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Source: Zoological Science, 27(11) : 842-850

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.27.842

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ZOOLOGICAL SCIENCE **27**: 842–850 (2010) © 2010 Zoological Society of Japan

Transposon-mediated Enhancer Detection Reveals the Location, Morphology and Development of the Cupular Organs, which are Putative Hydrodynamic Sensors, in the Ascidian *Ciona intestinalis*

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The adult of the ascidian *Ciona intestinalis* has cupular organs, i.e., putative hydrodynamic sensors, at the atrial epithelium. The cupular organ consists of support cells and sensory neurons, and it extends a gelatinous matrix, known as a cupula, toward the atrial cavity. These characteristics are shared with sensory hair cells in the vertebrate inner ear and lateral line neuromasts in fish and amphibians, which suggests an evolutionary link between the cupular organ and these vertebrate hydrodynamic sensors. In the present study, we have isolated and investigated two transposon-mediated enhancer detection lines that showed GFP expression in support cells of the cupular organs. Using the enhancer detection lines and neuron marker transgenic lines, we describe the position, morphology, and development of the cupular organs. Cupular organs were found at the atrial epithelium, but not in the branchial epithelium. We found that cupular organs are also present along the dorsal fold and the gonoducts. The cells lining the pre-atrial opening in juveniles are presumably precursor cells of the cupular organ. To our knowledge, the present study is the first precise description of the ascidian cupular organ, providing evidence that may help to resolve discrepancies among previous studies on the organ.

Key words: Cupular organ, support cell, sensory cell, transposon, enhancer detection, *Minos*

INTRODUCTION

The cupular organ is presumably a sensory organ that is present in several species of urochordates (Fedele, 1920, 1923; Millar, 1953; Bone and Ryan, 1978; Mackie and Singla, 2004; Mackie and Burighel, 2005). The organ is defined as a part of the epithelium that has a structure named the cupula, made of gelatinous material elongated from the basement of the organ toward the atrial cavity. The basement of the cupular organ consists of two cell types: sensory cells and support cells. Sensory cells are primary sensory neurons that extend cilia into the cupula, while support cells are believed to support the cupula and sensory cells. The basic structure of the urochordate cupular organ is similar to the sensory hair cells of the vertebrate inner ear and the lateral line neuromast in amphibians and fish (Dijkgraff, 1963, 1964; Shelton, 1971; Elepfandt, 1988; Montgomery et al., 2000), which suggests that they have a common evolutionary origin.

Despite the importance of the urochordate cupular organ for understanding the evolution of hydrodynamic sensors in chordates, the organ has not yet been precisely described. There is, for example, an unresolved controversy with respect to the location of the cupular organs in the urochordate body. In *Ciona intestinalis*, a cosmopolitan species of ascidians, the cupular organs are found in the epithelium of the atrial cavity (Millar, 1953). In another ascidian species, *Corella eumyota*, the location of the cupular organs is somewhat different from that in *Ciona intestinalis*; the cupular organs are located along the dorsal fold, which is the line connecting the brain and the gonoducts, but the organ was not found in the atrial epithelium (Mackie and Singla 2004). In addition, the development of cupular organs, namely when and how the organ is formed, has not been fully described. Although a description of the urochordate cupular organs was done based on bright field microscopy and electron microscopy, it is difficult to describe the development of the cupular organs with these methods. as these organs are small and semi-transparent.

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doi:10.2108/zsj.27.842

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A method of visualizing the cupular organ would effectively overcome this issue. In zebrafish, the lateral line neuromasts were identified using transgenic lines that specifically recognize sensory cells or support cells (Parinov et al., 2004; Xiao et al., 2005; Haas and Gilmour, 2006). A transgenic line of urochordates expressing a reporter gene in the cupular organs would be a powerful tool for analyzing the development and morphology of this organ. Various technical innovations to promote genetic studies have recently been achieved in *Ciona intestinalis*, as exemplified by germline transgenesis with a Tc1/*mariner* superfamily transposon, *Minos* (Sasakura et al., 2003a, b). The *Minos* system has been used in genetic techniques, such as insertional mutagenesis and enhancer detection (Awazu et al., 2004; Sasakura et al., 2005; Awazu et al., 2007; Sasakura, 2007; Sasakura et al., 2008; Hozumi et al., 2010). Enhancer detection is a useful way to create transgenic lines that show reporter gene expression in a specific organ or tissue. In *Ciona intestinalis*, enhancer detection was reported with a *Minos* vector containing a *green fluorescent protein* (*gfp*) gene and the promoter of *Ci-TPO*, a gene encoding thyroid peroxidase (Ogasawara et al., 1999). A high-throughput enhancer detection system that utilizes the remobilization of *Minos* in the *Ciona* genome has been established (Awazu et al., 2007; Sasakura et al., 2008). The established enhancer detection lines have provided us with useful markers for various tissues and organs (Sasakura et al., 2008).

In the present study, we characterized two enhancer detection lines, E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62, that show GFP expression in support cells of the cupular organs at the adult stage. Using these lines as markers, we describe here the location, morphology, and development of the cupular organs.

MATERIALS AND METHODS

Wild-types and transgenic lines

Wild-type *Ciona intestinalis* was collected from or cultivated in Onagawa Bay (Miyagi), Maiduru Bay (Kyoto) and Usa Bay (Kochi). E[MiTSAdTPOG]10 was isolated by remobilization of the *Minos* transposon by the microinjection of transposase mRNA into the eggs (Awazu et al., 2007; Sasakura, 2007). EJ[MiTSAdTPOG]62 was isolated using the jump-starter method (Sasakura et al., 2008). The transgenic line Tg[MiCib2TBK]2 was created by electroporation of the *Minos* vector pMiCib2TBK and transposase mRNA (Matsuoka et al., 2005). These lines were cultured using the inland culture system as described previously (Joly et al., 2007). EJ[MiTSAdTPOG]62 was crossed with Tg[MiCib2TBK]2 to create double transgenic animals.

Immunostaining

Tissue fragments containing cupular organs were collected surgically from adults of EJ[MiTSAdTPOG]62 and E[MiTSAdTPOG]10 with the aid of GFP expression. The tissue fragments were fixed with 3.7% formaldehyde in seawater. The fixed specimens were dehydrated with ethanol, embedded in polyester wax, and sectioned at a thickness of 4 μm. The sliced sections were subjected to immunostaining, as previously described (Horie et al., 2008a, b). A rat anti-GFP monoclonal antibody (GF090R, Nacalai Tesque) and a mouse anti-acetylated α-tubulin antibody (Sigma clone 6-11B-1; Crowther and Whittaker, 1994; Konno et al., 2010) were used as the primary antibodies. As the secondary antibody, an Alexa 488-conjugated antirat IgG goat antibody (Invitrogen) and an Alexa 555-conjugated antimouse IgG goat antibody (Invitrogen) were used.

Identification of *Minos* insertion sites

Thermal asymmetric interlaced PCR (TAIL-PCR) was done as described previously (Sasakura et al., 2003, 2005). PCR products were subcloned into pGEMT vector (Promega), and their sequences were determined. The nucleotide sequence was BLAST-searched against the GHOST database (Satou et al., 2002, 2005) to determine the *Minos* insertion sites of E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62.

Juveniles of enhancer detection lines were subjected to digestion in 50 ml of 1 × TE solution containing 0.2 mg/ml Proteinase K for 3 h at 50°C, followed by 15 min at 95°C to inactivate Proteinase K. One μl of the extract was used for the PCR analyses to test whether juveniles had the identified transposon insertions in their genomes. The primers were 5'-acgaaatgcattggtatgtgttatc-3' and 5'-attccgaaattgccataacacc-3' for E[MiTSAdTPOG]10, and 5'-acgaaatgcattggtatgtgttatc-3' and 5'-acagtaaacaaaacacttgaac-3' for EJ[MiTSAdTPOG]62. As a positive control, a genomic region encoding *Ci-snail* was amplified with the following primers: 5'-tgatgtatcgccaccacaac-3' and 5'-gaagtgctccaagagaactg-3'. PCR was done with ExTaq DNA polymerase (Takara Bio), and the PCR conditions were 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by a final extension for 10 min at 72°C.

Reverse transcription (RT)-PCR

Total RNA was isolated from the atrial epithelium or orange-pigmented organs by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). A 1.5-μg amount of total RNA was treated with DNase I (Invitrogen). The RNA was reverse-transcribed using oligo dT primers and Superscript III reverse transcriptase (Invitrogen), and the cDNA was subjected to PCR amplification using ExTaq DNA polymerase (Takara). cDNAs of KH.C1.950, *Ci-HSPA12*, KH.C1.988 and *Ci-Ef1α* were amplified with the following primers: for KH.C1.950, 5'-taatagtaaaagatgcatttcaac-3' and 5'-atacctgtatgaggtccgaatgtc-3' ; for *Ci-HSPA12*, 5'-aatcaaaaaacaccaactattg-3' and 5'-gatagcaggcacagtaacg-3'; for KH.C1.988, 5'-cattagatatgcaatttaaacag-3' and 5'-cattcacaatgcgagaagctg-3' ; for *Ci-Ef1α*, 5'-ttggacaaacttaaggccgagc-3' and 5'-gtctccagcaacataacctctc-3'. The PCR conditions were 50 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by a final extension for 10 min at 72°C.

The 5' end cDNA sequence of *Ci-HSPA12* was amplified using the GeneRacer Kit (Invitrogen) with the primers 5'-tgcttgtcgcataaactgtttggcc-3' and 5'-cgactggagcacgaggacactga-3' (1st PCR) and 5'-aattctcatattcgtgccaagctcg-3' and 5'-ggacactgacatggactgaaggagta-3' (2nd PCR). The PCR products were subcloned into pGEMT vector (Promega). cDNA containing the entire open reading frame of *Ci-HSPA12* was also amplified using the primers 5'-aagagatctatgcgcaagtggtcgggaggc-3' and 5'-aagctcgagttaggcaaagaaatccgctcg-3'. The PCR product was cleaved with *Bgl*II and *Xho*I and was subcloned into pBluescript II KS(–).

RESULTS

Isolation of enhancer detection lines expressing GFP in the cupular organs

Two enhancer detection lines, E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62, were isolated by the remobilization of *Minos* copies in the *Ciona* genome (Awazu et al., 2007; Sasakura et al., 2008). Both enhancer detection lines showed an identical GFP expression pattern at the adult stage; namely, GFP was expressed in the prebranchial zone of the pharynx and the atrial epithelium (Fig. 1). In the prebranchial zone, GFP was expressed in the apical region from the ring of tentacles (Fig. 1A and A'). In the atrial epithelium near the atrial siphon opening, there was a row of GFP-positive cells that formed a ring (Fig. 1B, arrows). Part

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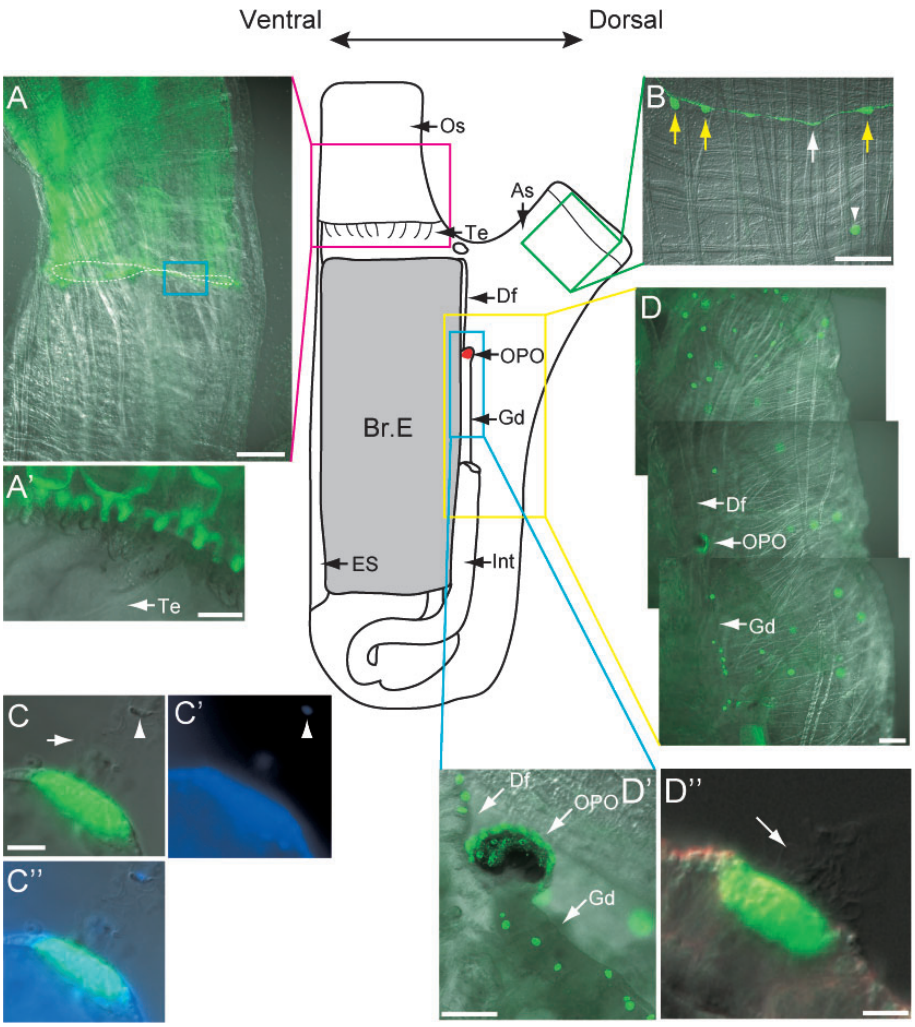

Fig. 1. GFP expression in the enhancer detection lines E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 in *Ciona intestinalis* adult**.** A schematic image of the mature adult body is shown in the center. Branchial epithelium (Br. E) is shown in gray. As, atrial siphon; Df, dorsal fold; En, endostyle; Gd, gonoduct; OPO, orange-pigmented organ; Os, oral siphon; Int, intestine. **(A)** The oral siphon of EJ[MiTSAdTPOG]62 at the adult stage. This is a merged image of bright field and fluorescence images. The apical plane is toward the top. Bar = 200 μm. GFP expression is shown as green fluorescence. The position of the tentacles is shown as a dotted line. (A') A higher magnification of the region indicated by a blue rectangle in A. Te, tentacle. Bar = 50 μm. **(B)** GFP expression around the atrial siphon opening. A white arrow indicates the ring-shaped GFP expression, and yellow arrows indicate clusters of GFP-expressing cells in the ring. A cupular organ is seen as a spot of GFP expression (arrowhead). The apical plane is toward the top. Bar = 200 μm. (C) Higher magnification images of the cupular organ, lateral view. **(C)** A merged image of DIC and GFP fluorescence images. An arrow indicates the cupula. (C') DAPI fluorescence. Wandering cell indicated by an arrowhead. (C'') The merged image of C and C'. Bar = 10 μm. **(D)** GFP expression around the atrial epithelium of the adult EJ[MiTSAdTPOG]62. This is a merged image obtained using DIC and fluorescence images. The dorsal direction is to the right and the ventral direction is toward the left. Bar = 200 μm. (D') A magnified image of the region around the orange-pigmented organ. The cupular organs are present along the dorsal fold and gonoduct. GFP expression was observed at the surface of the orange-pigmented organ (ORO). Bar = 200 μm. (D'') A higher magnification image of a cupular organ on the surface of the dorsal vessels. A cupula was projected from the basement (arrow). Bar = 10 μm.

of the ring was knobbed (Fig. 1B, yellow arrows). At the atrial epithelium posterior to the ring, spotted GFP signals were observed (arrowhead of Fig. 1B). When the spot was observed under higher magnification, we observed that a cupula extended toward the atrial cavity, suggesting that these spots correspond to the cupular organs (Fig. 1C). GFP was expressed at the base of the cupular organs. Previous reports suggested that there are some wandering cells in the cupula (Millar, 1953; Bone and Ryan, 1978). Accordingly, we found DAPI-positive wandering cells in the cupulae (Fig. 1C', arrowhead). Spotted signals were also present along the dorsal fold and gonoducts (Fig. 1D and D'). At higher magnification, these spots showed extended cupulae, suggesting that they were also the cupular organs (Fig. 1D''). At the end of the spermduct, there was an orange-pigmented organ. GFP expression was observed on the surface of the organ (Fig. 1D and D'). Details regarding the orange-pigmented organ are described below.

Enhancer detection lines express GFP in support cells of the cupular organs

We next performed detailed observation of the cupular organs in the enhancer detection lines. Semi-thin sections of cupular organs were immunostained with an anti-GFP antibody and an anti-acetylated tubulin antibody, a marker of cilia (Fig. 2A–C). There were gaps between GFP-positive zones, suggesting the presence of GFP-negative cells filling the gaps (Fig. 2A, arrowheads). Immunostaining of acetylated tubulin showed that the GFP-negative cells extended cilia toward the apical plane of the cupular organs (Fig. 2B and C, white arrow). These cells may be the sensory cells of the cupular organ, as previously suggested by Bone and Ryan (1978), who proposed that sensory cells extend cilia from deeply invaginated locations in the cells. It is therefore likely that GFP-negative and acetylated tubulin-positive cells are sensory cells, while GFP-positive cells are support cells.

In order to clearly define sensory cells and support cells, we created double transgenic animals by crossing EJ[MiTSAdTPOG]62 and Tg[MiCib2TBK]2. Because Tg[MiCib2TBK]2 expresses Kaede in neurons and cells with cilia under the control of the *Ci-β2tubulin cis* element, sensory cells in the cupular organs should be Kaede-positive in the line, and

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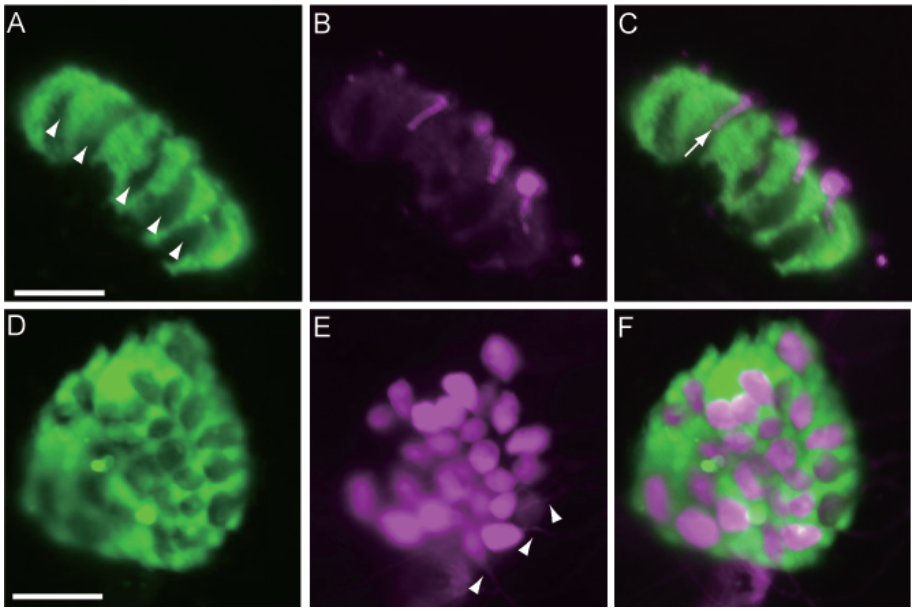

Fig. 2. GFP expression in support cells of cupular organs. (A–C) Images of an immunostained section of a cupular organ are shown. GFP (green) and acetylated-α-tubulin (magenta) are visualized. **(A)** GFP signal. There are GFP-positive and -negative (arrowheads) regions at the basement of the cupular organ. Bar = 20 μm. **(B)** Acetylated-α-tubulin. **(C)** A merged image of A and B. Cilia are extended from the deep zone of the GFP-negative cells (white arrow). (D–F) A cupular organ in a double transgenic animal obtained by a cross of EJ[MiTSAdTPOG]62 and Tg[MiCib2TBK]2. GFP (green) and Kaede (magenta) is visualized. **(D)** GFP fluorescence in support cells. Bar = 20 μm. **(E)** Kaede fluorescence in sensory cells. Arrowheads indicate axons. **(F)** A merged image of D and E.

thus should be distinguishable from support cells. The cupular organs were isolated from the double transgenic animals, and observed under fluorescent microscopy after Kaede fluorescence was photo-converted to red with UV light (Fig. 2D–F). The cupular organs expressed Kaede-derived red fluorescence exclusively in GFP-negative cells (Fig. 2D–F), indicating that GFP-negative cells are sensory cells and GFP-positive cells are support cells. Kaede-positive sensory cells exhibited axons (Fig. 2E), as Bone and Ryan 1978 found that sensory cells are neurons. We concluded that the E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 lines are the enhancer detection lines that entrapped an enhancer that is responsible for the expression in support cells of cupular organs.

Size variation of the cupular organs in the atrial epithelium

We found that various-sized cupular organs are present in the atrial cavity (Fig. 3A–E). The number of sensory cells was counted among the cupular organs; there

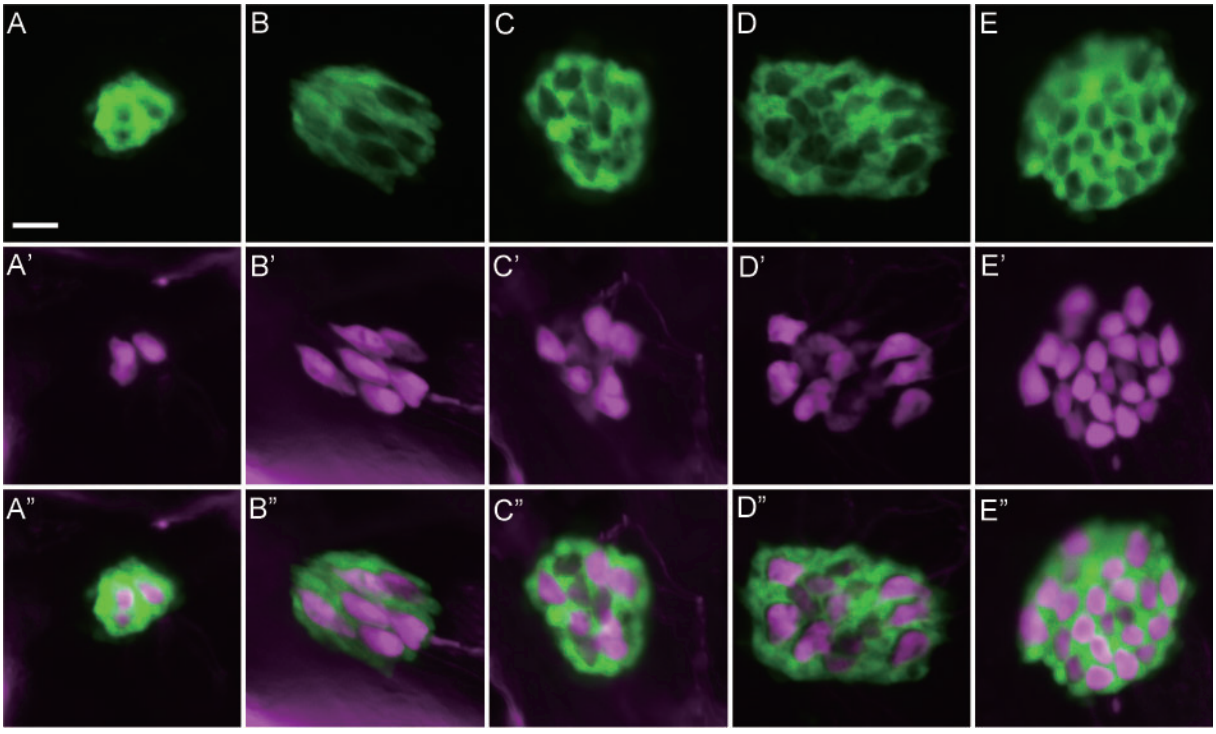

Fig. 3. Cupular organs of various sizes at the atrial epithelium. GFP (green) is expressed in support cells and Kaede (magenta) is expressed in sensory cells of cupular organs. **(A–E)** GFP fluorescence. Bar = 10 μm. (A'–E') Kaede fluorescence. (A''–E'') Merged images of A–E and A'–E', respectively. The numbers of sensory cells in A''–E'' were three, six, ten, 14 and 22, respectively.

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were 2 to 25 sensory cells in each cupular organ (Fig. 3A'–E'). The number of sensory cells tended to increase as the size of the cupular organs increased. Sensory cells in a cupular organ were separated well by support cells. The number of support cells could not be counted, as they were clustered tightly and the boundaries between support cells were difficult to detect. Small and large cupular organs were found in the entire zone of the atrial epithelium.

As mentioned above, the cupular organs were also present along the dorsal fold and gonoducts (Fig. 1D and D'). The number of sensory cells in the cupular organs in this region ranged from 4 to 17 in the mature adult. These cupular organs were comparatively smaller than those in the atrial cavity.

E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 express GFP in the orange-pigmented organ

GFP expression was observed on the surface of the orange-pigmented organ in the two-enhancer detection lines (Fig. 4). The orange-pigmented organ contained about 12 fine-pointed tubules (Millar, 1953). Each tubule was hollow and had a very narrow terminal opening; these openings can be seen as GFP-negative zones in the enhancer detection lines (arrowheads in Fig. 4A; Millar, 1953). Cells with strong GFP signals surrounded the edge of the terminal openings (Fig. 4B). Observation of the double transgenic animals between EJ[MiTSAdTPOG]62 and Tg[MiCib2TBK]2 showed that neuronal cells surrounded the GFP-positive cells at the opening (Fig. 4C–F). The neurons did not express GFP, and they extended axons toward the base of the orange-pigmented organ (Fig. 4C and D, arrows).

GFP expression pattern of E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 during development

GFP expression of the E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 enhancer detection lines during development was observed to determine the timing of the cupular organ formation. Larvae of these lines did not show GFP expression (data not shown). At the juvenile stage, both lines

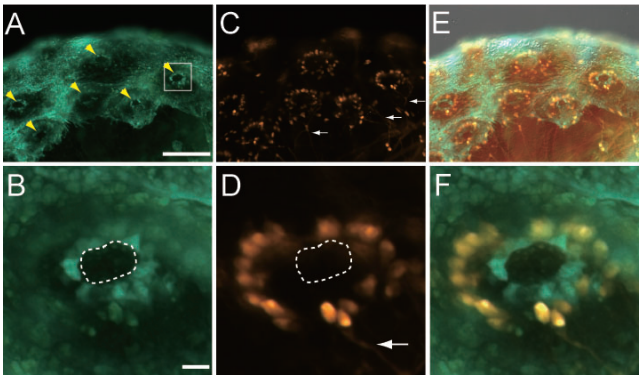

Fig. 4. GFP and Kaede expression in the orange-pigmented organ in a double transgenic animal between EJ[MiTSAdTPOG]62 and Tg[MiCib2TBK]2. (A–B) GFP fluorescence. Bars indicate 100 μm in **(A)** and 10 mm in **(B)**. Arrowheads indicate the narrow terminal openings for sperm ejection. The dotted line indicates the edge of a narrow terminal opening. **(C–D)** Kaede fluorescence. Arrows indicate axons. **(E–F)** Merged images of A–D and DIC images.

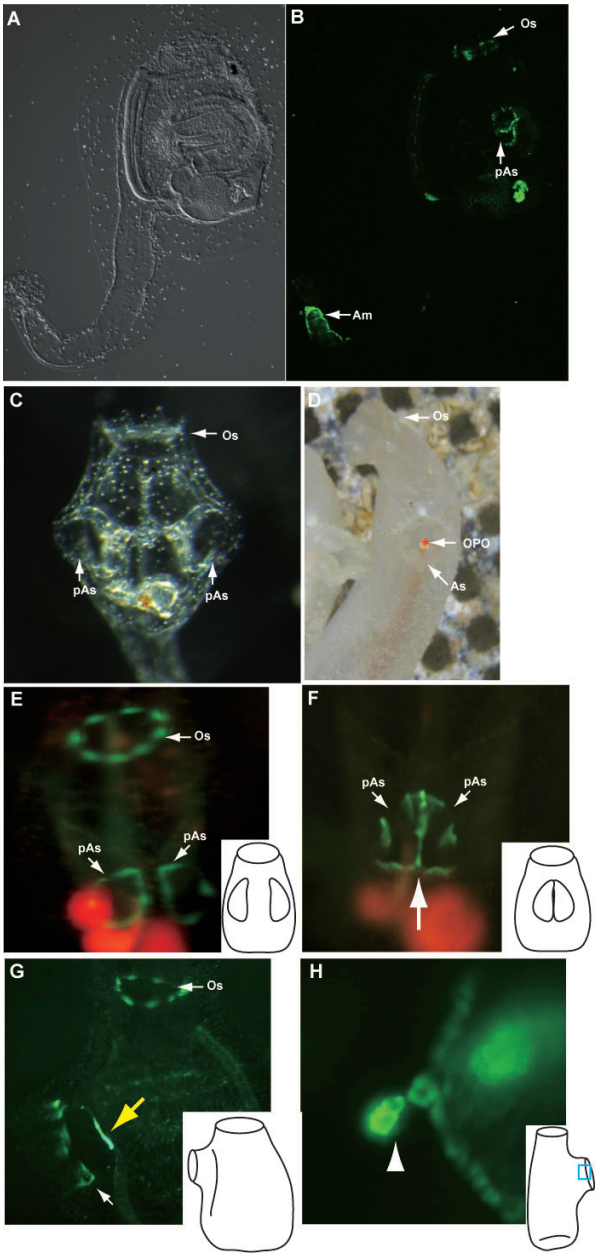

Fig. 5. (A–B) GFP expression of EJ[MiTSAdTPOG]62 at the juvenile stage, lateral view. **(A)** A bright field image. **(B)** A GFP fluorescence image. GFP expression is observed at the end of the ampulla (Am) and the edge of the oral (Os) and pre-atrial siphons (pAs). **(C)** A juvenile *Ciona intestinalis*, dorsal view. Two pre-atrial siphons are observed. **(D)** An adult *Ciona intestinalis*, dorsal view. An atrial siphon (As) is observed. An orange-pigmented organ (OPO) is seen from the atrial siphon opening. **(E)** A juvenile EJ[MiTSAdTPOG]62 before the fusion of pre-atrial siphons, dorsal view. Two pre-atrial siphons can be seen with GFP fluorescence. A schematic diagram of this juvenile is shown in the inset. **(F)** A juvenile EJ[MiTSAdTPOG]62 during the fusion of pre-atrial siphons, dorsal view. Two pre-atrial siphons are united. The center of the united pre-atrial siphons (large arrow) shows GFP fluorescence. **(G)** A juvenile EJ[MiTSAdTPOG]62 during the fusion of pre-atrial siphons, lateral view. The ventral side is toward the right. The center of the united pre-atrial siphons (big arrow) moves toward the ventral side. A small arrow indicates GFP fluorescence near the atrial siphon opening. **(H)** A cupular organ (arrowhead) pinched off from the ring at the atrial siphon opening.

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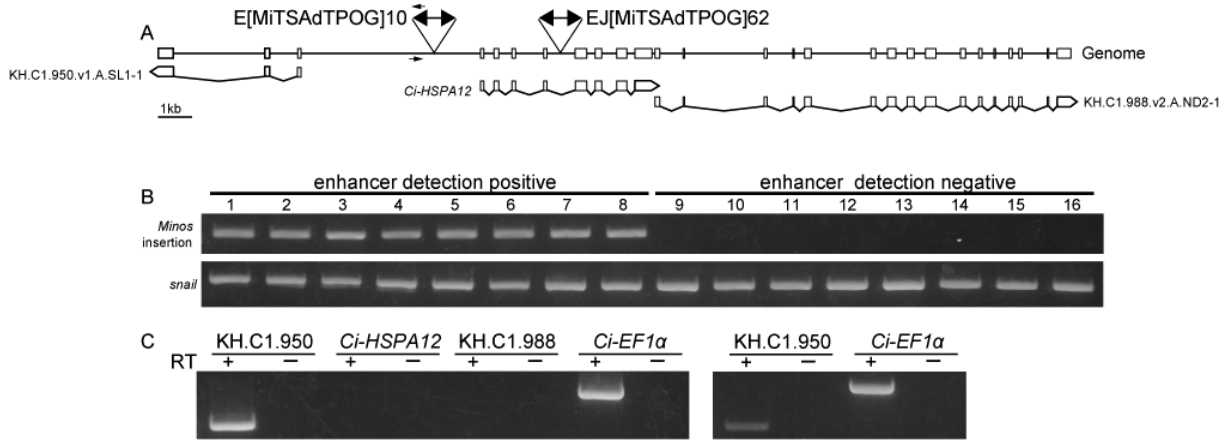

Fig. 6. Location of genes near the insertion sites of *Minos* in E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62, and their expression in cupular organs. **(A)** The insertion sites of *Minos* in E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 are indicated by double-head arrows. Three genes, KH.C1.950, *Ci-HSPA12* and KH.C1.988, are present near the insertion sites. Boxes indicate exons. As for KH.C1.950 and KH.C1.988, a gene model from the Ghost database is shown. For *Ci-HSPA12*, the gene model was constructed using the sequence of cDNA containing the full open reading frame of this gene, which was isolated and characterized in this study. **(B)** *Minos* insertions near *Ci-HSPA12* are responsible for enhancer detection of E[MiTSAdTPOG]10. Juveniles of E[MiTSAdTPOG]10 were subjected to PCR with the primers shown as small arrows in (A). "GFP-positive" indicates juveniles with an enhancer detection GFP expression pattern, and "GFP-negative" indicates juveniles lacking GFP expression. As a positive control, a genomic region encoding *Ci-snail* was subjected to PCR (*snail*). **(C)** Expression of genes in cupular organs, as revealed by RT-PCR. (Left) RNA was isolated from atrial epithelium with the cupular organs. RT-PCR of following genes was performed: KH.C1.950, *Ci-HSPA12*, KH.C1.988 and *Ci-Ef1α*. (Right) KH.C1.950 is expressed in atrial epithelium that does not contain the cupular organ.

showed GFP expression at the end of the ampulla and at the edge epithelia of the oral and pre-atrial siphon openings (Fig. 5A and B). No signal corresponding to a cupular organ was found at the juvenile stage. Two pre-atrial siphons were fused by the second ascidian stage to form an atrial siphon (Fig. 5C and D; Chiba et al., 2004). During the fusion of the pre-atrial siphons, the joined part at the center of the united pre-atrial siphons moved toward the ventral side, and the part was united to the dorsal midline of the branchial epithelium to form the dorsal fold and gonoduct (Fig. 5E–G). After the fusion of the pre-atrial siphons, several cupular organs were formed from the GFP-positive ring of the atrial siphon opening (Fig. 5H). GFP expression at the edge of the atrial siphon remained a ring-shaped signal around the atrial siphon opening.

Minos insertions in an hsp12 homologous gene are responsible for the enhancer detection of the cupular organ

Minos insertion sites of E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 were determined by TAIL-PCR (Liu et al., 1995; Sasakura et al., 2003b). The two enhancer detection lines had insertions in an identical gene, *Ci-HSPA12* (Wada et al., 2006). *Ci-HSPA12* encodes heat shock protein 12 ortholog (Wada et al., 2006). E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 have insertions at about 1.3-kb upstream from the first exon and the fourth intron of *Ci-HSPA12*, respectively (Fig. 6A). In order to confirm that *Minos* insertions in this gene are responsible for the enhancer detection, the correlation between enhancer detection and the presence of the insertions was investigated by genomic PCR. All of the juveniles that inherited the enhancer detection had the *Minos* insertions at *Ci-HSPA12*, while animals without the enhancer detection did not have the insertion (Fig. 6B), suggesting that insertions at the *Ci-HSPA12* are strongly linked to the enhancer detection.

Next, the expression of genes that are present around the insertion site was analyzed to determine whether they are expressed in the cupular organs. There are two more genes (KH.C1.950 and KH.C1.988; Satou et al., 2008) near the insertion sites in addition to *Ci-HSPA12* (Fig. 6A). KH.C1.950 encodes a protein that does not show notable similarity to known proteins, and KH.C1.988 encodes a protein homologous to neprilysin, a peptidase involved in degradation of amyloid-b peptide (Iwata et al., 2005). Tissue fragments of atrial epithelium containing the cupular organs were isolated from the enhancer detection lines with the aid of GFP expression, and the expressions of *Ci-HSPA12*, KH.C1.950 and KH.C1.988 were investigated by reverse transcription (RT–) PCR. A ubiquitous gene, *Ci-EF1α,* was also analyzed as a positive control. KH.C1.950 and *Ci-EF1α* were amplified from the cDNA of the tissue fragments, while *Ci-HSPA12* and KH.C1.988 were not (Fig. 6C). RT-PCR of the orange-pigmented organ amplified *Ci-HSPA12* (data not shown), suggesting that our PCR analysis of this gene was effective. Therefore, we concluded that *Ci-HSPA12* is not expressed in the cupular organ. It was found that KH.C1.950 is not specifically expressed in the cupular organ, as transcripts of this gene were also amplified from the tissue fragments of atrial epithelium that did not contain a cupular organ (Fig. 6C, right).

DISCUSSION

In the present study, we isolated enhancer detection lines that show GFP expression in the support cells of the cupular organs in the adult stage of *Ciona intestinalis*, thus providing a valuable opportunity for studying the cupular organs in this ascidian. A previous report has found that the

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cupular organs were present at the atrial cavity in *Ciona* (Millar, 1953). We found that the cupular organs are also present along the dorsal fold and gonoducts. The presence of the cupular organs along the dorsal fold was reported in a different ascidian species, *Corella eumyota* (Mackie and Singla 2004), suggesting that the presence of the cupular organs in the dorsal fold is common among ascidians. The cupular organs are small and semi-transparent, and it is thus difficult to find all of them using bright field or electron microscopy. In this report, we were able to describe the cupular organs in the entire body of the *Ciona* adult with the aid of reporter gene expression.

A previous study reported that there are usually 15 to 20 sensory cells in a cupular organ in *Ciona intestinalis* (Millar, 1953). We found in the present study that the number of sensory cells ranged from 2 to 25. Since small cupular organs tend to have small numbers of sensory cells, it is possible that such small cupular organs are immature; the number of sensory cells may increase as the organs develop. Precursor cells of sensory cells may be present in a cupular organ, and support cells are strong candidates for the precursors. In the zebrafish neuromasts, support cells are precursors of sensory cells (Ghysen and Dambly-Chaudiere, 2007), suggesting that a similar mechanism may be at work in ascidian cupular organ formation. Sensory cells may inherit GFP protein from support cells. However, a sensory cell with GFP fluorescence was not found in this study. Differentiation of sensory cells from support cells may take a long enough time for degradation of GFP to be under detection level. In each cupular organ, sensory cells are situated at regular distances from each other. There is likely a mechanism by which the sensory cells are situated at this regular distance from one another, such as a lateral inhibitory mechanism during the differentiation of sensory cells (Sternberg, 1988).

At the juvenile stage, E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 showed GFP expression at the ampulla and the edge of the oral and pre-atrial siphons. At this stage, no cupular organ was found. The cupular organs were formed during the transition from the juvenile to the adult stage, when the two atrial openings were united. We found that the cupular organs were formed from the GFP-positive cells near the atrial siphon opening, suggesting that these cells are precursors of the cupular organs. Likewise, the middle part of the united pre-atrial siphon openings joined to the dorsal midline of the branchial epithelium, and the cells in this joint region may be precursors of the cupular organs along the dorsal fold and gonoducts.

Because the cupular organs are formed after the second ascidian stage, their function may not be essential for the sessile lifestyle, but they may be required for adjustment at a later stage, when the body size is greatly increased. The presence of the cupular organs along the gonoduct implies that their function is related to reproduction. The similarity of the ascidian cupular organ to the vertebrate sensory hair cells and lateral line neuromast of fish and amphibians suggests that the ascidian cupular organ may also be a sensing organ (Bone and Ryan, 1978; Mackie and Burighel, 2005). One candidate for their function is sensing water flow in the atrial cavity (Bone and Ryan, 1978). Sensing water flow in a large adult body might require specific structures like cupular organs, and gentle water flow in the atrial cavity is required for reproduction, namely spawning gametes to the outside of the body.

E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 enhancer detection lines express GFP in the orange-pigmented organ at the end of the spermduct in addition to the cupular organs. The orange-pigmented organ shows similarities to the cupular organs, in that neuronal cells are present in both organs and neurons do not express GFP in the enhancer detection lines. In addition, the orange-pigmented organ is formed along the dorsal fold and gonoducts, as are the cupular organs. These similarities suggest that the cells expressing GFP at the orange-pigmented organ may play roles similar to those of the support cells of the cupular organs. The function of the support cells of the cupular organs is thought to be secretion of the cupula (Bone and Ryan, 1978) and physical support of the sensory cells. We could not find a cupula-like structure in the orange-pigmented organ. Support cells in the orange-pigmented organ may function in the support of sensory cells, and may also function as precursors of neuronal cells, as discussed above.

E[MiTSAdTPOG]10 and EJ[MiTSAdTPOG]62 entrapped enhancer(s), which are responsible for expression in the support cells of the cupular organs. It is likely that the enhancers entrapped in these lines are the same, as their causal insertions are proximal to each other and their GFP expression pattern is identical throughout development. The causal insertions of the enhancer detection lines are those targeting *Ci-HSPA12*. It is surprising that *Ci-HSPA12* is not expressed in the cupular organs. It is unclear how an enhancer regulates the expression of the *gfp* gene at the locus of *Ci-HSPA12* in the cupular organs without driving *Ci-HSPA12* in the organ. It is necessary to determine whether the causal enhancer is present close to or distal from the insertion site.

The two enhancer detection lines created in this study have insertions in the same gene. Recently, a method of enhancer detection using transposase-expressing lines was established (Sasakura et al., 2008; Hozumi et al., 2010). In this technique, a transposase-expressing line is crossed with a transgenic line that has a *Minos* insertion in the genome to create double-transgenic animals. In the germ cells of the double-transgenic animals, *Minos* insertions are remobilized to create new insertions. Once double-transgenic animals are created, as many enhancer detection lines as desired can be isolated simply by crossing the double-transgenic animals with wild-type animals. This reduces the labor required for the creation of enhancer detection lines and helps to efficiently isolate enhancer detection lines that can cover insertions in the same gene. With this high-throughput enhancer detection system, saturated enhancer detection of *Ciona* has become possible. The present study also demonstrates that enhancer detection is useful for identifying and analyzing tissues/organs that have yet to be fully described. Enhancer detection will be useful for further elaborating what is known about the adult body structure.

ACKNOWLEDGMENTS

We thank Kazuko Hirayama, Yasuyo Kasuga, Yoko Kitagawa,

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Yasutaka Tsuchiya, Toshihiko Sato, Hideo Shinagawa, Yoshiko Harada, Hiromi Takahashi, Kazuo Inaba, Yasunori Saito, Hiroshi Kubota, Yutaka Satou and Narudo Kawai for their kind technical support. We are grateful to all staff members at the Maizuru Fishery Research Station of Kyoto University and the Education and Research Center of Marine Bio-resources, Tohoku University, for the collection of *Ciona* adults. We also thank Dr. Shigeki Fujiwara for providing us with wild-type *Ciona intestinalis*. This study was supported by Grants-in-Aid for Scientific Research from JSPS and MEXT to TH, NS and YS. YS was supported by the NIG Cooperative Research Program (2008-B02 and 2009-A18). This study was also supported by the National Bioresource Project and by Global COE Program A06 at Kyoto University. The nucleotide sequence of the *Ci-HSPA12* cDNA was deposited in the DDBJ/EMBL/GenBank database under accession number AB482184.

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(Received May 11, 2010 / Accepted June 4, 2010)

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