Neurula rotation determines left-right asymmetry in ascidian tadpole larvae

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SUMMARY
Tadpole larvae of the ascidian Halocynthia roretzi show morphological left-right asymmetry. The tail invariably bends towards the left side within the vitelline membrane. The structure of the larval brain is remarkably asymmetric. nodal, a conserved gene that shows left-sided expression, is also expressed on the left side in H. roretzi but in the epidermis unlike in vertebrates. We show that nodal signaling at the late neurula stage is required for stereotypic morphological left-right asymmetry at later stages. We uncover a novel mechanism to break embryonic symmetry, in which rotation of whole embryos provides the initial cue for left-sided expression of nodal. Two hours prior to the onset of nodal expression, the neurula embryo rotates along the anterior-posterior axis in a counterclockwise direction when seen in posterior view, and then this rotation stops when the left side of the embryo is oriented downwards. It is likely that epidermis monocilia, which appear at the neurula rotation stage, generate the driving force for the rotation. When the embryo lies on the left side, protrusion of the neural fold physically prevents it from rotating further. Experiments in which neurula rotation is perturbed by various means, including centrifugation and sandwiching between glass, indicate that contact of the left epidermis with the vitelline membrane as a consequence of neurula rotation promotes nodal expression in the left epidermis. We suggest that chemical, and not mechanical, signals from the vitelline membrane promote nodal expression. Neurula rotation is also conserved in other ascidian species.

KEY WORDS: Left-right asymmetry, nodal, Brain asymmetry, Neurula rotation, Ascidians

INTRODUCTION
The adult body of most animals shows stereotypic left-right (L-R) asymmetry. In the early stages of animal embryogenesis, the morphology is bilaterally symmetrical, but later the symmetry becomes broken along the L-R axis (Hirokawa et al., 2006; Raya and Belmonte, 2006). Left-sided expression of the nodal gene precedes the onset of morphological L-R asymmetry in chick and mouse embryos (Levin et al., 1995; Collignon et al., 1996; Lowe et al., 1996). Involvement of the nodal protein, a member of the transforming growth factor beta (TGFβ) superfamily, in polarization of the L-R axis is conserved among many vertebrates, including Xenopus and zebrafish (Burdine and Schier, 2000; Whitman and Mercola, 2001).

The earliest event in the L-R determination process in mouse embryos is nodal flow, which is a leftward flow of fluid in the node. Cells of the node have monocilia, which are motile and generate a leftward flow of liquid that acts as a braking mechanism for acquisition of L-R symmetry in the mouse (Nonaka et al., 1998). The cilia tilt posteriorly and rotate, thereby generating a directed flow (Nonaka et al., 2005; Okada et al., 2005). The planar cell polarity (PCP) pathway and hydrodynamic forces are involved in this tilting of the nodal cilia (Guirao et al., 2010; Borovina et al., 2010). Through this system, the L-R symmetry of the embryo is broken using the polarity of the anterior-posterior (A-P) axis and a stereotypic direction of rotation of the motile monocilia. This leftward flow in a cavity within the embryo is also known to occur in other vertebrates (Okada et al., 2005). However, in Xenopus and zebrafish, some reports have indicated that specification of L-R asymmetry depends on a very early asymmetric signal, such as differential ion flux created by H+/K+-ATPase activity (Levin et al., 2002; Qiu et al., 2005; Kawakami et al., 2005).

The morphology of ascidians, which are the closest relatives to vertebrates, shows L-R asymmetry in both the larval and adult stages (Hirano and Nishida, 2000; Boorman and Shimeld, 2002). In the tadpole larvae of Halocynthia roretzi, morphological L-R asymmetry is represented in two ways. First, the larval tail always bends towards the left side within the limited perivitelline space. Second, the brain structure is remarkably asymmetric, the brain vesicle and sensory pigment cells being located on the right side of the midline (Morokuma et al., 2002; Tamiguchi and Nishida, 2004). It has been shown that ascidian genes nodal and Pitx, which encodes a transcription factor downstream of nodal, are expressed on the left side of the embryo at the neurula and initial tailbud stages, although the expression is restricted to the epidermis and is not evident in the mesoderm, unlike in vertebrates (Morokuma et al., 2002; Shimeld and Levin, 2006; Yoshida and Saiga, 2008). There is no cavity within ascidian embryos in which liquid flow is generated by ciliary movements.

In the present study we first investigated the involvement of nodal signaling in the formation of morphological L-R asymmetry in Halocynthia roretzi, and then attempted to clarify the initial process through which bilateral symmetry is broken. Prior to nodal expression, neurulae rotate with stereotypic orientation along the A-P axis within the vitelline membrane and the rotation always stops when the left side of the neurula is oriented downwards. We term this phenomenon ‘neurula rotation’. We demonstrate that neurula rotation, which is likely to be driven by epidermal monocilia, specifies the future L-R axis and suggest that contact between the vitelline membrane and left-side epidermal cells triggers nodal expression in the left-side epidermis.

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Accepted 15 February 2012
MATERIALS AND METHODS

Animals, embryos and nodal inhibitor

Adults of the ascidian *Halocynthia roretzi* were collected and kept in tanks. Eggs were spawned under temperature and light control, fertilized with a suspension of non-self sperm, and allowed to develop in Millipore-filtered seawater containing 50 μg/ml streptomycin and 50 μg/ml kanamycin at 13°C. To inhibit the nodal signaling pathway, embryos were treated with 5 μM SB431542 (Sigma), which blocks the TGFβ type I receptors ALK4, ALK5 and ALK7 for activin and nodal ligands, without inhibiting other ALK family members that bind to BMP ligands (Inman et al., 2002). The drug has been used in previous ascidian studies (Hudson and Yasuo, 2005; Hudson and Yasuo, 2006; Hudson et al., 2007; Yoshida and Saiga, 2011). Vitelline membranes were removed with fine tungsten needles. Eggs demuded just after fertilization were reared with the supernatant of a homogenate of the cleaving eggs to facilitate normal neural tube closure (Nishida and Satoh, 1985).

Fixation of embryo orientation using centrifugal force or sandwiching

To maintain embryos in fixed orientations within the vitelline membrane we used gentle centrifugation. At the neurula rotation stage (15 hours), embryos were placed in a tube and immediately centrifuged in an angled rotor at 2000 rpm (300 g) for 1.5 hours to prevent any further change in embryo orientation. As a second method for fixing their orientation, neurula embryos with the vitelline membrane were gently sandwiched between a glass slide and a coverslip so that embryos physically contacted with the vitelline membrane at both sides. The extent of pressure was controlled with a spacer of Vaseline between slide and coverslip. To sandwich naked embryos and to prevent adhesion of the embryos, slide and coverslip were coated with gelatin by placing them in a solution of 0.1% gelatin and 0.1% formaldehyde in distilled water, then allowed to dry and washed thoroughly with water as described previously (Zalokar and Sardet, 1984).

Scanning electron microscopy

Embryos were fixed with 2.5% glutaraldehyde and 1% paraformaldehyde in buffer containing 0.5 M NaCl and 0.1 M MOPS (pH 7.5) at 4°C for 16 hours and then devitellinized. After three washes with PBS, the embryos were dehydrated through 30%, 50% and 70% ethanol, and then stored in 70% ethanol at –30°C until use. After complete dehydration of the embryos with ethanol, the solution was substituted by isoamyl acetate. Critical-point drying was performed using CO2 with a Hitachi HCP-2 apparatus. Samples were coated with a thin layer of gold and observed with a scanning electron microscope (JEOL JSM-5800 and Hitachi SU6600).

Whole-mount in situ hybridization

Detection of miRNA for *Hr-nodal* (Morokuma et al., 2002) and *Hr-ETR-1* (Yagi and Makabe, 2001) was performed by whole-mount in situ hybridization with digoxigenin-labeled antisense RNA probes in accordance with Wada et al. (Wada et al., 1995), except that washing with 0.2× SSC containing 0.1% Tween 20 was omitted.

RESULTS

L-R asymmetry in *Halocynthia* tadpole larvae

Morphological L-R asymmetry is represented in two ways in tadpole larvae (Morokuma et al., 2002). As the larval tail elongates, in most cases the tail bends towards the left side within the limited perivitelline space (90-100% depending on egg batches and spawning season; Fig. 1A,B). When the dorsal midline was visualized by detecting the expression of the nervous system-specific *ETR-1* gene by in situ hybridization, this process was easily recognizable (Fig. 1C-E). Second, the larval brain is highly asymmetric, the brain vesicle and sensory pigment cells of the otolith and ocellus being positioned on the right side in most cases (Fig. 1A). This asymmetry was readily visible in sectioned embryos (Fig. 1F). Prior to emergence of this morphological asymmetry, expression of *nodal* and the downstream gene *Pitx* occurs in the left-side lateral epidermis from the late neurula to early tailbud stage (Morokuma et al., 2002). This expression of *nodal* was initiated at ~17 hours of development, became maximal at 18 hours, and was abolished by 23 hours at 13°C (Fig. 1G-I).

Inhibition of nodal signaling disrupts L-R asymmetry in tadpole larvae

To examine the function of nodal signaling in generation of the L-R asymmetric morphology, embryos were treated with a nodal receptor inhibitor. The treatment was initiated every 2 hours from 10 hours (gastrula) to 22 hours (middle tailbud) after fertilization, and continued until observation (Fig. 2). Tail bending orientation within the vitelline membrane was observed and scored just before hatching (35 hours), and the position of the brain vesicle was observed after hatching using the trunk and tail fins on both the dorsal and ventral sides and three palps (two in dorsal and one in ventral positions at the anterior tip of the trunk) as references for the midline. Larvae treated with the solvent DMSO as a control showed bending of the tail towards the left side in more than 95% of cases (Fig. 2B). When treatment with SB431542 was initiated at the 64-cell stage, just before gastrulation, tail morphogenesis was

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Fig. 1. L-R asymmetry in *Halocynthia roretzi* tadpole larvae. (A) A larva within the vitelline membrane just before hatching. Dorsal view. In most larvae, the tail bends towards the left side within the limited perivitelline space. The brain vesicle (red dotted circle) and sensory pigment cells of the ocellus and ocellus are located on the right side of the midline (yellow line). (B) In rare reversed larvae, the tail bends towards the right side. The brain vesicle and sensory pigment cells are positioned on the left side of the midline. (C-E) *ETR-1* expression during tail elongation. *ETR-1* is expressed in the central nervous system on the dorsal midline, showing that the tail is bending to the left. (F) Transverse section of the trunk showing asymmetric positioning of the brain vesicle. Yellow line indicates the midline connecting the dorsal and ventral fins. The green dotted circle indicates the margin of the brain. An otolith pigment cell is present within the brain vesicle. (G-I) Temporal profile of *nodal* gene expression. Dorsal views. Expression of *nodal* is initiated at ~17 hours after fertilization at 13°C, reaches a maximum at 18 hours, and is then gradually reduced. Scale bars: 100 μm.
abnormal (data not shown). This is consistent with the observation that nodal signaling is involved in cell fate specification of the secondary muscle and secondary notochord precursor cells, and the cells in the dorsal neural tube during gastrulation (Hudson and Yasuo, 2005; Hudson and Yasuo, 2006; Hudson et al., 2007). The abnormal tail morphogenesis became less marked with later initiation of the drug treatment. Approximately half the larvae treated prior to nodal gene expression had tails with a reversed bend (Fig. 2A, red). Thus, the direction of tail bending became randomized (Fig. 2C,D). The ratio of larvae showing reversed versus left tail bending was reduced with later initiation of drug treatment. These results suggest that nodal signaling is essential for stereotypic L-R asymmetry of tail bending.

More than 90% of control larvae showed the brain vesicle on the normal, right side of the trunk (Fig. 2F). Brain asymmetry was also affected by SB431542 treatment initiated before nodal expression, similar to the observed temporal sequence for tail bending. However, one difference was that most larvae showed positioning of the brain vesicle on the midline just beneath the dorsal fin, instead of randomized asymmetric positioning (Fig. 2E, green). Therefore, the larval brain had lost L-R asymmetry in the absence of nodal signaling (Fig. 2G-I).

These results suggest that expression of the nodal gene in the left-side epidermis from the late neurula to early tailbud stage is pivotal for generation of stereotypic L-R asymmetry in terms of both tail bending and brain morphology. Initiation of treatment with the nodal receptor inhibitor before the stage of nodal expression had no effect on the intensity and area of nodal gene expression at the tailbud stage (n=43/45 cases), suggesting that the expression is not autoregulated, in contrast to the situation in vertebrate embryos (Burdine and Schier, 2000; Meno et al., 1999; Schier and Shen, 2000).

Neurula rotation

Halocynthia embryos have a large perivitelline space, the egg diameter being 280 μm and that of the vitelline membrane 560 μm. Time-lapse video revealed that the late neurulae rotated within the vitelline membrane along the A-P axis, and that after the rotation every neurula lay with the left side oriented downward (Fig. 3A,B and supplementary material Movie 1). This neurula rotation took place at ~15 hours of development, i.e. 2 hours before nodal expression, and lasted 2-7 minutes. Most of the neurula rotated counterclockwise when viewed from the posterior. The speed of rotation was ~30-45° per minute, and the movement was hardly discernible without time-lapse. We scored the rotational movements of 192 embryos captured by time-lapse (Fig. 3C and supplementary material Movie 2). At the start of recording, more than half (58%) of the neurulae were ventral side down and dorsal side up (Fig. 3C, top left, blue). They rotated 90° counterclockwise and stopped when the left side had moved beneath the embryo. In 12% of cases, the embryos lay on the right side and rotated 180° counterclockwise (green), whereas 8% of embryos lay on the left side from the beginning and did not rotate (yellow). In 22% of cases, the embryos lay on the dorsal side at the beginning. Among them, 10% rotated 90° clockwise and then settled on the left side (red). The other 12% rotated 90° counterclockwise, lay on the right side, most paused, then reinitiated 180° of counterclockwise rotation and eventually settled on the left side (pink). In these cases, it is likely that the initial 90° rotation had been passive, and that the direction of rotation was random. This is because the neural fold protrudes at this stage (Fig. 3C and see also Fig. 4A), making dorsal-side-down embryos unstable and causing them to fall on their left or right side randomly. Whenever they fell on their right side, the active counterclockwise rotation appeared to start after a short pause.

After the completion of neurula rotation, we rotated the embryos 180° by trundling the vitelline membrane so that they lay on the right. Unexpectedly, they always started to rotate back 180° in a counterclockwise direction and settled on their left side again (supplementary material Movie 3). This ability lasted for ~3 hours...
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after the neurula rotation stage at 15 hours (until 18 hours, as the time for the next epidermal cell divisions approached), suggesting that the driving force responsible for the rotation remained active for a long period.

We wondered whether neurula rotation can occur under natural conditions in the ocean where seawater waves would rock the developing embryos. To test this, numerous embryos were placed in a container (25 cm × 35 cm) on a laboratory shaker (1.7 Hz, 100 shakes per minute) before the start of neurula rotation, and agitation was maintained until rotation had been completed. After agitation, the orientation of the embryos was immediately observed (within 1 minute). All of the embryos were on their left side (anterior end of the neural plate) of the neurula embryos. Embryos without an asterisk are oriented dorsal side down. (A) Embryos just before the neurula rotation stage at ~15 hours of development (a snapshot of supplementary material Movie 1). Asterisks indicate the dorso-anterior position (anterior end of the neural plate) of the neurula embryos. Embryos rotated 180° in a counterclockwise direction when seen in posterior view. Blue, embryos with the dorsal side up at the start of rotation; they rotated 90° in a counterclockwise direction. Red, embryos with the dorsal side down; they rotated 270° in a counterclockwise direction. Yellow, embryos with the left side down; they did not rotate. Rotation movements of these various embryos are shown in supplementary material Movie 2. Scale bars: 100 µm.

Presence of monocilia on surface epidermis cells
We observed embryos using scanning electron microscopy. A single cilium ~5 µm in length was present on each of the epidermal cells (Fig. 4A–C). The cilia were present over the entire embryo surface, except in the regions of the closing neural fold and the anterior midline (Fig. 4A, green area). Most of the cilia were positioned at the center of the cell surface with a slight bias towards the posterior direction (less than 10% of the cell diameter) (supplementary material Fig. S1). By contrast, there was a significant bias in the orientation of the cilia. Measuring the angle between the A-P axis and the orientation of the cilia at the base revealed that most cilia tilted in the clockwise direction when seen in posterior view (Fig. 4D,E and supplementary material Fig. S2).

Cilia were not observed before the start of neurula rotation (Fig. 4F). At the stage when rotation took place, short cilia ~3 µm in length appeared (Fig. 4G, yellow bars), and these lengthened to a maximum of 5 µm in 3 hours (Fig. 4H). At 3.5 hours after the rotation (18.5 hours of development), cilia were no longer observed, as the time for the next epidermal cell divisions at 19 hours approached (Fig. 4I). These observations imply that the cilia are responsible for driving neurula rotation, with a good correlation between when cilia are present and the capacity for rotational movement (3 hours after the neurula rotation stage, see above).

Test cells move around within the perivitelline space (supplementary material Movie 1). We hand-centrifuged embryos to collect the test cells away from the embryos (supplementary material Fig. S3A). Even in this situation, embryos rotated and then ceased rotation when the left side became oriented downwards, suggesting that test cells are dispensable for rotational movement and its cessation.

Fixation of neurula orientation by centrifugal force
To determine the significance of neurula rotation in establishment of larval L-R asymmetry, centrifugation and sandwich experiments were carried out. First, we used gentle centrifugation to retain embryos in a fixed orientation. At neurula rotation stage, left-side-down embryos were placed in a centrifuge tube, transferred to an angled rotor, and gently centrifuged at 2000 rpm for 1.5 hours. In another experiment, we rotated the embryos 180° by trundling the vitelline membrane so that they lay on the right, and then immediately centrifuged them (Fig. 5A). After centrifugation, only embryos that had retained the same orientations to those before centrifugation were selected and cultured. Left-side-down (or, more accurately, left-side-centrifugal) larvae preferentially showed normal tail bending toward the left side in 89% of cases, whereas right-side-down larvae showed tail bending in the reverse direction in 84% of cases (Fig. 5A). Similarly, left-side-down larvae had their brain vesicle on the normal right side in 67% of cases. By contrast, the brain vesicle was reverse positioned in 65% of the right-side-down larvae (Fig. 5B). Consistent with the morphological asymmetries, left-side-down embryos showed nodal expression on the left side, whereas right-side-down embryos expressed nodal on the right side in most cases (Fig. 5C,C’).
These results suggested that L-R polarity is determined by the orientation of embryos at the neurula stage. There are two possible explanations for the specification of this polarity. One is that it is determined by the relationship between the direction of gravity (or centrifugal force) and embryo orientation. In this case, the down side of embryos would be specified as the left. Alternatively, contact of the epidermis with the vitelline membrane is important, in which case the contact side would be specified as the left.

**Perturbation of L-R asymmetry by sandwiching of embryos within the vitelline membrane**

To distinguish between the above two possibilities, we sandwiched the neurula within the vitelline membrane so that its orientation was fixed and the embryo contacted with the vitelline membrane on both sides. Embryos within the vitelline membrane were gently sandwiched between a glass slide and a coverslip for 1.5 hours. Regardless of the orientation of the embryos (left-side down or right-side down), the orientation of tail bending became totally randomized (Fig. 5D). The position of the brain vesicle varied. Approximately half the larvae had the brain vesicles on the midline, whereas the other half showed normal or reversed positions (Fig. 5E). Most interestingly, expression of *nodal* was observed on both sides in every case, although the intensities of *nodal* expression on the left and right sides were not always equal (Fig. 5F). Therefore, it appears that the direction of gravity is not important for establishment of L-R polarity, as *nodal* was expressed on the upward side and the downward side in the sandwiched embryos. Instead, it seems highly likely that the side of the embryo in contact with the vitelline membrane is specified as the side that expresses *nodal*.

Embryos were also sandwiched in a dorsal-side-up or ventral-side-up orientation. In such cases, the embryos failed to complete neural tube closure and developed into larvae with abnormal tail and brain vesicle morphologies. *nodal* expression was absent in most embryos (*n=21/33* cases; Fig. 5G). This suggests that dorsal and ventral midline epidermis cells are not competent to express the *nodal* gene, consistent with the observation that in normal embryos *nodal* is expressed in the lateral epidermis but not in the broad midline area (see Fig. 1H). In some cases, embryos expressed *nodal* on both the left and right sides, but not on the dorsal and ventral sides (*n=5/33* cases; Fig. 5G’). In these cases, the embryos might have been squeezed to a greater extent such that parts of the lateral epidermis on both sides also came into contact with the vitelline membrane.

We then investigated the period when embryos are most sensitive to contact with the vitelline membrane by sandwiching them left side down for 30 minutes at various stages and observing the direction of tail bending at the prehatching stage (Fig. 5H). Randomization of tail bending was most evident when embryos were sandwiched for 30 minutes starting 0-10 minutes after the neurula rotation stage and released at 30-40 minutes (shown as 0 in Fig. 5H). This suggests that embryos are most sensitive at and after the neurula rotation stage, although contact for 30 minutes might not be enough to exert a full response because the proportion of reversed embryos was less than half of the total embryos examined even in sandwiching at this stage.

**Contact with the vitelline membrane after rotation is important for nodal expression**

The possibility that contact of epidermis cells with the vitelline membrane is crucial for *nodal* gene expression was investigated further. When the vitelline membrane was removed manually with fine tungsten needles just after fertilization and the embryos were cultured in agar-coated dishes, *nodal* expression was abrogated (*n=43/43* cases). These embryos developed the epidermal cilia at the neurula stage, but the tails grew straight and the brain vesicles were located on the midline (supplementary material Fig. S4). When the vitelline membrane was removed just before neurula...
rotation, \textit{nodal} expression was not observed \((n=0/38\) embryos). Removal just after rotation yielded the same results \((n=0/27)\), whereas removal 2 hours after rotation resulted in \textit{nodal} expression in 41 of 42 embryos \((98\%)\). Therefore, the rotational movement itself is not sufficient for \textit{nodal} expression, and contact with the vitelline membrane for a period is essential.

Our next question addressed the nature of the trigger for \textit{nodal} expression: mechanical stimulation at the point of contact or chemical stimulation from the vitelline membrane? Naked embryos placed on agar did not express \textit{nodal} (Fig. 6A). To mimic physical contact, naked embryos were gently sandwiched between glass sheets coated with gelatin to prevent embryo adhesion (Fig. 6B). However, \textit{nodal} was not expressed. Numerous follicle cells are attached to the outside of the vitelline membrane (supplementary material Fig. S3B). To examine whether these cells secrete chemical signals, we removed the cells by brief treatment with protease (supplementary material Fig. S3C). The vitelline membrane, from which the follicle cells had been removed just after fertilization and just before the neurula rotation stage, promoted \textit{nodal} expression on both sides of sandwiched embryos in all cases (Fig. 6C,D). Many test cells reside in perivitelline space. We removed most of the test cells through a hole made in the vitelline membrane at the early neurula stage (supplementary material Fig. S3D). These embryos still expressed \textit{nodal} in all cases (Fig. 6E). Although the evidence is far from conclusive, it seems likely that chemical signals present in the vitelline membrane, but not from follicle or test cells, promote \textit{nodal} expression in the epidermis.

\textbf{Neurula rotation in other ascidian species} We investigated whether other ascidians also show neurula rotation. All four ascidian species examined, i.e. \textit{Ciona intestinalis} (supplementary material Movie 4), \textit{Phallusia mammillata} (supplementary material Movie 5), \textit{Corella inflata} (http://celldynamics.org/celldynamics/gallery/movieWindows/timelapse/corella02.html), filmed by Drs K. Sherrard and G. von Dassow, Center for Cell Dynamics, University of Washington) and \textit{Styela clava} (Fink, 1991), rotate specifically at the late neurula...
Neural tube rotation and L-R asymmetry in ascidians

DISCUSSION

In this study, we showed that nodal signaling is indispensable for the stereotypic morphological L-R asymmetry of tadpole larvae, and that the left-sided expression of nodal is promoted by contact of the left epidermis with the vitelline membrane as a result of neurula rotation. Neurula rotation is conserved among all five ascidian species investigated so far.

nodal signaling and the asymmetric morphology of larvae

The larval tail bends towards the left side in the limited space within the vitelline membrane. The orientation of tail bending was randomized by treatment with a nodal receptor inhibitor prior to nodal expression. It is possible that nodal signaling sets stereotypic polarity along the L-R axis by initially biasing the bending of the tail slightly towards the left side at the initial tailbud stage, so that the tail elongates in the same direction only along the inner surface of the vitelline membrane. It is not possible to conclude for certain whether the polarity of the L-R axis is actually randomized in inhibitor-treated embryos as well as sandwiched embryos, or whether the embryos develop without L-R asymmetry and the tail is forced to bend randomly because of spatial constraints within the perivitelline space.

The brain vesicle and pigment sensory cells are located on the midline when there is no nodal signaling. Labeling of a single bilateral blastomere at the two-cell stage has demonstrated that morphological asymmetry of the brain arises through clockwise rotation of the neural tube by ~45°, when seen in posterior view, at the late tailbud stage (Taniguchi and Nishida, 2004). Therefore, nodal signaling appears to control the occurrence and orientation of neural tube rotation. Without nodal signaling, this rotation may not occur. Recently, it has been reported that in Ciona, nodal signaling is required for right-sided positioning of ocellus sensory pigment cells and formation of the photoreceptor through suppression of Rx gene expression on the left side of the brain vesicle (Yoshida and Saiga, 2011). Our results for Halocynthia are consistent with these findings. nodal is also expressed in the left-side brain vesicle at the late tailbud stage in Ciona, and this is also true for Halocynthia (J. Morokuma and H.N., unpublished). Yoshida and Saiga (Yoshida and Saiga, 2011) have suggested that the nodal expressed in the brain vesicle plays a role in the formation of brain asymmetry. However, our results do not support this proposal. The period during which embryos were sensitive to the nodal receptor inhibitor ended just after nodal expression in the left epidermis at the initial tailbud stage, whereas nodal expression in the left side of the brain vesicle starts later at the late tailbud stage (Fig. 2C). Our results for Halocynthia embryos suggest that nodal signaling in the brain vesicle at the late tailbud stage is dispensable for right-sided positioning of the sensory pigment cells and brain cavity, although we did not monitor the formation and location of photoreceptor cells.

Epidermal cilia and neurula rotation

Cilia grow on the surface of the epidermis at the neurula rotation stage. We have never succeeded in observing the presence and movement of these short and thin cilia at the light microscopy level, even with the use of a high-speed camera and image processing. We injected fluorescent beads (the same as used to visualize mouse nodal flow) into the perivitelline space or dropped beads onto devitellinized embryos and performed time-lapse recording. However, no constant and meaningful movement of the beads was observed. But we do believe that these cilia are motile because some devitellinized embryos were observed to suddenly start to move around on agar at and after the neurula rotation stage, a period that coincides with the stage at which epidermal cilia are present, although they failed to adequately rotate. It is likely that these epidermal cilia generate the driving force for neurula rotation, and that curvature of the inner surface of the vitelline membrane is required for rotation in a fixed position without any change in location.

Mouse nodal cilia tilt posteriorly and rotate in a clockwise direction (Shiratori and Hamada, 2006). If ascidian cilia tilt and rotate in a similar way, the direction of neurula rotation would be counterclockwise (Fig. 7), although it seemed that each cilium

Fig. 6. Requirement of the vitelline membrane and dispensability of follicle cells for nodal expression. (A) Naked eggs without the vitelline membrane fail to express nodal. At the bottom right is indicated the number of embryos that showed nodal expression, similar to the corresponding photograph, out of the total number observed. (B) Vitelline membrane was removed just before neurula rotation, and then the embryo was sandwiched between a glass slide and a coverslip coated with gelatin. These embryos never expressed nodal. (C) nodal expression was observed on both sides in all embryos with the vitelline membrane and with follicle cells when they were sandwiched. (D) Similarly, nodal expression was observed on both sides in all embryos with the vitelline membrane and without follicle cells. (E) Test cells are also dispensable for nodal expression.

Fig. 7. Model of neurula rotation. Cilia are present over the entire surface of the embryo except for the closing neural fold at the neurula rotation stage. These cilia appear to generate the driving force for stereotypic counterclockwise rotation of the whole embryo when viewed from the posterior pole. When the left side of the embryo is oriented downwards, the small protrusion of the neural fold (arrowhead), which lacks cilia, becomes an obstacle to further embryo rotation.
shows no or a limited posterior bias in its positioning on the surface of each epidermal cell. It is still unclear whether ascidian epidermal cilia rotate or beat. The observation that most of the cilia were tilted in the clockwise direction (Fig. 4E) might indicate that they beat in this direction. In ascidians, it has been reported that ion flux at the neurula stage is required for L-R asymmetry of Pitx expression (Shimeld and Levine, 2006). It is possible that ion flux controls the beat direction of cilia or the direction of tilt of rotation cilia.

Cessation of neurula rotation when the left side is oriented downwards

Neurula rotation always stops once the left side of the embryo faces down. Test cells in the perivitelline space are not involved in the rotational movement or its cessation. We assume that when an embryo lies on its left side, the small protrusion of the neural fold (Fig. 7, arrowhead) presents a physical obstacle to further rotation. This model is supported by three pieces of evidence. First, cells of the neural fold lack cilia and do not generate driving force, making the fold an insurmountable obstacle to further rotation (Fig. 4A, green). Second, the dorsal-side-down orientation is unstable because when embryos lay on their dorsal side at the start of rotation they appeared to fall onto their right or left side at the first step of rotation (Fig. 3C, pink and red). Third, it is plausible that the cilia continuously generate the driving force of rotation for 3 hours until the next cell division approaches and cilia are retracted, and that a certain physical obstacle prevents further rotation. This is because after the completion of neurula rotation, when we rotated the embryos so that they lay on their right, they always rotated back 180° in a counterclockwise direction and settled on their left side again (supplementary material Movie 3). This accords with the observation that cilia remain present for 3 hours after the neural rotation stage.

Contact of the left epidermis with the vitelline membrane promotes nodal expression

At 2 hours after neurula rotation, expression of nodal appears in left-side epidermis. Experiments involving centrifugation and sandwiching suggest that contact of the left epidermis with the vitelline membrane promotes left-sided nodal expression. The following evidence further supports this conclusion. nodal and Pitx are expressed in the epidermal layer of ascidians, which directly contacts with the vitelline membrane, but not in the mesoderm as observed in vertebrates. Similarly, rotational motion itself is not sufficient for nodal expression, as removal of the vitelline membrane just after rotational motion has completed prevented nodal expression. Experiments involving sandwiching and removal of the vitelline membrane at various stages indicated that contact between the epidermis and vitelline membrane must be sustained for some period of time in order to promote nodal expression.

Our next question was whether the cue for nodal expression is mechanical or chemical in nature. Simple compression of naked embryos between glass sheets coated with gelatin was unable to induce nodal expression. Follicle cells on the outside of the vitelline membrane, as well as test cells in the perivitelline space, were dispensable. Therefore, we propose that a chemical signal presented by the vitelline membrane might induce nodal expression in the epidermal cells that are in contact with it.

Neurula rotation in ascidians

All of the ascidian species that we have investigated show neurula rotation. However, embryos of other ascidian species can rotate more than 360°. In Ciona intestinalis, embryos tended to stop rotating when the tail starts to bend towards the ventral side, although this requires further confirmation. It has been reported that Ciona shows another significant difference: in devitellinized Ciona embryos, nodal and Pitx are expressed autonomously on both sides without signal from the vitelline membrane (Shimeld and Levin, 2006; Yoshida and Saiga, 2008). It is plausible that embryos of Halocynthia and Ciona utilize different mechanisms to promote conserved left-sided expression of nodal through neurula rotation.

It appears that at least the rotational movement at the neurula stage is conserved in ascidians, and that the rotation is probably driven by epidermal monocilia. Most non-chordate deuterostomes develop embryos and larvae that swim with cilia, such as the blastula and pluteus of sea urchins, the tornaria of hemichordates, and Amphioxus embryos (Satoh, 2009). By contrast, ascidian larvae swim with their tail. Ascidians might reutilize their ancestral epidermal cilia for neurula rotation, but not for swimming. In comparing the mechanisms of L-R asymmetry determination in vertebrates and ascidians it is intriguing to note that, in vertebrates, cilia generate a flow of liquid within the embryo, whereas in ascidians cilia might generate the actual driving force responsible for rotation of the entire embryo.

Acknowledgements

We thank the staff of the Asamushi Research Center for Marine Biology and the Otsuchi International Coastal Research Center for help in collecting ascidian adults and the staff of the Seto Marine Biological Laboratory for their assistance in maintaining them; Yasunobu Nabeshima (Research Institute of Environment, Agriculture and Fisheries, Osaka Prefectural Government) for providing us with seawater; Dr Naohito Takatori (Osaka University) for critical reading of the manuscript; and Dr Kyojuke Shinohara (Osaka University) for help in observation of ciliary movements with high-speed camera and image processing. Supplementary Movies 4 and 5 showing neurula rotation in Ciona intestinalis and Phallusia mammilata were kindly provided by Dr Ralf Schnabel and Andreas Hejnol (Institut für Genetik, TU Braunschweig, Germany) and Dr Takefumi Negishi (UMR7009/CNRS, Observatoire Oceanologique, France), respectively.

Funding

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) [22370078 to H.N.].

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.076083/-/DC1

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