Development 139, 2156-2160 (2012) doi:10.1242/dev.080234

Retinoic acid-driven *Hox1* is required in the epidermis for forming the otic/atrial placodes during ascidian metamorphosis

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SUMMARY

Retinoic acid (RA)-mediated expression of the homeobox gene *Hox1* is a hallmark of the chordate central nervous system (CNS). It has been suggested that the RA-*Hox1* network also functions in the epidermal ectoderm of chordates. Here, we show that in the urochordate ascidian *Ciona intestinalis*, RA-*Hox1* in the epidermal ectoderm is necessary for formation of the atrial siphon placode (ASP), a structure homologous to the vertebrate otic placode. Loss of *Hox1* function resulted in loss of the ASP, which could be rescued by expressing *Hox1* in the epidermis. As previous studies showed that RA directly upregulates *Hox1* in the epidermis of *Ciona* larvae, we also examined the role of RA in ASP formation. We showed that abolishment of RA resulted in loss of the ASP, which could be rescued by forced expression of *Hox1* in the epidermis. Our results suggest that RA-*Hox1* in the epidermal ectoderm played a key role in the acquisition of the otic placode during chordate evolution.

KEY WORDS: *Hox1*, Retinoic acid, Ascidian, Placode, Atrial siphon, *Ciona intestinalis*

INTRODUCTION

Hox1 plays a key role in anterior-posterior axis specification of the CNS in chordates (McGinnis and Krumlauf, 1992), and its expression is regulated by retinoic acid (RA) (Gavalas and Krumlauf, 2000). It has been suggested that this RA-*Hox1* network functions in the general ectoderm of chordates (Holland, 2005), which gives rise to both the epidermis and the nervous system. Indeed, in extant cephalochordates and urochordates, RA-mediated *Hox1* regulation is observed in the epidermis in addition to the CNS (Schubert et al., 2004; Ikuta et al., 2004; Kanda et al., 2009). In vertebrates, RA-*Hox1* is necessary for formation of the otic placode (Hans and Westerfield, 2007; Makki and Capecchi, 2010), a chordate-specific structure in the cranial epidermal ectoderm (Shimeld and Holland, 2000). Because the RA-*Hox1* network is crucial for specification of the CNS (Mark et al., 1993), which sends inductive signals to the overlying otic placode, the role of this network in otic placode development has generally been thought to be indirect. However, a recent study has indicated that mouse *Hoxa1* is expressed in the otic epithelium (Makki and Capecchi, 2010). Thus, it is possible that the epidermal RA-*Hox1* network contributed to the evolutionary innovation of the otic placode in higher chordates; however, the relationship between the epidermal RA-*Hox1* network and the otic placode remains unclear.

Here, we report the epidermal functions of the RA-*Hox1* cascade in the urochordate *Ciona intestinalis*. RA-*Hox1*-deficient animals do not form an atrial siphon placode (ASP), which is homologous to the vertebrate otic placode (Kourakis and Smith, 2007). Tissue-specific expression analysis of *Hox1* showed that the RA-*Hox1* cascade primarily functions in the epidermis to form the ASP. This study raises the possibility that RA-*Hox1* in the epidermal ectoderm played a key role in the acquisition of the otic placode during chordate evolution.

MATERIALS AND METHODS

Transgenic lines

An enhancer detection line EJ[MiTSAdTPOG]124 was created using the jump-starter method (Sasakura et al., 2008). Tg[MiTSAdTPOG]8 (Awazu et al., 2007) was used as a transposon donor. The *Minos* insertion site of EJ[MiTSAdTPOG]124 was determined by thermal asymmetric interlaced (TAIL)-PCR (Liu et al., 1995). Two transgenic lines, Tg[MiCiTnIG]2 and Tg[MiCiTnIGCiprmG]2, were used as muscle marker lines (Joly et al., 2007; Sasakura et al., 2008). Three transgenic lines, Tg[MiCiEpiIG]3, Tg[MiCiEpiIG]4 and Tg[MiCiCesACsCesA-CiEpiIG]4, were used as marker lines for the ASP (Joly et al., 2007; Sasakura et al., 2009; Sasakura et al., 2010). The GFP fluorescent images were pseudocolored green.

Plasmids

pRN3CiHox1

The cDNA containing the full open reading frame (ORF) of *Ci-Hox1* was amplified by RT-PCR with primers 5′-CCGGATCCCATGAA-TTCGTACATGAAATACC-3′ and 5′-TTTCACGTGACTATAT-TCATGTCC-3′. The amplified band was subcloned into the *Bgl*II and blunted *Eco*RI sites of pBS-RN3 (Lemaire et al., 1995) to create pRN3CiHox1.

pSPeCFP-ter

The ORF of eCFP was amplified by PCR with primers 5′-CTGGAATTCTTGTACAGCTCGTCC-3′ and 5′-AGCGGCCGCTATGG-TGAGCAAGGGCGA-3′. The PCR fragment was inserted into the *Not*I and *Eco*RI sites of pSP-eGFP (Sasakura et al., 2003) to create pSPeCFP-ter.

pSPeCFPCiHox1

An *Eco*RI fragment of *Ci-Hox1* cDNA was inserted into *Eco*RI site of pSP-eCFP-ter to create pSPeCFPCiHox1. A *Bam*HI fragment of a cis element of *Ci-CesA*, *Ci-AKR* and *Ci-β2TB* were isolated from pCesA(–2080)-GFP

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Accepted 8 April 2012

(Sasakura et al., 2005), pSPCiAKRK (Hozumi et al., 2010) and pSPCiβ2TBK (Horie et al., 2011), respectively. These cis elements were subcloned into *Bam*HI site of pSPeCFPCiHox1. A cis element of *Ci-TTF1* (Satou et al., 2001) was amplified from genomic DNA with primers 5′-TTTTGCGGCCGCCATCTCACAGCAAAGTTTCCAG-3′ and 5′-AAAAGCGGCCGCTAGTTCATGGTTAGCAATGAC-3′, digested with *Not*I and subcloned into the *Not*I site of pSPeCFPCiHox1.

Microinjection

The sequence of the antisense morpholino oligonucleotide (MO) for *Ci-Hox1* is 5′-AAACTTTACAACTTACTGCTTTTCG-3′. *Ci-Hox1* mRNA was synthesized with Megascript T3 kit (Ambion), poly A tailing kit (Ambion) and cap structure analog (New England Biolabs) using pRN3CiHox1 as a template. The concentrations of MO, mRNA and plasmid DNA in the injection medium were 0.5 mM, 200 ng/μl and 1-5 ng/μl, respectively. The sequence of the *Raldh* antisense MO is 5′-GTACTGCTGATACGACTGAAGACAT-3′. Embryos were treated with U0126 at a concentration of 10 μM.

Quantitative RT-PCR

Total RNA was extracted from juveniles using the AGPC (acid guanidinium-phenol-chloroform) method (Chomczynski and Sacchi, 1987). Genomic DNA was digested with DNaseI (Takara Bio). Reverse transcription was performed with Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primers. Quantitative reverse-transcription (RT)-PCR was carried out with SYBR Premix Ex Taq II (Takara Bio) and a Thermal Cycler Dice Real Time System TP800 (Takara Bio) following the manufacturer's instructions. *EF1α* was used as a normalizer gene. The PCR primers for *Ci-Hox1* were 5′-AAGCCAACTGTGTTACCACATG-3′ and 5′-ATGTTGTGGCGGATCTTGAAG-3′, and for *EF1α* they were 5′-CATGTCACGGACAGCGAAACG-3′ and 5′-CAATGTGTGTTG-AGGCATTCCAAG-3′.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Yoshida and Sasakura, 2012).

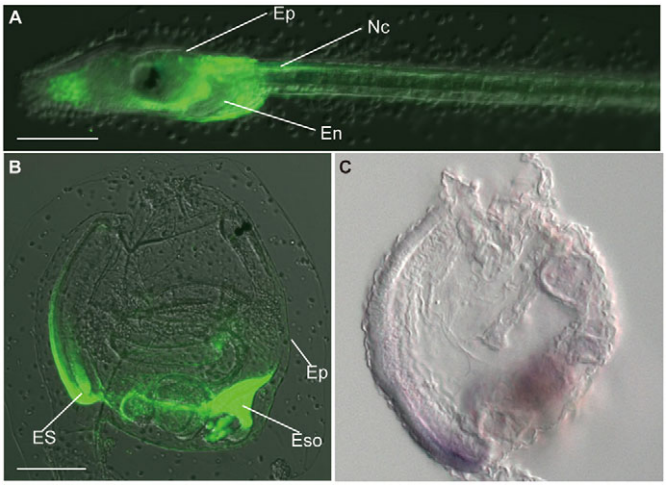

Fig. 1. GFP expression in EJ[MiTSAdTPOG]124 enhancer detection line. (**A**) Lateral view of a *Ciona intestinalis* larva (2 days post-fertilization) of EJ[MiTSAdTPOG]124 enhancer detection line. Scale bar: 100 μm. (**B**) Lateral view of a EJ[MiTSAdTPOG]124 juvenile. GFP is expressed in the posterior part of the endostyle (Es) and digestive tube, including the esophagus (Eso). Scale bar: 100 μm. (**C**) *Ci-Hox1* expression at the juvenile stage. *Ci-Hox1* is expressed in the posterior part of the endostyle and posterior part of the intestine including the esophagus. En, endoderm; Ep, epidermis; Nc, nerve cord.

RESULTS AND DISCUSSION

Loss of *Ciona Hox1* function results in loss of the gill slit and body wall muscle, and disrupted atrial siphon muscle formation

To investigate the role of the epidermal RA-*Hox1* network in chordates, we examined the function of RA-*Hox1* in urochordates, the closest evolutionary relatives of vertebrates (Delsuc et al., 2006). Previously, we used transposon-mediated enhancer trapping to create a green fluorescent protein (GFP)-enhancer trap reporter line, EJ[MiTSAdTPOG]124, in the ascidian *Ciona intestinalis* (Sasakura et al., 2008; Ikuta et al., 2010). In these animals, GFP is expressed in the same pattern as endogenous *Ciona intestinalis* (*Ci*-) *Hox1* (Fig.

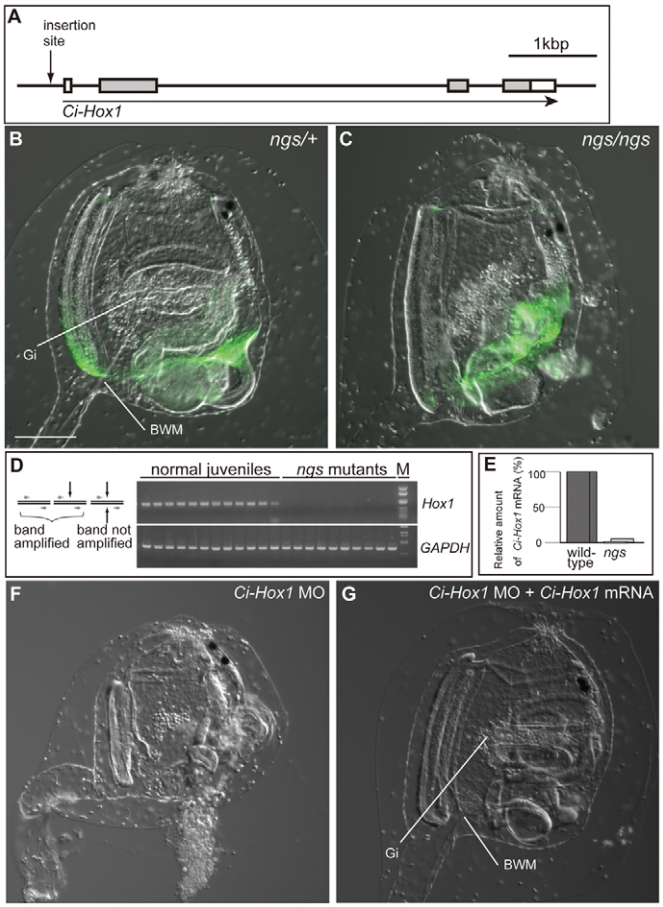

Fig. 2. *Ci-Hox1* is the affected gene in *ngs* mutants. (**A**) Insertion site of *Minos* in the EJ[MiTSAdTPOG]124 transgenic line. Exons are indicated by boxes. Gray and white boxes correspond to the open reading frame and untranslated regions, respectively. (**B**,**C**) A wild-type heterozygous *Ciona intestinalis* juvenile (left) and an *ngs* mutant (right). Scale bar: 100 μm. (**D**) Genomic PCR of normal and *ngs* mutant juveniles. The scheme of the experiment is shown on the left. Bars represent chromosomes and PCR primers are shown by gray arrowheads. Transposon insertions are shown by black arrows. PCR bands were not amplified from the genome of homozygous animals, because long transposon insertion interfered with PCR amplification. Normal juveniles showed PCR bands, whereas *ngs* mutants showed no PCR band. The lower column is the *GAPDH* loading control. (**E**) Quantitative RT-PCR of *Ci-Hox1* transcripts. Experiments were performed in duplicate. In *ngs* mutants, the relative level of *Ci-Hox1* mRNA was ~2-5% that of normal juveniles. (**F**,**G**) Juveniles injected with *Ci-Hox1* antisense MO (left) or both *Ci-Hox1* antisense MO and *Ci-Hox1* mRNA (right). Gi, gill slit; BWM, body wall muscle.

1) (Ikuta et al., 2004) owing to a transposon insertion 192 bp upstream of the *Ci-Hox1* transcriptional start site (Fig. 2A). To test whether the insertion disrupts function of the *Ci-Hox1* promoter and generates a loss-of-function allele, we generated homozygous animals by crossing two heterozygous EJ[MiTSAdTPOG]124 animals (Fig. 2B-D). The homozygous animals showed notable phenotypes after metamorphosis, including loss of gill slits and body wall muscle (BWM) (Fig. 2B). The mutant was named *no gill slit* (*ngs*), after its gill-less phenotype. The *ngs* mutant phenotype showed the expected Mendelian frequency for a single recessive mutation (supplementary material Table S1), suggesting that a single gene underlies the observed phenotype.

To determine whether *Ci-Hox1* is the gene for which function is abrogated in *ngs* mutants, we examined *Ci-Hox1* expression levels in *ngs* mutant versus control animals by quantitative RT-PCR. *ngs* mutants showed a dramatic decrease in *Ci-Hox1* expression (Fig. 2E). We also knocked down *Ci-Hox1* function using an antisense MO that disrupts *Ci-Hox1* splicing (supplementary material Fig. S1). *Ci-Hox1*-MO animals phenocopied *ngs* mutants (Fig. 2F) and could be rescued by co-injection of *Ci-Hox1* mRNA (Fig. 2G). These results indicate that mutation of the *Ci-Hox1* gene underlies the *ngs* phenotype, and that *Ci-Hox1* is required for juvenile gill slit and BWM formation.

To determine whether *Ci-Hox1* plays a role in the development of other muscle tissues, we knocked down *Ci-Hox1* in transgenic lines expressing GFP in muscles (supplementary material Fig. S2A) (Joly et al., 2007; Sasakura et al., 2008). Formation of the atrial siphon muscle (ASM) was abnormal in these animals, whereas the oral siphon and heart muscles formed normally (supplementary material Fig. S2A,B, Table S2). Although GFP-positive muscle cells were present in the region of the presumptive ASM, they failed to form a ring-shaped, functional ASM muscle. Co-injection of *Ci-Hox1* mRNA rescued the *Ci-Hox1* MO phenotype (supplementary material Fig. S2C), demonstrating that *Ci-Hox1* is required for proper formation of the ASM. Next, we examined whether the BWM and ASM are related to one another as previously suggested (Hirano and Nishida, 1997; Stolfi et al., 2010) by observing formation of the BWM by time-lapse imaging. We found that, indeed, the BWM formed as an extension of the ASM (supplementary material Movie 1). Thus, the absence of the BWM in *Ci-Hox1*-deficient juveniles is likely to be due to disruption of ASM formation, and a primary function of *Ci-Hox1* is ASM formation. Because ASM and heart muscle originate from the same blastomeres (Hirano and Nishida, 1997; Stolfi et al., 2010), *Ci-Hox1* should affect ASM formation after the two muscle cells separate.

Ci-Hox1 is necessary for formation of the epidermal structure homologous to the otic placode

In ascidian juveniles, two atrial siphons are formed from the ASP, two thickenings of the larval epidermal ectoderm (Fig. 3A,B). The two atrial siphons then fuse at the midline to form one adult atrial siphon (Berrill, 1947). It has been suggested that the ascidian ASP is homologous to the vertebrate otic placode (Manni et al., 2004; Mazet and Shimeld, 2005; Mazet et al., 2005; Kourakis et al., 2010). In addition, a previous study showed that the ASP is also required for formation of the gill slit (Kourakis and Smith, 2007). To test whether ASP formation is affected in *Ci-Hox1*-deficient animals, we made use of epidermal GFP transgenic lines (Sasakura et al., 2010) in which the disc-like oral siphon primordium and the ASPs emit stronger GFP fluorescence than do the neighboring epidermal cells (Fig. 3C). In *Ci-Hox1* knockdown animals, the ASP was lost, whereas formation of the oral siphon primordium was normal (Fig. 3D; supplementary material Table S3). This phenocopy could be rescued by co-injection of *Ci-Hox1* mRNA (Fig. 3E; supplementary material Table S3), indicating that the *Ci-Hox1* MO is specific. Taken together, our results demonstrate that *Ci-Hox1* is essential for formation first of the ASP and then of the gill slit and ASM/BWM.

Ci-Hox1 is expressed in several tissues at the larval stage, including the epidermal ectoderm, CNS and endoderm (Ikuta et al., 2004). To determine which expression domain of *Ci-Hox1* is

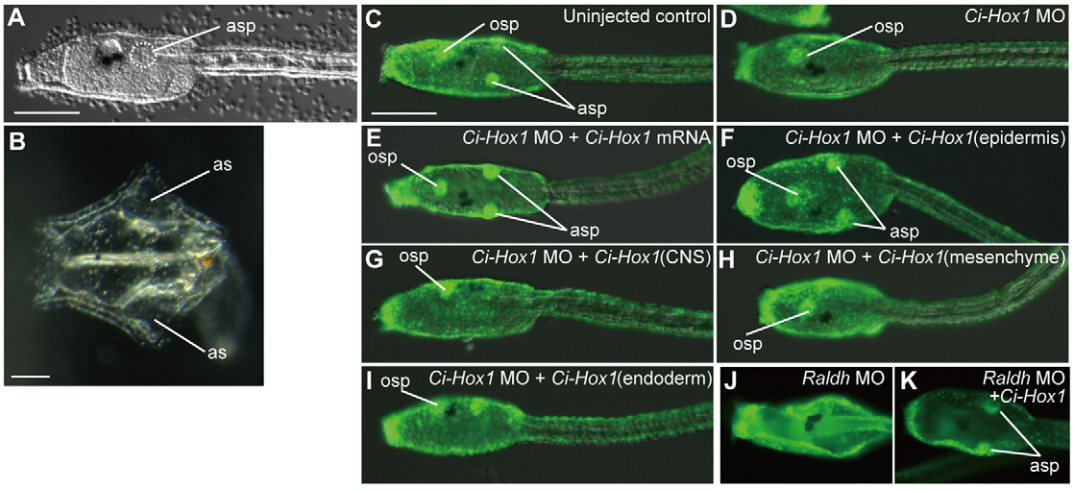

Fig. 3. *Ci-Hox1* is essential for formation of the ASP. (**A**) Lateral view of *Ciona intestinalis* larva with an ASP indicated by a dotted line. (**B**) Dorsal view of a juvenile. (**C**) An epidermal GFP transgenic line. (**D**) A larva injected with *Ci-Hox1* antisense MO. No ASP was formed. (**E**) A larva simultaneously injected with *Ci-Hox1* antisense MO and *Ci-Hox1* mRNA. Two ASPs were formed. (**F**) A *Ci-Hox1*-MO-injected larva in which *Ci-Hox1* is overexpressed in the epidermis. ASPs were formed. (**G**) A *Ci-Hox1*-MO-injected larva in which *Ci-Hox1* was overexpressed in the CNS. ASPs were not formed. (**H**) A *Ci-Hox1*-MO-injected larva in which *Ci-Hox1* was overexpressed in the mesenchyme. ASPs were not formed. (**I**) A *Ci-Hox1*-MO-injected larva in which *Ci-Hox1* was overexpressed in the endoderm. ASPs were not formed. (**J**) A *Raldh*-MO-injected larva. ASPs were not formed. (**K**) A *Raldh*-MO-injected larva in which *Ci-Hox1* was overexpressed in the epidermis. Two ASPs were formed. as, atrial siphon; osp, oral siphon primordium. Scale bars: 100 μm.

Fig. 4. A model summarizing the relationship between *Ci-Hox1* and the inductive signal of the ASP. RA drives *Ci-Hox1* expression (purple) in the epidermis. The epidermis acquires competence to receive the inductive signal, and two ASPs are formed. In *Ci-Hox1*-mutant or morphant embryos, the epidermis cannot receive the inductive signal, and no ASP was formed.

responsible for ASP formation, we generated tissue-specific *Ci-Hox1* expression constructs and tested their ability to rescue the *Ci-Hox1*-MO ASP phenocopy. Strong rescue was observed when *Ci-Hox1* was expressed in the epidermal ectoderm (Fig. 3F; supplementary material Table S3). This result indicates that expression of *Ci-Hox1* in the epidermal ectoderm is required for formation of the ASP. As two properly positioned ASPs were formed even though *Ci-Hox1* was overexpressed throughout the embryo body, which was shown by rescue experiment of *Ci-Hox1* MO phenocopy with *Ci-Hox1* mRNA, RA-driven *Ci-Hox1* might render the epidermis competent to respond to the ASP-inducing signals. In contrast to the epidermis, expression of *Ci-Hox1* in the CNS and mesenchyme failed to rescue the MO phenocopy (Fig. 3G,H; supplementary material Table S3). A moderate rescue was observed when *Ci-Hox1* was expressed in the endoderm (Fig. 3I; supplementary material Table S3), suggesting that *Ci-Hox1* in the endoderm has a role in formation of the ASP.

Kourakis and Smith (Kourakis and Smith, 2007) previously suggested that FGF/MEK signaling after the early tailbud stage serves to induce the ASP. If *Ci-Hox1* gives the epidermal ectoderm competence to respond to the inductive signal of the ASP, expression of *Ci-Hox1* in the epidermis should be independent of the inductive signal. *Ci-Hox1* expression is observed in the epidermis at the early tailbud stage, which is earlier than the induction of the ASP, suggesting that *Ci-Hox1* expression is independent of the inductive signal. To address the independence between *Ci-Hox1* in the epidermis and inductive signaling of ASP, we treated embryos with U0126 from the early tailbud stage, causing loss of the ASP (supplementary material Table S4). Microinjection of *Ci-Hox1* mRNA failed to overcome the effect (supplementary material Table S4), suggesting that inductive signaling of the ASP is not mediated by *Ci-Hox1* in the epidermal ectoderm.

Retinoic acid-driven epidermal expression of *Hox1* is necessary for ASP formation

The expression of *Ci-Hox1* in the epidermal ectoderm is directly upregulated by RA (Ishibashi et al., 2005; Kanda et al., 2009). To test the possibility that RA is involved in ASP formation, we disrupted RA synthesis by knocking down the gene encoding retinal dehydrogenase (*Raldh*), an RA synthesis enzyme (Niederreither et al., 1999), with an antisense MO. *Raldh*-MO larvae exhibited loss of the ASP (Fig. 3J; supplementary material Table S5), suggesting that RA is necessary for ASP formation. When *Ci-Hox1* was overexpressed in the epidermis of *Raldh*-MO larvae, the phenotype was rescued and ASP formation was observed (Fig. 3K; supplementary material Table S5). These results indicate that RA functions in ASP formation via epidermal expression of *Ci-Hox1*.

Conclusions

We conclude that RA-driven *Hox1* expression in the epidermal ectoderm is essential for organizing the ASP/otic placode in the urochordate *Ciona intestinalis* (Fig. 4). In addition, this network functions directly in the ASP/otic placode to pattern it. Similarly, in amphioxus, RA functions in patterning of the epidermal sensory organ (Schubert et al., 2004), from which placodes are thought to originate (Holland and Holland, 2001). In vertebrates, otic placode formation depends on signals from a properly patterned CNS (Hans and Westerfield, 2007), and is therefore indirectly dependent on RA and *Hox1*. However, expression of *Hoxa1* in the otic epithelium (Makki and Capecchi, 2010) raises the possibility that the RA-*Hox1* network might also function in the epidermal ectoderm to form the otic placode in vertebrates. Furthermore, *Hox1* expression in the epidermal ectoderm is observed in hemichordates (Aronowicz and Lowe, 2006). Thus, the role of RA-*Hox1* in specification of the epidermal sensory organ might have been inherited from the deuterostome ancestor of chordates and recruited for otic placode formation in the urochordate/vertebrate lineages. Because both the dorsal position of the CNS and the epidermally specialized placodes are hallmarks of chordates, the RA-*Hox1* network appears to have played key roles in these evolutionary innovations crucial for acquiring the chordate body plan. Our study also suggests that *Ci-Hox1* in the endoderm functions in ASP formation. In *Ciona*, the inductive signal for ASP formation is thought to be mediated by fibroblast growth factor (FGF) signaling (Kourakis and Smith, 2007). Although the source of the FGF signal has not been uncovered, the endoderm is a good candidate. Because *Ci-Hox1* is probably the competence factor for ASP/otic placode formation, *Ci-Hox1* might be upstream of the transcription factor genes expressed preferably in the placode, such as *FoxIa*, *Pax2/5/8*, *eyes absent* and *Six1/2* (Mazet and Shimeld, 2005). This issue also needs to be investigated for understanding the mechanisms underlying formation of the ASP/otic placode.

Acknowledgements

We thank the National Bioresource Project, MEXT, and all members of the Maizuru Fishery Research Station of Kyoto University and the Education and Research Center of Marine Bioresources of Tohoku University for providing us with *Ciona* adults.

Funding

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to Y.S., T.H., N.S. and S.F. Y.S. was supported by the National Institute of Genetics Cooperative Research Program [2009-B01].

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.080234/-/DC1

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