Animal pole determinants define oral–aboral axis polarity and endodermal cell-fate in hydrozoan jellyfish *Podocoryne carnea*

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Abstract

Cnidarians, in contrast with bilaterians, are generally considered to exhibit radial symmetry around a single body axis (oral–aboral) throughout their life-cycles. We have investigated how the oral–aboral axis is established in the hydrozoan jellyfish *Podocoryne carnea*. Vital labeling experiments showed that the oral end of the blastula derives from the animal pole region of the egg as has been demonstrated for other cnidarian species. Gastrulation is restricted to the oral pole such that the oral 20% of blastula cells give rise to endoderm. Unexpectedly, bisection experiments at the 8-cell stage showed that animal regions are able to develop into normally polarized larvae, but that vegetal (aboral) blastomeres completely fail to develop endoderm or to elongate. These vegetal-derived larvae also failed to polarize, as indicated by a lack of oral-specific RFamide-positive nerve cells and a disorganized tyrosinated tubulin-positive nerve net. A different result was obtained following bisection of the late blastula stage: aboral halves still lacked the capacity to develop endoderm but retained features of axial polarity including elongation of the larva and directional swimming. These results demonstrate for the first time in a cnidarian the presence of localized determinants responsible for axis determination and endoderm formation at the animal pole of the egg. They also show that axial polarity and endoderm formation are controlled by separable pathways after the blastula stage.

Keywords: Axis determination; Endoderm; Determinant; *Podocoryne carnea*; Cnidaria

Introduction

Members of the phylum Cnidaria (jellyfish, hydra, corals and sea anemones) are morphologically divergent but are all generally considered to be “diploblasts” with only two germ layers, endoderm and ectoderm and to have a radially symmetric body with a single body axis referred to as the oral–aboral axis. These characters of the cnidaria contrast with those of its sister group the bilateria, whose members have three germ layers and bilateral body patterns. It is widely believed that the bilateral body plan was invented in the bilaterian clade and a common ancestor of bilaterians and radiata (cnidaria and ctenophores) had a radial body pattern. An alternative viewpoint supported by some recent molecular data is that the cnidaria have lost ancestral bilaterality, explaining why some cnidarians exhibit elements of a biradial or bilateral body organization (Hyman, 1940) and exhibit bilateral expression of *bmp* and *hox* genes during embryogenesis (Finnerty et al., 2004; Ball et al., 2004). The issue of whether diploblasty is ancestral has also been recently questioned. Orthologues of genes that are involved in mesoderm formation in bilaterians are expressed in precursors of striated muscle or subpopulation of endoderm in cnidarians (Spring et al., 2001, 2002; Müller et al., 2003; Martindale et al., 2004). These observations suggest that studies of axis and germ layer establishment in bilaterians will provide insight into the body plan of the common eumetazoan ancestor as well as into the evolution of early developmental mechanisms. The existing studies of embryonic axis specification in Cnidaria mostly concern members of the class Hydrozoa. Hydrozoans often have a larval form called the planula, which exhibits an oral–aboral polarity manifested by elongated morphology, directed locomotion and a graded pattern of cell differentiation. In all cases examined so far, the oral end (future
polyp hypostome) has been found to derive from the animal pole of the egg, irrespective of cleavage and gastrulation patterns that are highly variable among hydrozoans (Teissier, 1931, 1933; Freeman, 1980, 1983, 1990). The animal pole is marked by the position of the female pronucleus and a polar body, and by the initiation site of the first mitotic cleavage furrow (Rappaport, 1963; Campbell, 1974). It is not thought, however, that the embryonic oral–aboral axis has already been determined in the egg, since in *Phialidium gregarium* (order Hydroida, Leptomedusae) the position of the future oral end of the larva corresponds to the site of the first cleavage furrow determined in the egg, since in Phialidium, it is confined to the future oral end. This suggests that endoderm formation and the axis polarity are likely to be independently regulated in Cnidaria.

Very little is known about the molecular mechanisms underlying the specification of the body axis, although it appears likely that the Wnt/β-catenin pathway is involved. In *Hydra* (Hydrozoa), molecules in this pathway, including β-catenin, are expressed in the hypostome region of the polyp and at the region of asexual buds (Hobmayer et al., 1996, 2000). In *Nematostella victensis* (Anthozoa), β-catenin protein is localized to nuclei on the future oral side of the embryo after the 32-cell stage, and has been suggested to play a role in endoderm segregation, similar to that reported for mesoderm/endoderm in deuterostomes (Wikramanayake et al., 2003). It is possible that RNAs localized in the egg somehow activate the Wnt/β-catenin pathway in the embryo. In *Podocoryne carnea* (Hydrozoa), two maternal RNAs transcribed from a posterior hox gene cnox4-Pc and a Brachyury orthologue Pc-Bra (Yanze et al., 2001; Spring et al., 2002) are localized to the animal pole of the egg. In this study, we have examined axis specification experimentally during embryogenesis in *Podocoryne* to understand the origin of the oral–aboral axis and its relationships to endoderm formation in a cnidian species.

**Materials and methods**

**Animal culture and dissection**

*P. carnea* (class Hydrozoa, order Hydroidea), which are found as polyp colonies on shells of the snail *Nassarius rutilatii*, were collected near Roscoff (France). They were cultured on glass slides and maintained in artificial sea water at 16–20°C. The polyps were fed on nauplii larvae of *Artemia salina* every 2 or 3 days. Released medusae were collected into filtered sea water (FSW). Spawning was induced by light stimulation after the cultures were kept for several hours in the dark. Fertilized eggs were collected and reared at 18°C in FSW. The animal–vegetal polarity of 2- to 8-cell stage embryos was identified by the location of polar bodies and cleavage pattern. For isolation experiments, embryos were dissected under an inverted microscope using glass needles.

**Microscopy for living animals**

Specimens were embedded in 0.3–0.5% low melting point agarose in FSW to arrest locomotion, mounted on chamber slides and then observed under Nomarski microscopy. For time-lapse imaging, the chamber slides were sealed with Vaseline and pictures were taken once every 30 s with an automated CCD camera.

**Cell labeling**

A 0.5% solution of *M. 2 × 10^6* dextran conjugated to tetramethylrhodamine (D-7139, Molecular Probes, Eugene, USA) was centrifuged briefly to remove insoluble particles, then microinjected using a Femtotjet microinjector (Eppendorf). Roughly 0.1 to 1 pl was injected into each blastomere depending on its size. Injected embryos were reared in FSW in glass dishes. Damaged embryos were removed a few hours after injection. The distribution of the labeled cells was observed under a fluorescence microscope. To mark the polarity of bisected embryos, a small drop of 1% DiI (Molecular probe) dissolved in DMSO was placed on the surface of target cells using a microinjection needle. The labeled specimens were immediately washed with fresh FSW before the DiI solution diffused.

**Immunohistochemistry and endoderm-specific alkaline phosphatase detection**

Immunohistochemistry to detect tyrosinated tubulin (TT) and RFamide neuropeptide was performed as previously described (Grimmelikhuijzen, 1985; Groger and Schmid, 2001; Müller et al., 2003). Endoderm-specific alkaline phosphatase (AP) was detected at 38 hpf as previously described in another species (Whittaker and Meedel, 1989). AP activity is found exclusively in endoderm and it becomes detectable by 30 hpf in normal embryos.

**Results**

**Podocoryne embryonic development**

In *Podocoryne*, the animal pole of fertilized eggs can be distinguished by the presence of the nucleus and the polar body (Fig. 1A). The furrow of the first cleavage is unipolar, beginning from the animal pole and progressing to the vegetal pole (Fig. 1B). Subsequent cleavages occurred about every 30 min (Fig. 1C). Embryos become blastula with blastocoel (coeloblastula) about 3–4 h postfertilization (hpf) (Fig. 1D). They elongate along the animal–vegetal axis after about 6 hpf (Fig. 1E). The oral–aboral polarity is morphologically clear after 10 hpf; epithelialized blastoderm cells become elongated apicobasally at the oral end of the embryo, and the blastula is normally tapered towards the oral end (Fig. 1F). Gastrulation begins around 13–14 hpf and seems to take place exclusively at the oral end (Fig. 1G). During gastrulation, the embryos develop cilia and swim unidirectionally with their aboral end in front. Gastrulation is completed by 24–28 hpf and gives rises to a planula (Fig. 1H). The planula has an oral (=posterior)–aboral (=anterior) axes. Our observations showed that in *Podocoryne* the pattern of early cleavages is variable (Fig. 1I). One of the typical patterns was regular radial divisions; each division occurring perpendicular to the previous division. The second cleavage plane was perpendicular to that of the first division and parallel to the animal–vegetal axis, while the third cleavage plane was perpendicular to the animal–vegetal axis such that the blastomeres at the 8-cell stage were arranged in rows. The other typical pattern was irregular radial in which the second and subsequent cleavage planes were twisted to variable degrees with respect to each other so that the blastomeres at 4-cell stages were staggered.
Correspondence between the animal pole and the oral end

To determine the relationship between egg polarity and the oral–aboral axis of the blastula, we injected rhodamine-conjugated dextran (DR) solution into single blastomeres of 4- or 8-cell stage embryos showing regular radial division patterns. When DR was injected into one blastomere at the 4-cell stage, DR-positive cells in the blastula formed a contiguous column of DR-positive cells running along the body (Fig. 2A). When DR was injected into one of the animal blastomeres at the 8-cell stage, the descendants were always found at the oral end at the late blastula stage (Fig. 2B), while the descendants of vegetal blastomeres were found at the aboral end (data not shown). In both cases, the labeled cells formed a contiguous region parallel to the oral–aboral axis. Given that the 8-cell stage blastomeres were arranged in rows with respect to the animal–vegetal axis, these observations show that the animal pole of the Podocoryne egg gives rise to the oral end of the blastulae, and vegetal pole to the aboral end. The same results were obtained with embryos that showed irregular radial divisions except that the DR-positive regions were arranged spirally around the axis, reflecting the twisted divisions.

Oral–aboral axis and endoderm fate

Two possible patterns of endoderm migration were conceivable: (1) the endodermal cells migrate into blastocoel exclusively from the oral-end of blastulae (i.e. unipolar gastrulation) or (2) the migration begins from the oral end then spread along the longitudinal axis (multipolar ingressing initiating at the oral end). We used time-lapse imaging during gastrulation to distinguish between these possibilities (Fig. 2C, Supplemental material). The oral end of blastulae was identified by the apicobasal elongation of the epithelial cells (9.5–11.5 hpf). At 13.5 hpf, endodermal cells started to migrate at the oral pole. They migrated continuously into blastocoel through the oral pole, where no clear boundary between two layers could be observed. The endodermal cells first filled the blastocoel at the oral end (15.5 hpf), then moved aborally (17.5 hpf). The lateral boundary between endoderm and ectoderm remained clear throughout gastrulation, and no cells were observed to cross this boundary. Small clump of cells sometimes flaked off from the blastoderm into blastocoel before gastrulation began and formed small epithelialized vesicles.

The presumptive endoderm territory in blastulae was determined by DR-labeling. DR was injected randomly into single blastomeres at 32-cell stage. Distribution of DR-positive cell was first recorded at late blastula stage, when the orientation can be distinguished morphologically. The specimens were allowed to develop to the planula stage and the presence of DR cells in endoderm and ectoderm was recorded. When the oral region of the blastulae was labeled (Fig. 2D), the DR-positive cells were found in both endoderm and ectoderm (Fig. 2E). In contrast, when the aboral region of the
blastula was labeled, DR-positive cells were found only in ectoderm (Figs. 2F, G). These results indicate that endoderm fate is restricted to cells at the oral end of the blastula. To map the region in the blastula that becomes endoderm, the distributions of the DR-positive cells in the late blastulae were measured as the distance from the oral end relative to body length (Fig. 3A). DR-positive endodermal cells were present only when there were labeled cells in roughly 20% of the oral end in the blastulae. One such specimen was followed through gastrulation and later found to have DR-signal exclusively in the endoderm. These results show that endoderm cells originate exclusively from the oral 20% of the blastula (Fig. 3B). To summarize, our analyses indicate that gastrulation in Podocoryne proceeds as follows: (1) The oral end of the blastula elongates apicobasally; (2) Endodermal cells migrate into the blastocoel exclusively from the oral end; (3) Once inside the blastocoel, the endoderm cells move in an aboral direction; (4) Endoderm derives from the oral

Fig. 2. The animal–vegetal polarity of the egg corresponds to its oral–aboral axis. (A) A single blastomere was labeled with DR at 4-cell stage. The descendants of the labeled cell formed a contiguous region spanning the entire axis. (B) An animal blastomere was labeled at 8-cell stage. Its descendants were located in contiguous region spanning the oral region of the blastula. (C) A time-lapse observation of gastrulation. Numbers indicate hours postfertilization (hpf). (D–G) Blastulae were randomly labeled by injecting DR into a blastomere at 32-cell stage. (D) A blastula that had labeled at the oral end. (E) The same embryo as panel D at the planula stage. DR-positive cells were found in both endoderm and ectoderm. Dispersed groups of cells were seen in endoderm. (F) A blastula that had been labeled at the aboral end. (G) The same embryo as panel F at the planula stage. The DR-signal was detected exclusively in the ectoderm. Correspondence between Oral is to the top in panels A, B and D–G and to the right in panel C. Scale bar: 20 μm.

Fig. 3. (A) Endoderm fate mapping at the blastula stage. Distribution of DR along the axis in blastulae is represented as black bars. The fate of the DR-positive cells is indicated on the left. Numbers on each side of the bars indicate aboral and oral limit of DR distribution. (B) Schematic representation showing the correspondence between egg polarity, the oral–aboral polarity and the endoderm fate. The animal pole of fertilized eggs corresponds to the oral end at the blastula stage and the vegetal pole to aboral end. The oral 20% region of blastula gives rise to endoderm, while the aboral 80% only gives rise to ectoderm.
20% of blastula, while the aboral 80% region give rise only to ectoderm.

Localized determinants in cleavage stage embryos specify axial properties

To investigate the mechanism of oral–aboral axis determination, we isolated various parts of Podocoryne embryos at the 2- to 8-cell stage and examined how their axial properties including morphology, locomotion and cell differentiation were affected. First we cut 2- and 4-cell stage embryos laterally into two halves (Lat1/2 and Lat2/4, respectively). All isolates scored developed in a normal manner and formed planula larvae (Table 1). Next we cut 8-cell stage embryos (showing regular radial division patterns) through the equatorial plane to yield isolates containing the animal four or the vegetal four blastomeres (Am4/8 and Vg4/8, respectively) (Fig. 4A). At 10 hpf, all Am4/8 isolates had formed normal blastulae with thickened oral epithelia (Fig. 4B, Table 1). They gastrulated normally. In contrast, all Vg4/8s failed to elongate and remained as a spherical coeloblastula. The blastoderm epithelialized but showed no sign of the thickening (Fig. 4C, Table 1). At 30 hpf, the Am4/8s formed planulae indistinguishable from normal planulae (Fig. 4D). The Vg4/8 isolates were still coeloblastulae and showed no apparent oral–aboral polarity (Fig. 4E). Several thickened regions developed in the ectoderm of these isolates, displaying morphology typical of the aboral ectoderm in control embryos at this stage. The Vg4/8 isolates had motile cilia, but their twirling behavior (Fig. 4D, arrow) rendered it impossible to identify the oral or the aboral end from the swimming direction. We conclude that determinants present in animal blastomeres at the 8-cell stage are necessary for the establishing of axial properties and endoderm formation at the presumptive oral pole.

The animal determinants for endoderm and polarity are probably localized before the 8-cell stage, since compatible results were obtained by isolating single cells from 4-cell embryos showing irregular radial division. In these embryos, the second division partially segregates animal and vegetal components between the blastomeres (see Fig. 1B). As with the Vg4/8 isolates, 1 of 20 such “Lat1/4” isolates from five embryos failed to gastrulate (Table 1).

The development of axial polarity of Vg4/8 was further assessed using markers of neuronal differentiation that show organized patterns along the oral–aboral axis; tyrosinated tubulin (TT) and RFamide. In control planulae, TT-positive nerve rings were detectable in the ectoderm of the aboral region at 36 hpf, forming neurites which mainly extend perpendicular to the oral–aboral axis (Figs. 5A, B, arrow). Some neurites also extend longitudinally (Figs. 5B, arrowhead and H). In the oral region, all TT-positive nerves always projected unidirectionally towards the aboral end (Figs. 4H and 5C, arrowhead). The TT-positive neurons were multipolar as they were in the aboral end of normal planulae. RFamide antibody stained distinctive large spherical nerve cells that located exclusively at the oral end of planulae at 52 hpf, in addition to nerve cells that were present throughout the body (Fig. 5F). None of the Vg4/8s showed oralspecific RFamide-positive nerve cells (Fig. 5G). These antibody staining experiments indicate that the Vg4/8s lack the axis polarity and oral-specific cell differentiation in planula stage.

Table 1
Axis formation of isolated blastomeres

<table>
<thead>
<tr>
<th>Pattern of isolation</th>
<th>Stage</th>
<th>Direction</th>
<th>Oral–aboral axis</th>
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<td></td>
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<tr>
<td>Lat1/2</td>
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<tr>
<td>Lat2/4</td>
<td>4-cell</td>
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<td>9</td>
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<td>Latitudinal</td>
<td>20</td>
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<tr>
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<td>8-cell</td>
<td>Longitudinal</td>
<td>7</td>
</tr>
<tr>
<td>Vg4/8</td>
<td>8-cell</td>
<td>Longitudinal</td>
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Fig. 4. Animal pole blastomeres are required for establishing the oral–aboral axis. (A) Diagram of the isolation experiment. 8-cell stage embryos were dissected into animal (Am4/8) and vegetal (Vg4/8) halves, each composed of four blastomeres. (B) Am4/8 (10 hpf). It formed normal blastula. (C) Vg4/8 (10 hpf). The isolates neither elongated nor gastrulated. (D) Am4/8 (30 hpf). It became a normal planula. (E) Vg4/8 (30 hpf). There was no endoderm inside the blastocoel. Ectoderm has thickened in several regions (arrowhead). There was no sign of an oral–aboral axis. Oral is to the top in panels B and D. Scale bar: 20 μm.
The presumptive endoderm territory is fixed prior to gastrulation

To define the timing of the establishment of polarity, late blastulae (9 hpf), which already show a polarized morphology, were bisected into oral and aboral halves (Fig. 6B). Not only did both the oral- and aboral-1/2s continue to elongate, but they also swam directionally in line with their main axis (Figs. 6A–E, arrow). DiI labeling of the cut edge immediately after the dissection confirmed that the original polarity had been retained in both cases (Fig. 6B).

The blastula bisection experiments also showed that endoderm-forming potential is restricted to a small group of cells at the oral end of the blastula. All of the oral-1/2 isolates gastrulated normally and formed planulae comprised of both ectoderm and endoderm (Fig. 6C). The endoderm in oral isolates developed AP activity as in normal embryo (Fig. 6F). In contrast, the aboral-1/2 developed no detectable endoderm by 24 hpf (Figs. 6D, E) and endoderm-specific AP activity could not be detected at 38 hpf (Fig. 6G). Two types of morphology were observed, a coeloblastula-like form, which elongated but retained the blastocoel (Fig. 6D), and a collapsed-coeloblastula form, in which elongated ectoderm was folded and the internal cavity had collapsed (Fig. 6E). Different portions of blastulae were removed to define the distribution of endoderm-forming activity (Fig. 7A). Isolates containing the oral end always gastrulated to give endoderm and the ectoderm, even when as small as 1/5 normal size. In the resultant tiny planuloids (Fig. 5).
6H), the relative amount of endoderm:ectoderm was higher (en: ec (v/v) ≈ 1) than in normal embryos (Fig. 6A, en: ec (v/v) ≈ 0.25). Even smaller oral “tip” (1/8 – 1/10 cut from the oral end) gave rise almost exclusively to endoderm (Fig. 6I, en: ec (v/v) ≥ 20). In contrast, most of the isolates from aboral parts, including up to 4/5 of the embryo, failed to form endoderm formation. Thus, less than 20% of the oral end of the blastula is required for endoderm formation.

Although no endoderm was detected in aboral-1/2 isolates at 24 hpf, a small amount of endoderm did develop in some of these isolates by around 30–36 hpf, a process delayed by 14–22 h compared with endoderm formation in controls (Figs. 6J, 7B). Dil labeling of the cut edge showed that the “regenerated” endoderm formed from the original oral-most end (Fig. 6K). Trace amounts of endoderm were first detectable at 30 hpf, in 12% of aboral-1/2 isolates. At 36 hpf, a maximum proportion of 50% of isolates had formed endoderm. The amount of endoderm was significantly lower than in control embryos, but nevertheless, these isolates did finally express endoderm-specific alkaline phosphatase (AP) activity (data not shown). Interestingly, all aboral-1/2 isolates that regenerated endoderm showed a normal pattern of RFamide-positive nerve cells (data not shown, n = 27), whereas isolates with no endoderm showed no or few oral-specific RFamide-positive cells (Fig. 6L). This implies that the full expression of the oral identity in the ectoderm is dependent on the presence of endodermal cells. Numbers of TT-positive neurons extending perpendicular to the oral–aboral axis were observed in the entire body of the aboral-1/2 isolates and a few longitudinal neurons were also found (Fig. 6M), which is similar to the pattern of TT-positive neurons in the aboral region of normal embryos.

Discussion

Axis determination in cnidarians

Our bisection experiments clearly indicate that materials contained in animal blastomeres at 4- and 8-cell stage are necessary to establish the oral–aboral axis in Podocoryne (Fig. 8A), which can be termed an axis determinant. Embryos derived from vegetal halves lack all axial characteristics including polarized morphology and pattern of cell differentiation as well as endoderm formation. We conclude that the axis determinant must be localized to the animal hemisphere by the time of the

Fig. 6. Endoderm-forming fate is localized in the oral end of blastulae, while axial polarity is retained in the entire embryo. (A) Normal embryo (30 hpf). (B) Operations for generating oral and aboral halves at 10–11 hpf. (B, top) An oral-1/2 isolate (30 hpf). It has become a normal planula. (B, bottom) An aboral-1/2 isolate (30 hpf). The aboral cut surface on the oral half and the oral cut surface on the aboral half have been labeled with Dil. (C) Oral-1/2 isolate (30 hpf). (D) Aboral-1/2 isolate (30 hpf). (E) An alternative morphology of aboral-1/2. It is similar to panel D except that the blastocoel has collapsed. No endoderm can be seen in either case. Both have elongated longitudinally and swam. (F) Endoderm-specific alkaline phosphatase (AP) staining in oral-1/2 isolates (38 hpf). (G) AP staining in aboral-1/2 isolates (38 hpf). No signal can be seen. (H) An isolate from oral 1/5 region of blastulae (30 hpf). Endoderm:ectoderm ratio was approximately 1:0. (I) An isolate from oral tip (30 hpf). Very little ectoderm is produced and endodermal clumps are present and not covered by ectoderm. The endoderm:ectoderm ratio was larger than 20. Arrowheads indicate ectoderm clumps. (J) An aboral-1/2 isolate (38 hpf). Endoderm was regenerated from the oral end. (K) Dil label in specimen F. The original oral end (i.e., cutting edge) is labeled. (L) RFamide immunostain in an aboral isolate that lacked endoderm formation (52 hpf). A few RFamide-positive cells were found at the original oral end. (M) TT-immunostain in an aboral isolate that lacked endoderm (38 hpf). TT-positive neurites that ran perpendicular to the elongation were found throughout the body. A few longitudinal neurites were also present (arrowhead). ec: ectoderm, en: endoderm, bc: blastocoel, arrows: direction of swimming. Scale bar: 20 μm.
second cleavage, if not earlier. We also conclude that vegetal components do not play a major role in axis determination. These results are the first demonstration in cnidarians that axial properties are determined by localized factors.

Based on the results presented here (summarized in Fig. 8A), we propose a possible regulatory and determination mechanism for embryonic axis formation in *Podocoryne*. An axis determinant localized to the animal pole of fertilized eggs becomes segregated to the animal blastomeres as cleavage proceeds (1). It specifies the endoderm fate in blastulae, perhaps in an autonomous manner (2), as well as the oral–aboral polarity in non-autonomous manner, so that the animal pole becomes the oral-end (3). A weak capacity to regenerate endoderm is present (4). Oral regional identity and perhaps the endoderm territory are established based on the polarity (5) and most probably induction from endoderm is also required for this process (6). Our finding of localized determinants responsible for directing axial properties and endoderm fate was surprising because previous studies on hydrozoan early embryogenesis have demonstrated the regulation of the oral–aboral axis when a part of early embryo is isolated (Zoja, 1895; Hargitt, 1904; Mass, 1905; Hauenschild, 1954; Rappaport, 1969; Campbell, 1974; Freeman, 1980, 1981b, 1983, 1990). All other hydrozoan species examined so far demonstrate regulation of oral–aboral axial polarity when bisected before the 16-cell stage. In only one other study has autonomous fate determination been found: in bisected *Nanomia cara* (siphonophora, class hydrozoa) embryos, some oral and aboral structures develop autonomously; however, axial polarity is retained (Freeman, 1983; Primus and Freeman, 2004). Since the correspondence between the animal pole of the egg and the oral pole of the larvae appears to hold for all hydrozoans (Freeman, 1981a,b, 1990), it is reasonable to assume that they share a common mechanism for the initial steps of axis determination. A likely explanation for the variation is that the same axis determinant factor is more broadly distributed in hydrozoans that show axis regulation, while it is more tightly localized in *Podocoryne* eggs (Fig. 8B).

Egg polarity factors are known to play key initial roles in determining embryonic polarity in many model organisms including *Xenopus*, *C. elegans*, *Drosophila*, sea urchins and ascidians. Our finding predicts that molecules related to the egg polarity factors in the bilaterians may also play roles in early axis patterning in hydrozoans. The molecular basis of the cnidarian axis determination is not known. It has been suggested that β-catenin protein plays a conserved role for endoderm formation during the embryogenesis of *Nematostella* (Wikramanayake et al., 2003), although its function in axis polarity is not clear yet (Primus and Freeman, 2004). In this anthozoan, β-catenin protein becomes enriched on the presumptive oral side at the 16-to 32-cell stage, which is significantly later than the establishment of egg polarity. Our

Fig. 7. Endoderm fate is localized to the oral 20% of blastulae. (A) Endoderm formation in different types of isolates (at 29 hpf). (B) Endoderm formation in oral and aboral isolates from 24 to 52 hpf. The types of isolates are indicated on the left. (+): Endoderm filled blastocoel completely. (±): Endoderm was partly formed. (−): No endoderm was formed.

Fig. 8. (A) Schematic representation of bisection experiments and a model for axis determination in *Podocoryne*. (B) A model explaining the different results of bisection experiments in *Podocoryne* and *Phialidium*. 
results indicate that the axial determinant is expressed and localized in the animal pole of embryo by the second cleavage. This suggests that β-catenin is not the egg polarity determinant itself, rather it mediates its activity. Some maternally expressed mRNAs have been found to be localized to the animal pole of fertilized eggs in \textit{Podocoryne}. These include the products of the posterior type \textit{Hox} gene \textit{Cnox4-Pc} and the \textit{Brachyury} orthologue \textit{PcBra} (Yanze et al., 2001; Spring et al., 2002). Considering their conserved role in bilaterian axis patterning and gastrulation, these genes are good candidates to participate in the different aspects of axis formation in \textit{Podocoryne}.

\textbf{Endoderm formation}

As well as containing determinants that direct axial polarity, the animal pole region of the egg contains factors necessary for gastrulation and endoderm formation. At the blastula stage, a region covering 1/5 of the oral end is necessary and sufficient to form endodermal cells. This region coincides with the presumptive endoderm territory shown by DR-labeling. The number of endoderm cells that develop in each region is conserved in blastula fragments, indicating that endoderm fate is determined in a cell-autonomous manner at the late blastula stage. Nevertheless, a small amount of endoderm was able to form in 50% of the aboral fragments examined, suggesting that a non-autonomous regulation mechanism can compensate the missing endoderm. This regulatory activity is negligible compared to the endoderm determination process in normal development: it was significantly slower and only formed a tiny amount of endoderm. It is possible that a maternal endoderm determinant is localized to the animal pole of the early embryo that is subsequently inherited by their daughter cells in the oral region of the blastulae and autonomously determines the endoderm fate. An alternative possibility is that the maternal determinant (which could be the same as the polarity determinant) triggers a series of events throughout the embryo (ex. (3) and (4) in Fig. 8A), leading ultimately to the restriction of endoderm fate in a corresponding territory at the late blastula stage. The efficiency of the endoderm regeneration diminishes within several hours after initiation of gastrulation (data not shown). Similar results have been reported in \textit{Phialidium} (Thomas et al., 1987), in which no endoderm was formed from the aboral half of mid-gastrula.

Endoderm formation is regarded as an oral characteristic in some cnidian species. Our results indicate that the endoderm formation and axis polarity establishment, although highly related, are separable processes during embryogenesis. The axis of polarity is present in aboral isolates that lack endoderm. The distinct mechanisms involved help explain divergent pattern of endoderm formation among hydrozoan species (Campbell, 1974; Martin, 1997), which do not all coincide with the axis. For example, an early cleavage stage embryo of \textit{Nanomia} divides in a centro-peripheral direction to form inner and outer blastomeres that give rise to endoderm and ectoderm, respectively, while its oral and aboral identities are determined by animal–vegetal polarity (Freeman, 1983; Primus and Freeman, 2004). In \textit{Podocoryne}, the endoderm is necessary for determining full oral regional identity in planulae, indicating that it is certainly a component of the axis determination mechanism in this species. It is not clear whether a single egg polarity factor determines these two properties in \textit{Podocoryne} or two coincident egg polarity factors determine them independently.

\textbf{Regional identity in planulae}

The positions of cells along oral–aboral axis in blastulae and in planulae are not identical because the oral region of blastulae will migrate into blastocoeel and then move aborally to form endoderm. Therefore, the positional information of presumptive endodermal cells in blastulae is likely to be reorganized or lost during the gastrulation. The presence of endoderm is necessary for the acquisition of the oral regional identity in planulae, which is marked by the oral-specific RFamide-positive cells (Müller et al., 2003). These are present sporadically in aboral isolates when endoderm is not regenerated.

The germinale layer origin of the RFamide-positive nerve cells is not clear. A previous report argued that nematocytes and ganglion cells are formed from endoderm layer via interstitial cells (Thomas et al., 1987). RFamide-positive nerve cells may also derive from the endoderm. Another possible explanation is that the oral-specific RFamide-positive cells are formed from oral ectoderm following an induction from the endoderm. A similar observation has been reported in the context of hydra regeneration: \textit{predl-a} expression suggests that the apical endoderm bears head organizer activity during regeneration and budding of hydra polyps (Gauchat et al., 1998; Galliot and Miller, 2000). A conserved mechanism might be involved in oral/head formation in cnidarian embryo and planula formation.

In either case, the endoderm seems to play an important role for acquisition of oral regional identity of planulae.

The organization of the TT-positive nerve net suggests that aboral regional identity has expanded in aboral isolates from blastulae. Aboral regional identity might be a default state and it is negatively regulated by the oral regional identity.

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\textbf{Appendix A. Supplementary data}

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.01.012.

\textbf{References}


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