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 Hox1 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[MITSAZTPOG]124 was created using the jump-starter method (Sasakura et al., 2008). Tg[MITSAZTPOG]8 (Awazu et al., 2007) was used as a transposon donor. The Minos insertion site of EJ[MITSAZTPOG]124 was determined by thermal asymmetric interlaced (TAIL)-PCR (Liu et al., 1995). Two transgenic lines, Tg[MiCTnIG]2 and Tg[MiCTnIGCipprnG]2, were used as muscle marker lines (Joly et al., 2007; Sasakura et al., 2008). Three transgenic lines, Tg[MiCiEpilIG]3, Tg[MiCiEpilIG]4 and Tg[MiCiCesACsA-CiEpilIG]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’-TGAGCAAGGGCGA-3’. The amplified band was subcloned into the BglII and Blunt EcoRI sites of pBS-RN3 (Lemaire et al., 1995) to create pRN3CiHox1.

pSpecCFP-ter
The ORF of eCFP was amplified by PCR with primers 5’-CTGGAATTTCTGTACAGTGTCCG-3’ and 5’-AGCGGCGCTATGGA-TGAGCAAGGGCGA-3’. The PCR fragment was inserted into the NotI and EcoRI sites of pSP-eCFP (Sasakura et al., 2003) to create pSpecCFP-ter.

pSpecCFPCiHox1
An EcoRI fragment of Ci-Hox1 cDNA was inserted into EcoRI site of pSP-eCFP-ter to create pSpecCFPCiHox1. A BamHI fragment of a cis element of Ci-CesA, Ci-AKR and Ci-β2TB were isolated from pCesA(-2080)-GFP

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(Sasakura et al., 2005), pSPCiAKRK (Hozumi et al., 2010) and pSPCiJ2TBK (Horie et al., 2011), respectively. These cis elements were subcloned into BamHI site of pSpeCFCiHox1. A cis element of Ci-TTF1 (Satou et al., 2001) was amplified from genomic DNA with primers 5'-TTTGTGGCGCCGCATCTCACAGAAAGTTTCCAG-3' and 5'-AAAAGCGGCCGCTAGTTCATGGTTAGCAATGAC-3', digested with NotI and subcloned into the NotI site of pSpeCFPCiHox1.

**Microinjection**

The sequence of the antisense morpholino oligonucleotide (MO) for Ci-Hox1 is 5'-AAGCCAACTGTGTTACCACATG-3' 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/µg and 500 ng/µg, respectively. The sequence of the *Raldh* antisense MO is 5'-GTACTGTGATACGACTGAAGACAT-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'-ATGTTGTGGCGCCGCATCTCACAGAAAGTTTCCAG-3', and for EF1α they were 5'-CATGTGACGACAGGAAACG-3' and 5'-CAATGTGACGTGGTCATCAAGAC-3'.

**Whole-mount in situ hybridization**

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

**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. 1).
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.
responsible for ASP formation, we generated tissue-specific Ci-Hox1 expression constructs and tested their ability to rescue the Ci-Hox1-MO ASP phenoency. 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.

**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 Hox1 in the otic epithelium (Makki and Capecci, 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.

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**Competing interests statement**

The authors declare no competing financial interests.