



# Gene expression analysis of three homeobox genes throughout early and late development of a feather star *Anneissia japonica*

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## Abstract

Crinoids are considered as the most basal extant echinoderms. They retain aboral nervous system with a nerve center, which has been degraded in the eleutherozoan echinoderms. To investigate the evolution of patterning of the nervous systems in crinoids, we examined temporal and spatial expression patterns of three neural patterning-related homeobox genes, *six3*, *pax6*, and *otx*, throughout the development of a feather star *Anneissia japonica*. These genes were involved in the patterning of endomesodermal tissues instead of the ectodermal neural tissues in the early planktonic stages. In the stages after larval attachment, the expression of these genes was mainly observed in the podia and the oral nervous systems instead of the aboral nerve center. Our results indicate the involvement of these three genes in the formation of oral nervous system in the common ancestor of the echinoderms and suggest that the aboral nerve center is not evolutionally related to the brain of other bilaterians.

**Keywords** Echinoderm · Crinoid · Nervous system · Body patterning

## Introduction

Crinoids are considered as the most basal group of the extant echinoderms due to the fossil records (Paul and Smith 1984; Bottjer et al. 2006) and the molecular analyses (Scouras and Smith 2006; Janies et al. 2011). They are largely divided into two groups by their adult morphology, stalked sea lilies and stalkless feather stars. Feather stars are more dominant than sea lilies in the present seas, which have over 500 species and inhabit in both shallow and deep seas (Clark and Clark 1967). Morphological and molecular analyses indicate that feather stars are derived from ancestral sea lilies (Hyman 1955; Cohen et al. 2004; Rouse et al. 2013); however, many of the

“basal” body organizations are conserved, such as larval stalk, unique water vascular system that adapts to the suspension feeding, and well-developed aboral nervous system with a ganglion (Hyman 1955). For these reasons, feather stars can be considered as the important group to reveal evolutionary origin of the unique features of the echinoderm.

Many bilaterians (protostomes and deuterostomes) have a brain or a nerve center in their heads. In contrast, echinoderms usually do not have a clear head structure nor a brain. The main nervous system of the echinoderms consists of a unit of one nerve ring and five radial nerves (Hyman 1955; Brusca et al. 2016). Two such units exist in eleutherozoan echinoderms (starfish, brittle stars, sea urchins, and sea cucumbers): the predominant oral (ectoneural) nervous system and the inconspicuous deeper oral (hyponeural) nervous system (Supplementary Fig. 1a). The last group of the extant echinoderms, crinoids, also possesses these two nervous systems; however, these two systems do not play principal role. Instead, they have the third system, the aboral (entoneural) nervous system, which serves both motor and sensory functions in crinoids (Supplementary Fig. 1b). In addition, the aboral nervous system of the crinoids possesses a relatively large ganglion in the aboral center of their body, which works as the nerve center at least to integrate the motion of the arms (Marshall 1884).

The molecular mechanism of the anterior-posterior (A-P) regionalization of brain or central nervous system (CNS) is

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basically conserved in bilaterians (Reichert 2009), although morphology of CNS varies among phyla or classes. In the basal deuterostomes, hemichordates retain this molecular mechanism in their ectoderm (Lowe et al. 2003; Pani et al. 2012); however, no such mechanism was observed in echinoderms. A recent study on a stalked crinoid *Metacrinus rotundus* indicated the alteration of the expression and function of three brain regionalization-related homeobox genes *six3*, *pax6*, and *otx*; the three genes are involved in the A-P patterning of the larval endomesoderm instead of the ectodermal nervous systems (Omori et al. 2011). The study yet did not analyzed gene expression in the adult structures; thus, the expression and the function of these genes in the adult structures of the crinoids were still obscure.

*Anneissia japonica* is a feather star crinoid which inhabits the shallow seas of the Japanese temperate coast. Because of the ease of collection, this species has been chosen for the model to confirm the developmental process of feather stars from the fertilized egg through to the adult (Dan and Dan 1941; Kubota 1969, 1970; Shibata et al. 2008). The developmental process of *A. japonica* after the larval hatch is summarized in Fig. 1. This species only took doliolaria-type planktonic larva (Fig. 1a, b), instead of taking both diplerula and doliolaria-type larva as observed in those eleutherozoan echinoderms and a stalked crinoid (Garstang 1894; Nakano et al. 2003). Attachment of the planktonic larva with an anterior

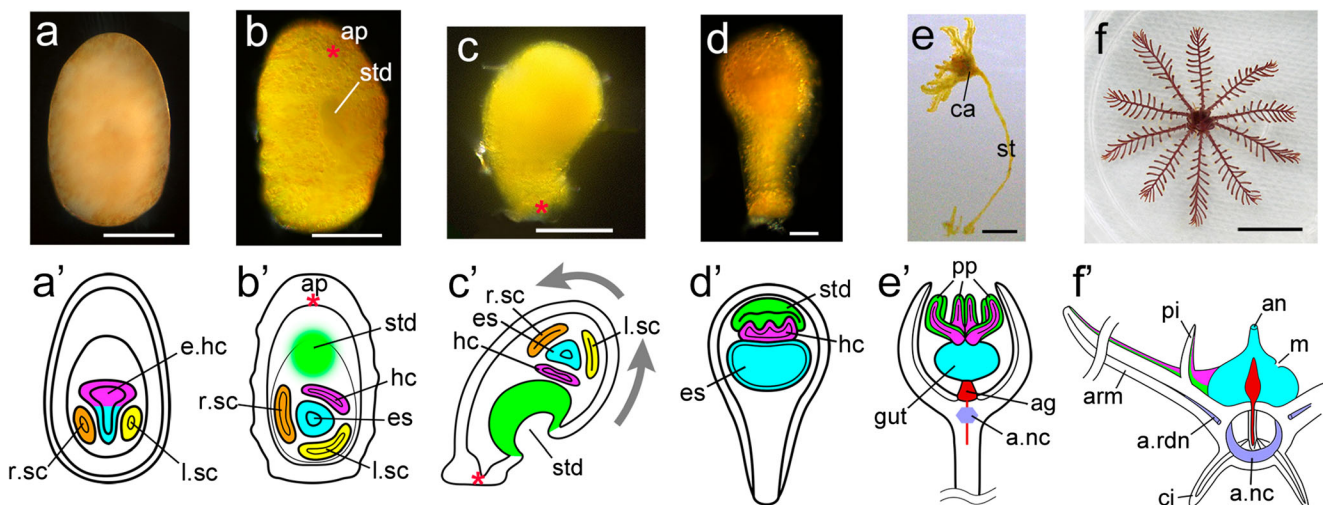
ventral surface and coincident rotation of the endomesodermal structures results in the change of body axis and relating tissue arrangement (Fig. 1c, d). The rearrangement of the body axis is widely known in the development of echinoderms, which makes it difficult to simply compare the body axis of echinoderms and that of other bilaterians to reveal the evolution of body patterning. *A. japonica* did not make a rudiment; thus, it is relatively easy to trace each tissue throughout the axis rearrangement.

To reveal the evolution of patterning of the nervous systems in crinoids, including the evolutionally origin of the aboral nerve center of crinoids, we cloned homologs of *six3*, *pax6*, and *otx* from *A. japonica* and examined temporal and spatial expression patterns of these genes. We improved in situ hybridization methods in the feather star and revealed the expression patterns of three homeobox genes from early planktonic stage to juvenile.

## Materials and methods

### Collection and larval culture of *Anneissia japonica*

Collection, keeping of adult specimens in the aquarium and/or in the sea, and culturing of larvae were conducted as described in Shibata et al. (2008).



**Fig. 1** Normal development of *Anneissia japonica* after larval hatch. Bright-field pictures (a–f) and schematic illustrations of the inner structures (a'–f') are shown for the six developmental stages. Structures in same colors in a'–f' have developmentally same origins. a, a' Semi-doliolaria larva. Ventral view. Two somatocoels were formed beside enterohydrocoel (e.hc). b, b' Doliolaria larva. Para-ventral (b) or ventral (b') view. An adhesive pit (ap), which is identical to the future attachment point (red asterisks), was formed on the anterior ventral surface, and the anterior part of the enterohydrocoel (e.hc) was separated to be a hydrocoel (hc). Relative positions of the endomesodermal tissues began to rotate. c, c' Attachment stage. An attachment point (red asterisks) is arranged to down. (gray arrows). Rotation of the endomesodermal tissues continued (gray arrows). d, d' Cystidean stage. Lateral view with the attachment

point to the bottom. Rotation of the endomesodermal tissues finished, which resulted to arrange the tissue order from top to bottom as stomodaeum (std), hydrocoel (hc), and enteric sac (es). e, e' Pentacrinoid stage. Lateral view with the mouth opening to the top. Primary podia (pp) were formed from the hydrocoel and the stomodaeum of the earlier stages. The aboral nerve center (a.nc) was also formed in the calyx (ca). f, f' Juvenile. Oral (ventral) view (f) or lateral view with the oral side up. Abbreviations: ag, axial gland; an, anus; a.nc, aboral nerve center; a.rdn, aboral radial nerve; ca, calyx; ci, cirrus; e.hc, enterohydrocoel; es, enteric sac; hc, hydrocoel; l.sc, left somatocoel; m, mouth; pi, pinnule; pp., primary podia; r.sc, right somatocoel; std., stomodaeum. Scale bars represent 100  $\mu$ m (a–d), 1 mm (e), or 1 cm (f)

## RNA extraction and cloning of the genes

Total RNA was isolated from different developmental stages of *A. japonica* using TRIzol reagent (Invitrogen). To remove any polysaccharides, we added final concentration of 1 M of ammonium acetate at the isopropanol precipitation. After the 70% ethanol washing, residual DNA was digested with DNase I (TaKaRa). cDNA was prepared from 500 ng total RNA using random hexamers in a 20- $\mu$ L reaction as described in the instructions of SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was dissolved in 80  $\mu$ L TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA).

Fragments of *six3*, *pax6*, and *otx* were isolated by PCR using sets of degenerated primers. The primer sequences are listed in Supplementary Table 1. After the PCR cycles, the products were subjected to gel-electrophoresis. DNA fragments which were extracted from the bands of the appropriate size were cloned into the *EcoRV* site of the pBluescript II SK (+) vector. Gene-specific primers for 5' and 3' rapid amplification of cDNA ends (RACE) analyses were then designed in each fragment. RACE analyses were performed using the GeneRacer kit (Invitrogen) with total RNAs which were extracted from planktonic or settled larvae of *A. japonica* as templates. The resulted sequences were confirmed by comparing conserved domains of each gene to those of other animals (see Supplementary Information for detail).

## Q-PCR

Primers were designed in the coding region of the target genes to obtain 102–115 base pair products (Supplementary Table 1). Mitochondrial cytochrome oxidase subunit I gene (*cox1*) was used as a reference of the Q-PCR. The Q-PCR reaction was carried out using LightCycler (Roche) following the instructions of GoTaq qPCR Master Mix (Promega). All Q-PCR experiments were performed twice using two groups of cDNA prepared separately. Relative concentrations of mRNA were normalized to the *cox1* values.

## WISH

Digoxigenin (DIG)-labeled antisense riboprobes were prepared using plasmids containing 472–892 bp fragments of the genes examined. The primers used for generating the probes are listed in Supplementary Table 1.

Fixation and storing of the samples were performed as described in Omori et al. (2011). The fixed specimens were rehydrated with a graded series of ethanol in PBST (phosphate-buffered saline containing 0.1% Tween 20) and treated with 2–10  $\mu$ g/ml proteinase K (ProK) in PBST at 37 °C for 20 min. The optimal concentration of ProK was 2  $\mu$ g/ml for the planktonic larvae, 5  $\mu$ g/ml for the settled larvae, and 10  $\mu$ g/ml for the juvenile. The ProK-treated specimens were

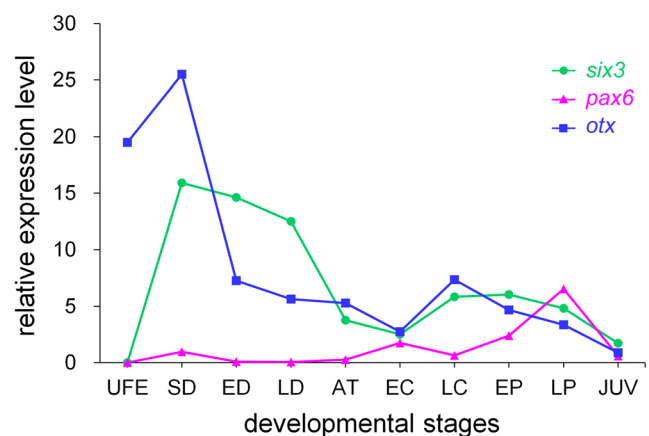
washed with PBST three times, re-fixed in 4% paraformaldehyde in PBST at 4 °C for 30 min, washed with PBST three times, and then incubated in hybridization buffer (50% formamide, 5 $\times$  SSC, 100  $\mu$ g/mL yeast RNA, 50  $\mu$ g/mL heparin, 1% Tween 20) at 50 °C for 4–6 h. The hybridization reaction was carried out in hybridization buffer containing 0.2  $\mu$ g/ml (for the larvae) or 0.5  $\mu$ g/ml (for the juveniles) DIG-labeled riboprobes at 50 °C for 5–10 days. Sample washing and immunodetection were carried out as described in Omori et al. (2011). Stained specimens were immersed in PBST and observed under the light microscopes. After the observation of whole specimens, the stained samples were dehydrated by acetone (5 $\times$  30 min), embedded in Technovit 8100 (Heraeus Kulzer), and cut into 7–10- $\mu$ m-thick slices. The dissected WISH samples were counterstained with Nuclear Fast Red and observed under light microscopes.

## Results

### Temporal gene expression profiling of *six3*, *pax6*, and *otx*

Temporal expression profiles revealed by the quantitative real-time PCR (Q-PCR) analysis (Fig. 2) showed that almost no maternal *six3* and *pax6* mRNAs existed in the unfertilized egg. *six3* was highly expressed in the semi-doliolaria and the doliolaria. The expression of *six3* declined after the larval attachment, following slight increase of the expression in the late cystidean and gradual decrease through to the juvenile.

*pax6* expression became detectable in the semi-doliolaria, although the expression level of *pax6* was under 1/10 of that of *six3* and *otx*. The expression level of *pax6* declined to almost 0 in doliolaria and gradually increased after the larval



**Fig. 2** Temporal expression profiles of *six3*, *pax6*, and *otx*. Values are represented as the relative RNA copy numbers when the *cox1* expression is considered as 100. The developmental stages we investigated are unfertilized egg (EGG), semi-doliolaria (SD), early and late doliolaria (ED, LD), attachment stage (AT), early and late cystidean (EC, LC), early and late pentacrinoid (EP, LP), and juvenile (JUV)

attachment. The peak of the expression was detected in the late pentacrinoid, and the expression slightly declined in the juvenile stage.

*otx* mRNA substantially existed in the unfertilized egg. The high-level expression of *otx* was also observed in the semi-doliolaria; however, the expression level was decreased in the early doliolaria. *otx* expression continued to decline by the early cystidean, which resulted the 1/10 of the expression level compared to that of the semi-doliolaria. The expression level of *otx* slightly increased in the late cystidean and gradually declined through to the juvenile.

Taken together, *six3* and *otx* had two peaks of gene expression (semi-doliolaria and cystidean-pentacrinoid). *pax6* showed low expression level in the planktonic stage and relatively high expression level after attachment.

### Spatial expression patterns of *six3*, *pax6*, and *otx* in the planktonic and settled larvae

In the semi-doliolaria, *six3* was expressed in the anterior ectoderm in a belt which horizontally surrounds the larva (Fig. 3a, double-arrowheads), and in the anterior region of the enterohydrocoel (Fig. 3a, arrow). The ectodermal expression in the banded region decreased slightly in the late doliolaria (Fig. 3b, double-arrowheads), while weak expression was also observed in the entire region of the anterior ectoderm. The ectodermal expression completely disappeared after the larval attachment. The *six3* expression was also observed in the hydrocoel in the late doliolaria (Fig. 3b, arrow), which derives from the anterior region of the enterohydrocoel (Dan et al. 1988). The endomesodermal expression in the hydrocoel also disappeared after the larval attachment. In the early attached larva, *six3* was expressed in a part of inner cell masses which was formed from invaginated stomodeum (Fig. 3c, arrowheads). These expression patterns were maintained in the early cystidean stage (Fig. 3d, arrowheads). The *six3*-positive cell masses developed into the epithelium which covers the oral surface of the podia (Fig. 3e, arrowheads).

*pax6* expression was observed in the anterior region of the enterohydrocoel in the early doliolaria (Fig. 3f, arrow) and in the hydrocoel in the late doliolaria (Fig. 3g, arrow). The expression pattern of *pax6* in the endomesoderm was the same as that of *six3* (Fig. 3a, b, arrows). After the larval attachment, the endomesodermal expression of *pax6* decreased to a non-detectable level, and the expression of *pax6* started in the inner cell mass derived from invaginated stomodeum (Fig. 3h, arrowhead). The expression area of *pax6* expanded in the cell mass in the early cystidean (Fig. 3i, arrowheads). The *pax6* expressing cell mass developed into the outer layer of the podia in the mouth-opening stage (Fig. 3j, arrowheads).

*otx* was broadly expressed in the ectoderm of the early doliolaria (Fig. 3k). The expression was especially stimulated in four circumferential belts and the most posterior wall (Fig.

3k, asterisks). The *otx*-negative cells were located in the positions of future ciliary bands. *otx* was also expressed in the posterior part of the enterohydrocoel (Fig. 3k, arrow), which is the precursor of the enteric sac. The expression in the ectoderm and the endomesoderm continued until the late doliolaria stage. *otx* was strongly expressed in the ciliary bands in the late doliolaria (Fig. 3l, asterisks) and in the enteric sac (Fig. 3l, arrow). After the larval attachment, the expression of *otx* observed in the planktonic larvae was diminished to a non-detectable level, and the expression of *otx* in the wall of the invaginating stomodeum was newly observed (Fig. 3m, arrowhead). The expression of *otx* in the stomodeum continued in the cell mass exhibiting a doughnut shape which derives from the wall of the stomodeum in the early cystidean (Fig. 3n, arrowheads). In the mouth-opening stage larva, *otx* was expressed in a ring surrounding the mouth (Fig. 3o, arrowheads).

### Spatial expression patterns of *six3*, *pax6*, and *otx* in the juvenile tissues

In the juvenile of *A. japonica*, *six3* expression was observed in the basal part of the podia (Fig. 4a, arrows; b) and the basal lamina of the food grooves which includes oral radial nerves (Fig. 4a, b, arrowheads). The oral ring nerve was also *six3* positive (Supplementary Fig. 4a). The *six3* expression in the podia was not observed in the podia around the mouth (Fig. 4a). In the calyx, *six3* was expressed in the axial gland (Fig. 4c, arrow). Faint expression of *six3* was also observed in the aboral ring nerve (Fig. 4c, arrowheads). The deep stain in the filament-like structures in the chambered organ (Fig. 4c, asterisks) was an artifact, which was also observed in the negative control sample (Fig. 4j, asterisks).

*pax6* was expressed in the outer layer of all the podia (Fig. 4d, e). In the calyx, *pax6* was expressed in the axial gland (Fig. 4f, arrow). No significant expression of *pax6* was observed in other tissues in the calyx.

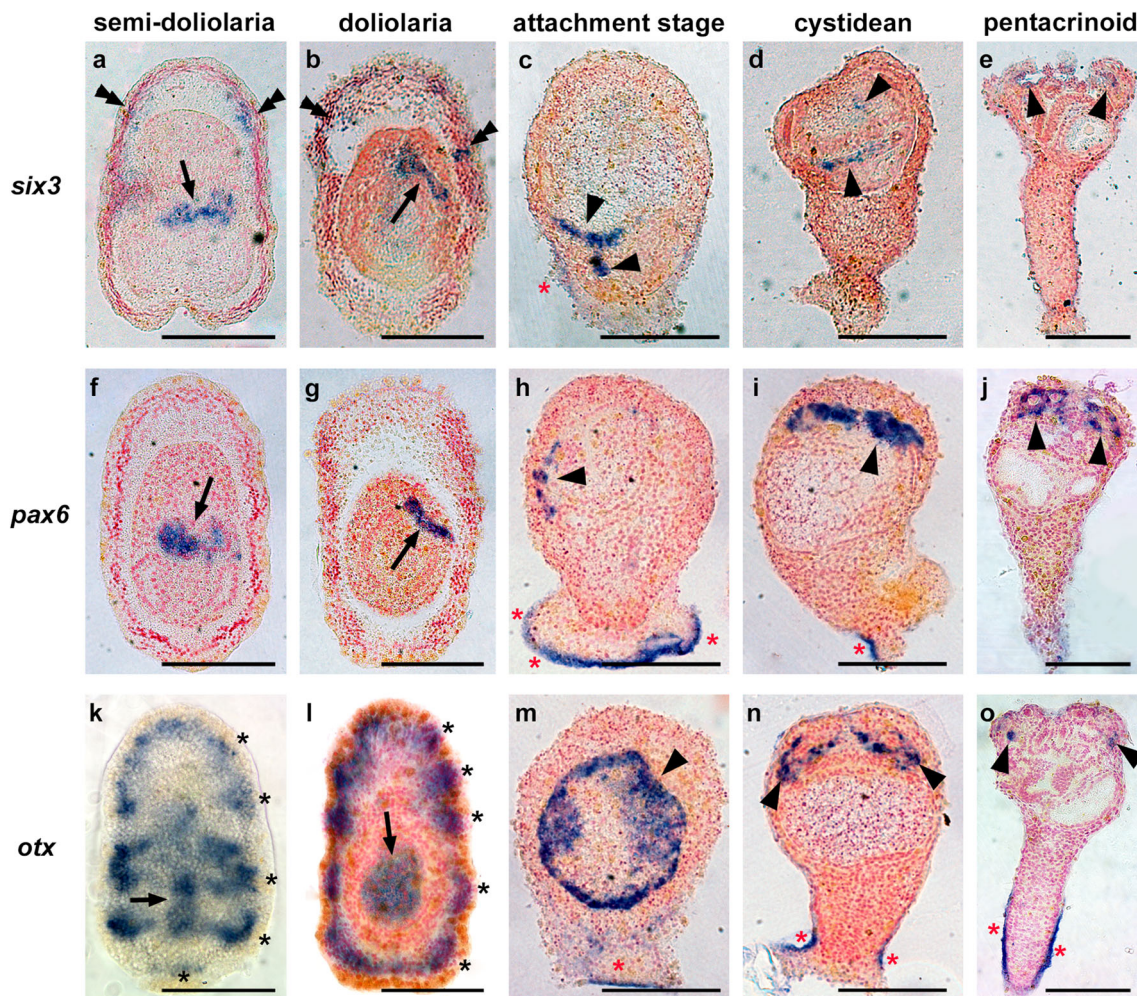
*otx* was expressed in the podia in the distal tip of the arms (Fig. 4g, h, arrows). Podia in the proximal part of the arms did not express *otx* (Fig. 4h, arrowheads). No significant expression of *otx* was observed in the calyx (Fig. 4i).

## Discussion

### Involvement of the three homeobox genes in the patterning of larval endomesoderm

In the planktonic larvae of *A. japonica*, *six3* and *pax6* were expressed in the anterior enterohydrocoel of early doliolaria and the hydrocoel of doliolaria, while *otx* was expressed in the posterior enterohydrocoel of early doliolaria and the enteric sac of doliolaria (Fig. 5a, b). As the enterohydrocoel is the



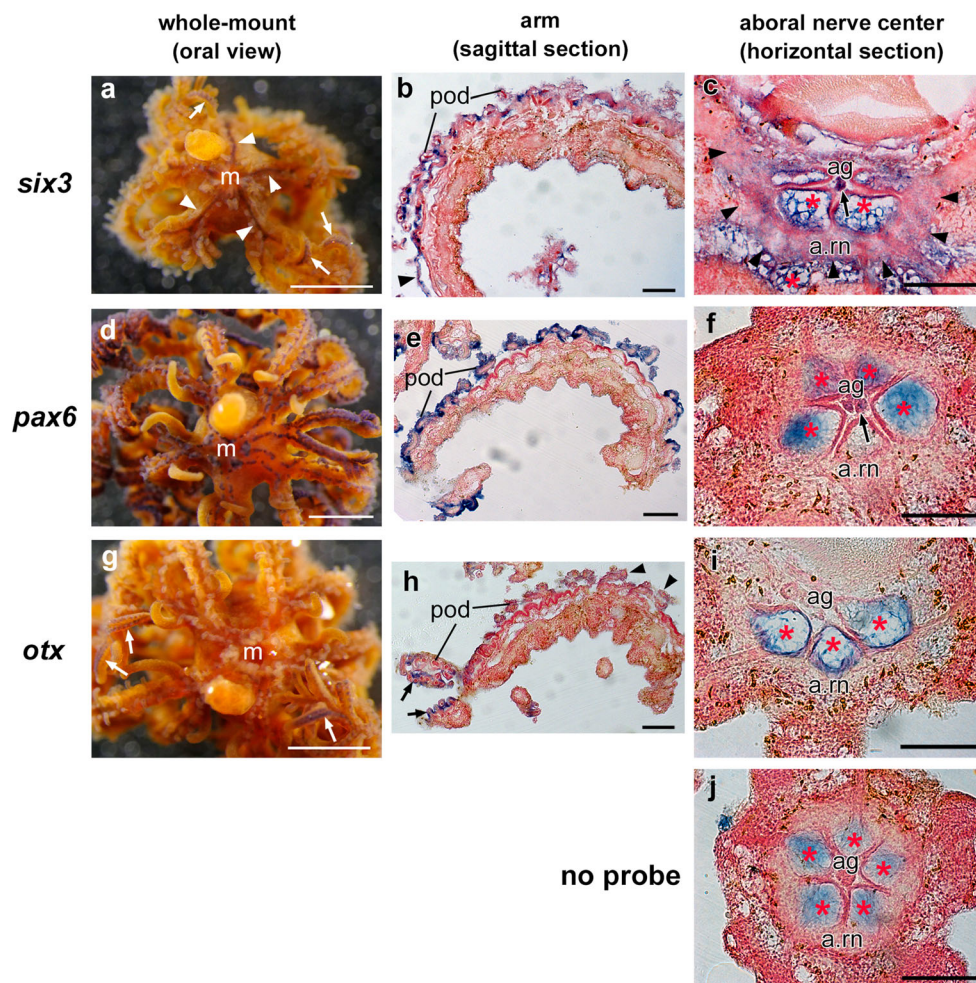


**Fig. 3** Spatial expression patterns of *six3* (a–e), *pax6* (f–j), and *otx* (k–o) throughout the larval development of *A. japonica*. Expression of the genes is represented by the sections of the WISH samples, which were counterstained with Nuclear Fast Red. The planktonic larvae (a, b, f, g, k, l) are viewed ventrally with the vegetal pole down. The settled larvae (c–e, h–j, m–o) are oriented with the attachment point to down. Red asterisks on c, h, i, m, n, o indicate non-specific signals which often appear around the attachment point. a *six3* expression in an early doliolaria. Expression in the anterior enterohydrocoel is indicated by an arrow. The ectodermal expression is represented by double-arrowheads. b *six3* expression in a doliolaria. Expression in the hydrocoel is indicated by an arrow. The ectodermal expression is represented by double-arrowheads. c *six3* expression in an early attached larva. Arrowheads represent the expression in the stomodeum. d *six3* expression in an early cystidean. Expression in the stomodeum derivative cells is represented by arrowheads. e *six3* expression in a late cystidean. The expression detected in the oral surface of podia is indicated by arrowheads. f *pax6* expression in an early doliolaria. The expression in the anterior enterohydrocoel is indicated by an arrow. g *pax6* expression in a doliolaria. The expression in the hydrocoel is indicated by an arrow. An arrowhead represents the expression in the stomodeum. h *pax6* expression in an early attached larva. An arrowhead represents the expression in the stomodeum. i *pax6* expression in an early cystidean. The expression in the stomodeum-derivative cells is represented by arrowheads. j *pax6* expression in a late cystidean. The expression in the outer layers of all podia is indicated by arrowheads. k *otx* expression in an early doliolaria. Asterisks depict the point of strong expression of the ectoderm. The expression in the posterior enterohydrocoel is indicated by an arrow. l *otx* expression in a doliolaria. Asterisks depict the point of ciliary bands, where the strong expression is observed. The expression in the enteric sac is indicated by an arrow. m *otx* expression in an early attached larva. An arrowhead represents the expression in the stomodeum. n *otx* expression in an early cystidean. The expression in the stomodeum derivative cells is represented by arrowheads. o *otx* expression in a late cystidean. Arrowheads depict the expression in the oral ring nerve. Scale bars represent 100  $\mu$ m

term for the closed archenteron of feather stars (Hyman 1955; Kubota 1969), these results suggest that *six3*, *pax6*, and *otx* are involved in the patterning of the archenteron of *A. japonica* planktonic larvae. In the planktonic larvae of a sea lily crinoid *Metacrinus rotundus*, *six3*, *pax6*, *otx*, and *hox* genes are involved in A-P patterning of the archenteron and its derivatives which include axohydrocoel, enteric sac, and left and right somatocoels

(Hara et al. 2006; Omori et al. 2011). While the precursor of the somatocoels has not been clearly detected in *A. japonica*, somatocoels of other feather star *Antedon adriatica* are known to be derived from posterior enterohydrocoel, which is separated by the anterior enterohydrocoel by a constriction that emerges on the middle of the enterohydrocoel (Hyman 1955). Our results suggested that the involvement of *six3*, *pax6*, *otx*, and *hox* genes





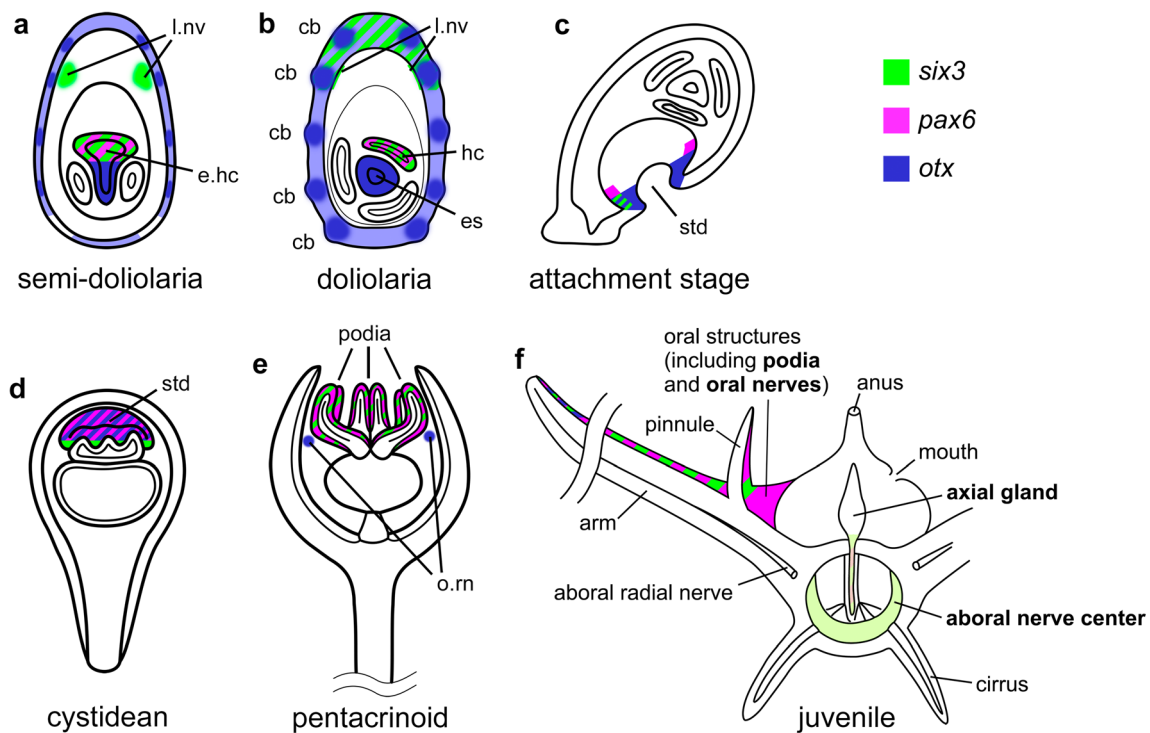
**Fig. 4** Spatial expression patterns of *six3* (a–c), *pax6* (d–f), and *otx* (g–i) in *A. japonica* juvenile. Ventral views of the WISH samples (a, d, g) or the dissected WISH samples which represent sagittal sections of the arms (b, e, h) and horizontal sections of the calyx (c, f, i, j). For the dissected arm samples (b, e, h), distal side of the sample is arranged to left, and ventral (oral) side is arranged to up. **a** A whole-mount image of the expression of *six3* in the juvenile. *six3* expression was detected in the basal part of the podia (arrows) and the basal lamina of the ambulacral grooves (arrowheads). **b** *six3* expression in the arm. Basal cells of the almost all podia (pod) on the arm were *six3* positive. An arrowhead represents *six3* expression in the basal lamina. **c** *six3* expression in the calyx. An arrow represents *six3* expression in the axial gland. Arrowheads depict expression of *six3* in the aboral ring nerve. **d** A

whole-mount image of the expression of *pax6* in the juvenile. All podia represented the strong expression of *pax6*. **e** *pax6* expression in the arm. The strong *pax6* expression in the podia was limited in the outer layer. **f** *pax6* expression in the calyx. An arrow represents *pax6* expression in the axial gland. **g** A whole-mount image of the expression of *otx* in the juvenile. Arrows indicate the *otx* expression in the distal podia. **h** *otx* expression in the arm. Arrows indicate the *otx*-positive podia on the distal part of the arm. Arrowheads indicate the *otx*-negative podia on the proximal part of the arm. **i** *otx* expression in the calyx. No significant signal was observed. **j** A calyx of the no probe negative sample. Red asterisks on c, f, i, and j indicate the non-specific signals. Abbreviations: ag, axial gland; a.rn, aboral ring nerve; m, mouth; pod, podia. Scale bars represent 1 mm (a, d, g), or 100  $\mu$ m (b, c, e, f, h, i, j)

in A-P patterning of the endomesoderm is a basal feature in crinoids, although external shapes of the early larvae are not similar between a sea lily and feather stars (Hyman 1955; Nakano et al. 2003),

*otx* expression in the endodermal tissues is widely observed in deuterostomes (Ang et al. 1994; Gan et al. 1995; Wada et al. 1996; Williams and Holland 1996; Mitsunaga-Nakatsubo et al. 1998; Tomsa and Langeland 1999; Harada et al. 2000; Shoguchi et al. 2000; Nielsen et al. 2003; Lowe et al. 2003; Hinman et al. 2003). In the embryos and larvae of an amphioxus *Branchiostoma floridae*, *six3* and *pax6* are expressed in

the anterior tip of the endoderm (Glardon et al. 1998; Kozmik et al. 2007), which is separated by the more posterior *otx*-expressing endodermal area within the progress of its development (Williams and Holland 1996). The expression of these three genes in the mesendoderm is also reported in some hemichordates (Lowe et al. 2003; Gonzalez et al. 2017). The similarity of the expression patterns of *six3*, *pax6*, and *otx* among crinoids, hemichordates, and amphioxus implies the involvement of these genes in A-P patterning of the endomesoderm in the basal deuterostomes. However, *pax6* is only expressed in the ectodermal tissues in ascidians



**Fig. 5** Schematic illustrations of the expression patterns of *six3*, *pax6*, and *otx* in the larvae and juvenile of *A. japonica*. Semi-doliolaria (a) and doliolaria (b) are the ventral view with the posterior side down. Thus, the left side of the figures corresponds to the right side of the larvae. Attachment stage (c), cystidean (d), pentacrinoïd (e), and juvenile (f) represent the lateral view with the attachment point to the bottom. Green-, pink-, and blue-colored areas represent the gene-expressing area

(Glaridon et al. 1997; Irvine et al. 2008), hemichordates (Lowe et al. 2003; Gonzalez et al. 2017), and a basal metazoan xenacoelomorph (Hejnol and Martindale 2008). Further studies on more species of deuterostomes and basal metazoans will reveal the basal function of these genes in body patterning.

### Ectodermal *otx* expression in the planktonic larvae and its putative function

In the doliolaria larva of *A. japonica*, *otx* was expressed in the circumferential ciliated bands. A doliolaria-type larva with some circumferential ciliated bands is known in crinoids, brittle stars, and sea cucumbers (Hyman 1955; Nakano et al. 2003). A sea cucumber *Psolus chitinoïdes*, which develops into doliolaria directly without the auricularia stage, expresses *otx* in all three ciliary bands of the doliolaria (Lowe and Wray 1997). In contrast, a different species of sea cucumber *Apostichopus japonicus*, which develops into both auricularia and following doliolaria, does not express *otx* in the ciliary bands of doliolaria (Shoguchi et al. 2000). Different properties of *otx* expression in ciliary bands in the doliolaria stage of different species suggest that *otx* is not responsible for the maintenance of circumferential ciliary bands. In the hatching stage of *A. japonica*, the entire surface of the larva

is uniformly covered with cilia. The larva rearranges the ciliated cells to form the ciliary bands afterward. In the semi-doliolaria larva, the expression of *otx* is restricted to a prospective non-ciliary-band region. Taking the expression patterns of *otx* in the semi-doliolaria and the doliolaria together, it is suggested that *otx* is involved in the formation of ciliary bands by inducing cell migration of the ciliary cells. *otx* expression in the ciliary cells has been also observed in the polychaete protostomes (Arendt et al. 2001; Arenas-Mena and Wong 2007) and in the more basal metazoans like a sea anemone *Nematostella vectensis* (Mazza et al. 2007). The widely conserved *otx* expression pattern associated with the ciliary cells suggests an ancestral role of *otx* in forming the ciliary cells and/or regionalization of the ciliary band.

### Switching of the expression status of three homeobox genes after the larval attachment

After attachment of the larva, the expression of *six3*, *pax6*, and *otx* completely ceased in the regions where these genes expressed in the planktonic larvae. In parallel, these genes start to be expressed in the wall of stomodeum (Fig. 5c). The temporal expression profiles of three genes also showed the change of expression phase after the larval attachment (Fig. 2). It has been reported that the adult nervous system

begins to form at least 1 day after the larval attachment without any connection to the larval nerves (Nakano et al. 2009). These imply that the larval attachment triggers the transition of gene expression pattern to form adult structures.

The expressions of *six3*, *pax6*, and *otx* continued in the wall of the stomodeum in the cystidean (Fig. 5d), and the expression area of these genes shifted to the podia in the later stage (Fig. 5e). A previous immunohistochemical observation using sea star synaptotagmin antibody has suggested that primary oral nerves exist in the podia or in their precursors (Nakano et al. 2009). The *pax6* expression pattern in the podia is similar to the patterns of synaptotagmin immuno-reactivity, and *six3*-expressing cells on the surface of the podia were also located in the synaptotagmin-positive area (Figs. 3e, j and 4b, e, Supplementary figure 1c). In addition, *six3* expression was also observed in the oral nerves of the juvenile (Fig. 4a; supplementary Fig. 4a). These suggest that *six3* and *pax6* are involved in the formation of the oral nervous systems. It was also reported that *otx* is expressed in the oral ring nerve and the primary podia in sea urchins (Nielsen et al. 2003; Morris et al. 2004). In our results in *A. japonica*, *otx* was expressed in the ringed area around the mouth in the pentacrinoid (Fig. 3o, arrowheads), where the intense synaptotagmin immuno-reactivity was reported (Nakano et al. 2009). Taken together, *six3*, *pax6*, and *otx* are possibly involved in the formation of the oral nervous system in the post-attachment stages of *A. japonica*, and this property was probably acquired in the common ancestor of the echinoderms.

### Gene expression in the juvenile tissues and its evolutionally relationship among deuterostomes

*otx* was expressed in the outer layer of the podia in the juvenile of *A. japonica* (Fig. 4g, h). *otx* expression in the outer layer of the podia has been also observed in some eleutherozoan echinoderms (Lowe and Wray 1997; Morris et al. 2004), suggesting that this expression pattern was acquired in the common ancestor of extant echinoderms. However, the *otx* expression in the podia was restricted to the distal tip of the arms, in contrast to the uniform expression in the entire podia observed in the eleutherozoans (Lowe and Wray 1997). It has been reported that *pax6* is mainly expressed in the inner layer of podia in sea urchins (Czerny and Buslinger 1995; Ullrich-Luter et al. 2011), while *pax6* is expressed in the outer layer of podia in *A. japonica* (Fig. 4d, e). These differences may reflect the morphological difference of the podia. The podia of the crinoids contain many sensory papillae on their surface, which are not observed in the podia of the eleutherozoans. As *pax6* and *otx* are involved in the formation of optic sensory organs collaborating with *six3* in various phyla (Callaerts et al. 1997; Gehring and Ikeo 1999; Arendt 2003; Zuber et al. 2003; Martínez-Morales et al. 2004; Stierwald et al. 2004; Kozmik 2005), our result implies the existence of some optic sensory organs on the podia of *A. japonica*.

Further morphological and molecular studies will confirm the existence of podial sensory organs.

Crinoids have three nervous systems: oral, deeper oral, and aboral systems (Hyman 1955). Disruption of the aboral nerve center results in the disorganization of the arm movement, while removal of the oral and the deeper oral nervous systems does not affect its behavior (Marshall 1884). Thus, the aboral nervous system is considered as the main nervous system in crinoids (Hyman 1955). This evokes an idea that the aboral nerve center is evolutionally similar to the central nervous system of other animals. However, in the present study, homologs of the brain-patterning genes *pax6* and *otx* were not expressed in the aboral nerve center of *A. japonica* (Figs. 4f, i and 5f). Other brain-patterning gene homolog *six3* was weakly expressed in the whole aboral ring nerve (Figs. 4c and 5f); however, the expression was not biased in any axes. These imply that the aboral nervous system of crinoids is not homologous to the central nervous system of other animals. The arms of the crinoids play important roles in their feeding and movement. The aboral nerve center may originally develop in the basal echinoderm clade to function as the center for motor neuron which organizes the arm movement. Further morphological and molecular analyses will reveal the origin of the aboral nervous system.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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