga, 2007). Therefore, MOs against Ci-Hox1 and Ci-Hox3, or Ci-Hox1 and Ci-Hox5, were simultaneously introduced into fertilized eggs. In these cases too, no effects of the introduced MOs on neuronal marker gene expression or on larval morphology (Fig. 7G,H) were observed. By contrast, when either Hox10MO1 or Hox10MO2 was injected, the expression of Ci-vGAT in the dorsal visceral ganglion was downregulated in the larvae (Fig. 7E,F), whereas larvae injected with Hox10muMO exhibited normal expression of Ci-vGAT (data not shown). During normal development, Ci-Hox10 is expressed in a small subset of dorsal cells of the anterior nerve cord at the tailbud stage, but expression becomes undetectable by the larval stage (Ikuta and Saiga, 2007; Ikuta et al., 2004); the developmental fate of the Ci-Hox10 positive cells in relation to the larval CNS is not known. To assess the...
developmental fate, a lacZ reporter construct harbouring 5 kb of DNA from upstream of Ci-Hox10 was introduced into eggs. The construct recapitulated the endogenous expression of Ci-Hox10 in the anterior dorsal nerve cord with a frequency of 28% (n=21) for X-gal staining and 33% (n=32) for WISH at the late tailbud stage (Fig. 7L). At the larval stage, X-gal staining was detected in the dorsal visceral ganglion in 24% (n=21) of the injected specimens (Fig. 7J); however, the transcriptional signal of lacZ gene was not detected at this stage (n=21; Fig. 7L), and thus this is likely to represent carry-over of the β-galactosidase protein from the tailbud stage. These results suggest that the cells expressing Ci-Hox10 in the anterior dorsal nerve cord at tailbud stage contribute to GABAergic neurons in the dorsal visceral ganglion in the larva, and that the function of Ci-Hox10 might be required for the development of these neurons.

**DISCUSSION**

The present study outlines the functional analysis of the Hox genes expressed during larval development of the ascidian *Ciona intestinalis* in order to understand their roles in ‘chordate-type’ larval development. We have demonstrated that Ci-Hox12 and Ci-Hox10 play roles in tail and neuronal development, respectively. Furthermore, and rather surprisingly, knockdown of Ci-Hox1, 2, 3, 4 and 5 did not cause any consistent morphological defects.

**Involvement of Ci-Hox12 in tail morphology**

Upon the loss of Ci-Hox12 functions, the distinctive morphology of the tail – a tapered end with several columnar epidermal cells at the tip – is lost and the length of the tail is also shortened. We have demonstrated that under the regulation of Ci-Hox12, Ci-Wnt5 contributes to the formation of the columnar cells and the tapered tail shape, and Ci-Fgf8/17/18 contributes to the proper elongation of the tail. Although treatment with Fgf inhibitors could block the activity of any Fgf, Ci-Fgf8/17/18 is most likely involved in this process. This was supported by the observation that embryos injected with Fgf8/17/18MO had a shortened tail with tapered end (Fig. 3I, Fig. 6I). Recently, it has been reported that Ci-Fgf3/7/10/22 emanating from floor cells of the tail nerve cord directs notochord intercalation and, in turn, controls tail elongation through non-MAPK intercalation defects. However, Ci-Fgf8/17/18 was expressed only in a small number of cells in the tail tip epidermis and is expected to exert its effect in the close proximity of the tail tip, it is unlikely that Ci-Fgf8/17/18 affects notochord intercalation over the length of the tail during tail elongation. We speculate that Ci-Fgf8/17/18 might regulate tail elongation through the control of cell number or cell length along the AP axis of the posterior tail epidermis: if the function of Ci-Fgf8/17/18 were inhibited, the tail epidermis would not elongate accordingly. The notochord intercalation defect observed upon the administration of a high dose of Fgf signalling inhibitors in the present study (Fig. 3) could be attributed to a secondary effect due to insufficient space in the tail for the notochord cells to intercalate properly.

For the impaired elongation of the nerve cord, a possible candidate downstream of Ci-Hox12 that is involved in proper tail nerve cord elongation is Ci-EphrinA-d, which is expressed in the posterior half of the tail nerve cord and is downregulated upon knockdown of Ci-Hox12 (data not shown). Ephrin/Eph signalling has been noted to regulate cell shape, movement and attachment. Loss of Ci-EphrinA-d expression in the posterior nerve cord could induce a change in cell adhesion characters, which in turn might result in the loss of proper cellular contact between the posterior nerve cord and the tail end epidermis. Unfortunately, knockdown of Ci-EphrinA-d by MOs gave rise to such severe phenotypes of the embryo from early developmental stages onwards that the knockdown effect on nerve cord elongation could not be assayed (data not shown).

**Gene regulatory network of Hox, Fgf, Wnt and Cdx in the posterior body might be different in Ciona to in vertebrates**

In vertebrates, Fgf has been implicated in posterior body patterning via interactions with Wnt and retinoic acid, and in regulating the expression of Cdx, which in turn induces the expression of posterior Hox genes (Deschamps and van Nes, 2005; Keenan et al., 2006; Lohnes, 2003; Pilon et al., 2006; Shimizu et al., 2005). Regulation of Fgf and Wnt by Hox genes has never been reported in vertebrates. Gene regulatory relationships among Fgf, Wnt and Cdx in *C. intestinalis* seem to be different from those observed in vertebrates.

In the present study, Ci-Hox12 is required for the maintenance of expression of Ci-Fgf8/17/18 and Ci-Wnt5 in the tail tip epidermis. As the expression of Ci-Wnt5 at the tail tip was unaffected by treatment with an Fgf receptor inhibitor or injection of the Fgf8/17/18MO, and because expression of Ci-Fgf8/17/18 at the tail tip was detectable in Ci-Wnt5 knockdown embryos, the transcriptional regulation of these signalling molecules in the tail must be independent. Additionally, expression of Ci-Hox12 was not affected by treatment with SUS402, injection of the Fgf8/17/18MO or the knockdown of Ci-Wnt5 (see Fig. S2 in the supplementary material), which suggests that in *C. intestinalis* expression of the posterior Hox gene might not be regulated by Fgf or Wnt.
Fig. 7. Analysis of the effect of injection of MOs against Ci-Hox1, 3, 5 and 10 on neuronal development at the larva stage. (A-H) Two-colour fluorescence WISH in larva injected with MO(s) or in uninjected control larva for the expression of Ci-vACHTP (red) and Ci-vGAT (green), which are markers of cholinergic and GABAergic neurons, respectively, in the visceral ganglion and the anterior tail. Note that expression of Ci-vGAT in the dorsal visceral ganglion was downregulated in larvae injected with either Hox10MO1 or Hox10MO2 (open arrowhead in E and F, which should be compared with the positive signal in control sample indicated by the white arrowhead in A). For all panels, bright-field and two-colour fluorescence images are merged. (I-L) Expression of a lacZ reporter gene driven by a putative Ci-Hox10 enhancer shown by X-gal staining (I,J) and WISH (K,L) at the late tailbud stage (I,K) and the larval stage (J,L). Percentages of samples with positive reporter signals in the anterior nerve cord (black arrows in LK) and dorsal visceral ganglion (black arrow in J) are shown at the bottom right. For all panels, lateral views of the trunk to the anterior tail are shown. Anterior is to the left.

Alternatively Fgf and Wnt might redundantly regulate the expression of Ci-Hox12. It is also possible that Ci-Hox12 is downstream of the MAPK pathway and does not interact with Fgf, as expression of Ci-Hox12 was not affected by treatment with SU5402 but was downregulated by treatment with U0126 (see Fig. S2 in the supplementary material). Additionally, the expression domain of Ci-Hox12 is unlikely to overlap with that of Ci-Cdx (see Fig. S3 in the supplementary material), as the knockdown of Ci-Hox12 or blocking of Fgf signalling did not affect the expression of Ci-Cdx (see Fig. S3 in the supplementary material). It should be noted that the expression of Fgf, Wnt and Hox genes in the ascidian tail is similar to the expression of the gene counterparts in the tailbud of vertebrate embryos, although the tail end of the ascidian embryo is not such a pool of undifferentiated cells as the ascidian tailbud. In any case, the findings of this study point to novel interactions among the genes essential for chordate tail development.

Roles of Ci-Hox1, 2, 3, 4, 5 and 10 in Ciona development

The neural tube in ascidian larva, in which Ci-Hox1, 3, 5 and 10 are expressed, is an organ exhibiting regionalized structure, consisting of four regions along the AP axis: the sensory vesicle, the neck, the visceral ganglion and the tail nerve cord. As proposed previously, the neck, visceral ganglion and tail nerve cord could be homologous to the rhombospinal region of vertebrates (Ikuta and Saiga, 2007). In the vertebrate rhombomere, Hox genes have been reported to be key regulators for the differentiation of motoneurons. In many cases, knockout or knockdown of a Hox gene leads to striking defects in the character and distribution of motoneurons (Chandrasekhar, 2004; Guthrie, 2007). In the visceral ganglion and the anterior tail nerve cord of Ciona larva, GABAergic and cholinergic neurons have been suggested to be regulators of locomotor activity (Brown et al., 2005; Takamura et al., 2002; Yoshida et al., 2004). Although we presumed that the identity or location of these neurons could have been affected by the knockdown of Hox genes, expression patterns of Ci-vACHTP and Ci-vGAT, which mark cholinergic and GABAergic neurons, respectively, were unaffected in larvae injected with MOs against Ci-Hox1, 3 and 5, including after double knockdown of the co-expressed genes. Additionally, in the Ci-Hox1 null mutant line (kindly provided by Y. Sasakura, University of Tsukuba, Japan), expression of Ci-vACHTP and Ci-vGAT in the larval visceral ganglion and anterior nerve cord was not affected (data not shown). Furthermore, expression of the regional marker genes of the CNS along AP axis was unaffected in mid- or late-tailbud embryos injected with MOs against Ci-Hox1, 3 and 5 (see Fig. S1 in the supplementary material). It should be noted, however, that the developmental fates of the cells expressing Ci-Hox1, 3 and 5 in the CNS are currently unknown. There should be cells other than cholinergic and GABAergic neurons in the CNS around the junction of the trunk and the tail, but their character and location still remain to be described. Moreover, the innervation patterns of individual neurons in ascidian larva are still unclear. Therefore, the possibility cannot be excluded that functions of Ci-Hox1, 3 and 5 in the CNS might be uncovered by investigating the differentiation and/or innervation of individual cells expressing Hox genes.

By contrast, our present data suggest that cells expressing Ci-Hox10 in the anterior nerve cord at tailbud stages contribute to GABAergic neurons in the dorsal visceral ganglion of larva, and that Ci-Hox10 is involved in the development of these neurons. Currently it is not clear whether the downregulation of Ci-vGAT in the dorsal visceral ganglion upon knockdown of Ci-Hox10 results from cell death or a change in neuronal identity. Interestingly, it has
been previously reported that mouse Hox10 paralogous genes coordinateably regulate lumbar motoneuron patterning (Lin and Carpenter, 2003; Wu et al., 2008). For further study, detailed anatomical data in combination with molecular data of the larval ascidian CNS are required.

Lastly, morphological defects in regions other than neural tissues were not detected in the embryos and larvae injected with MOs against Ci-Hox1, 2, 3, 4, 5 and 10. Most of these genes have been reported to be expressed also in the mesoderm or endoderm during embryogenesis: Ci-Hox1 in larval endoderm; Ci-Hox4 in trunk lateral cells and the mesenchyme; Ci-Hox5 in trunk lateral cells; Ci-Hox10 in the posterior endoderm and the endodermal strand; and Ci-Hox2 in the larval ectodermal atrial primordia [in a previous study, it was described in trunk lateral cells (Ikuta et al., 2004)]. As the cells in these regions remain undifferentiated in embryogenesis and later contribute to the adult body (Hirano and Nishida, 1997; Hirano and Nishida, 2000), it is possible that the roles of Hox genes in these tissues become evident during or after metamorphosis. In spite of this possibility, Hox genes seem to have very limited roles, if any, in the patterning of mesodermal and endodermal tissues during larval development.

Conclusions

Taken together, the findings of this study suggest that, apart from Ci-Hox10 and 12, Ciona Hox genes do not play crucial roles in the morphogenesis of tadpole larva. It has been proposed that Hox genes were organized into a cluster in the ancestor of deuterostomes and that the Hox gene cluster became disorganized in echinoderm and urochordate lineages (Cameron et al., 2006; Ikuta and Saiga, 2005; Lemons and McGinnis, 2006; Seo et al., 2004). It is an indisputable fact that ascidian Hox genes are no longer all clustered and that the colinearity is residual. The present study suggests that their functional roles are also limited as far as larval development is concerned. Coordinated functions of Hox genes to regionalize the segmental structure along the AP axis have been mainly described in relatively slow-developing animals; thus, a correlation is present between the limited roles of Hox genes in ascidian embryogenesis and the rapid embryogenesis that forms a tadpole larva with a simple body plan lacking obvious segmental structures.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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