Spiral cleavages determine the left-right body plan by regulating Nodal pathway in monomorphic gastropods, *Physa acuta*

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ABSTRACT The handedness of gastropods is genetically determined, but the molecular nature of the gene responsible and the associated mechanisms remain unknown. In order to characterize the chirromorphogenesis pathway starting from the gene to the left-right asymmetric body plan, we have closely analyzed the cytoskeletal dynamics of the *Physa (P.) acuta* embryo, a fresh water non-dimorphic sinistral snail, during the early developmental stage by mechanically altering the handedness of the embryos at the critical spiral third cleavage. A fertile *situs inversus* was created and the nodal-Pitx gene expression patterns were completely mirror imaged to the wild type at the trochophore stage. Together with our previous work on *Lymnaea (L.) stagnalis*, we could show that chirality is established at the third cleavage, as dictated by the single handedness-determining gene locus, and then chirality information is transferred via subsequent spiral fourth and fifth cleavages to the later developmental stage, dictating the nodal-Pitx expression pathway. The cytoskeletal dynamics of manipulated and non-manipulated embryos of sinistral *P. acuta* and dextral dominant *L. stagnalis* are compared.

KEY WORDS: spiral cleavage, nodal pathway, chirality, shell coiling, gastropod
third to fifth cleavages (Morrill, 1982; Verdonk and van den Biggelaar, 1983; Meschcheryakov, 1990). The handedness of snails can be distinguished easily at the third cleavage (i.e., 4 to 8 cell stage) as a quartet of micromeres is rotated with respect to that of sister macromeres in a clockwise sense in a dextral embryo, while in an anticlockwise manner in a sinistral embryo, looking from the animal pole. The rotation direction of protruding micromeres is always referred as looking down from the animal pole. It had been believed that all the developmental steps for the sinistral and dextral embryos are mirror images of each other based on conventional microscopic observations (Crampton, 1894; Meschcheryakov and Belousov, 1975). However, by close analysis of the temporal and spatial behaviour of cytoskeletons of the dextral and the sinistral L. stagnalis during the early cleavages, we have revealed non-mirror-image cytoskeletal dynamics at the third cleavage (Shibazaki et al., 2004). A helical deformation of blastomere shape (spiral deformation, SD) was observed at the animal hemisphere in metaphase to anaphase, together with helical spindle orientation (spindle inclination, SI) in the case of dominant dextral embryos. In contrast, no SD and SI were observed for the recessive sinistral animals. Thus, the blastomere shape and the spindle orientation as well as the timing of helicity generation are different for the dextral and sinistral embryos within a species, and are far from enantiomorphs. By using congenic strains we had generated, we could further show that the dextral-specific cytoskeletal dynamics (SD and the consequent SI) is genetically strongly linked to the single handedness-determining gene (Shibazaki et al., 2004; Kuroda et al., 2009, Kuroda, 2014).

More recently, we succeeded in creating healthy and fertile creatures with body handedness reversed to what is inherited, by blastomere micromanipulation at the third cleavage stage. We have shown that the asymmetric Nodal pathway does operate for the determination of the asymmetric body plan in L. stagnalis as well. When chirality at the 8-cell embryos was reversed by micromanipulation, the nodal and Pitx expression sites were completely shifted to the mirror-image positions (Kuroda et al., 2009).

One may argue that the success in reversing genetically determined handedness by mechanical manipulation is due to the fact that L. stagnalis is dimorphic. This means that the species may somehow have a potential, by nature, to accommodate the reversing physical force. Further, it is interesting to see whether similar handedness-determining mechanisms for L. stagnalis operate in sinistral-dominant snails as well. Thus, in this study, we tried to create dextral snails of the genus Physa, a sinistral gastropod, whose dextral strains have not been found. We followed and compared the cytoskeletal dynamics at the third and fourth spiral cleavages in detail for the mechanically reversed and non-manipulated embryos. We cloned the P. acuta orthologues of nodal and Pitx genes and investigated the expression patterns by whole mount in situ hybridization at the late trochophore stage. We could create a mirror image animal whose strain does not seem to exist in the wild, and showed that the third cleavage determines the following l-r asymmetric development, passing chirality information to the fourth and fifth spiral cleavages and then to the Nodal-Pitx pathway, similar to L. stagnalis, but with opposite chirality. The implications of these results are discussed in perspective of spiralian and vertebrates l-r asymmetry determination.

**Results**

*Creating a reversed P. acuta individual by mechanically changing the 8-cell blastomere arrangement*

Micromanipulation on cleavage directions was performed using the methods we have developed previously for L. stagnalis (Kuroda...
et al., 2009). Wild P. acuta undergoes anticlockwise rotation at the third cleavage (Fig. 1 A-F). SD was observed as in dextral L. stagnalis, though in opposite direction. We have previously reported that the mirror image relationship holds in terms of blastomere shape and spindle orientation, for the dominant sinistral and dominant dextral snails across species (Shibazaki et al., 2004). Thus, as in the case of L. stagnalis, micromanipulation was applied from the metaphase ~ anaphase stage. A constant mechanical force was applied to the protruding micromeres at the animal pole of each blastomere in the directions opposite to the normal third cleavage (Fig. 1 H-K) until contacts between newly formed micromeres and macromeres were established (Fig. 1L).

Judging from the blastomere configurations and intercellular contacts, 62% (92/149) of sinistral embryos were successfully reversed to "dextralized" 8-cell stage embryos. We incubated the manipulated embryos up to 33-64 cell stage and transferred the manipulated embryos into glass capillary tubes filled with the natural capsular fluid (70/92). Seven out of the seventy embryos developed into a veliger stage (Fig. 1 M) and six of them developed to juvenile snails with completely reversed features (Fig. 1N). This can be compared with control juveniles without any manipulation and grown inside the capsules (Fig. 1O). It is clearly seen that the aperture is on the right-hand side of the body and the shell coils in the right-handed way for the reversed embryos (Fig. 1N), while in the left-hand side of the body and left-handed shell coiling for the control (Fig. 1O). The six manipulated juvenile snails were taken out of the capillary tubes and transferred to a small aquarium, out of which one was successfully reared to adult, which had a perfect mirror-imaged body of the control snail (Fig. 1 P-R). Thus, we could show that the chirality-reversed embryos at the 8-cell stage developed to a quite normal adult snail with the opposite handedness, i.e., situs inversus, although our current survival rate was low.

The chirality-reversed snail produced offspring of reverted chirality

We have managed to rear the manipulated snail to the stage that gives birth to offspring by self-crossing. The mechanical manipulation we carried out is the inversion of the blastomere arrangements at the third cleavage, and that should not affect DNA. We could confirm that all the offspring of the chirality-inverted animal displayed sinistral body handedness as genetically dictated (Fig. 2, 248/248 eggs). The results have clearly revealed that the chiral geometry at the third cleavage in fact determines the body handedness, and the role of handedness-determining gene is to produce the particular blastomere geometry.

The fourth spiral cleavage directions are determined by the third cleavage

Spiral cleavage is characterized by the alternating clockwise and anticlockwise rotation particularly during the third - fifth cycles (Verdonk and van den Biggelaar, 1983). We closely look into the fourth cleavage of embryos to see whether the alternating cleavage occurs after the mechanically reversed third cleavage (Fig. 3). The spiral cleavage steps for the control and reversed-chirality embryos were compared in detail by taking images of the two embryos together in one frame at the interval of 30 sec. It is not only the healthy looking embryos with proper compaction, into glass capillary tubes filled with the natural capsular fluid (70/92). Seven out of the seventy embryos developed into a veliger stage (Fig. 1 M) and six of them developed to juvenile snails with completely reversed features (Fig. 1N). This can be compared with control juveniles without any manipulation and grown inside the capsules (Fig. 1O). It is clearly seen that the aperture is on the right-hand side of the body and the shell coils in the right-handed way for the reversed embryos (Fig. 1N), while in the left-hand side of the body and left-handed shell coiling for the control (Fig. 1O). The six manipulated juvenile snails were taken out of the
easy to place the control and reversed embryos side by side in the
same orientation and at similar developmental stages, but these
figures conclusively show the mirror-image relationship in cleavage
patterns and different developmental behaviour in time course. Fig.
3 shows representative such images. Time for the fourth cleavage
was conveniently set to 0, when the blastomere compaction after
the third cleavage was about to end. The developmental stage for
the control (Con) and reversed (Rev) embryos in a frame is a little
offset, with slightly more advanced cleavage stage for Rev (4'30"
slower as compared with Con).

In the control embryos, first quartet macromeres (1Q) underwent
spiral cleavage in a clockwise manner (t = 13'00'', Fig. 3D Con)
to form second quartet micromeres (2q, t = 16'00", Fig. 3E Con).
At t = 24'30" (Fig. 3H Con), compaction after the first macromere
cleavage started, and still in the process of compaction, first quartet
micromeres (1q) went through spiral cleavage dextrotropically (t =
32'00", Fig. 3I Con) to produce 1q1 and 1q2.

Similar sequential cleavages of 1Q and 1q occurred for the
reversed embryos (t = 12'30", Fig. 3C Rev, and t = 22'00", Fig. 3F
Rev, respectively), but the rotation was in an anticlockwise sense
in both cases. The compaction after the macromere cleavage was
not obvious, and the second micromere cleavage started sooner.
Thus, the time duration between the 1Q and 1q divisions in the
fourth cleavage was much shorter for the reversed embryos (n = 3)
as compared with the control (n = 9). There seems to be a
slight difference in the cytoskeletal behavior of the fourth cleavage
between the reversed and control embryos. However, both kept
the features of alternating rotation direction in the successive third
and fourth cleavages, and of the non-synchronous division of 1Q
and 1q in the fourth cleavage.

Microfilaments and mitotic spindles at the fourth cleavage were
visualized by double staining of filamentous actin and microtubule
by fluorescently labeled phalloidin (green) and anti-β-tubulin an-
tibody (red), respectively (Fig. 4). At the stage of 1q cleavages
corresponding to Fig. 3F Rev and Fig. 3I Con, spindles are oriented
in an opposite sense: clockwise for the control (Fig. 4A) and anti-
clockwise for the mechanically reversed (Fig. 4B) embryos. Thus,
we can say that the spindle orientation at the fourth spiral cleavage
stage is controlled by spatial arrangement of blastomeres at the
previous spiral cleavage, i.e., the third cleavage.

Asymmetric nodal-Pitx expression system is conserved in
Physa embryogenesis and the expression patterns are regu-
lated by the spiral cleavage direction at the 8 cell stage

To visualize the expression patterns of nodal and Pitx genes
in P. acuta, we cloned the orthologues and determined the amino
Chirality inversion at the 8-cell stage embryos resulted in situs inversus in P. acuta. Unlike L. stagnalis, P. acuta is not dimorphic and does not seem to adopt a series of opposite-sense spiral-rotation, i.e., anti-clockwise, clockwise and anti-clockwise rotation for the third, fourth and fifth cleavages, respectively, in the wild (Schilthuizen and Davison, 2005). Although dextral shells/individuals were reported as a very rare collectors’ item in 19th and early 20th centuries (Williams, 1887; Pelseneer, 1920), these might have been a result of malformation and not of genetic changes. No dextral strains of P. acuta have been reported. Even so, cytoskeletal dynamics was not upset by the forced dextrality at the third cleavage and performed the almost normal subsequent spiral cleavages. Thus, the creation of mirror-image individuals is not restricted to species which exhibit dimorphism in the wild, and it may be workable in other members of spiralia.

Spiral cleavage where the cleavage planes are at oblique angles to the animal-vegetal axis of the egg occurs during the second (or may be even the first?) to fifth cleavages in alternating rotation direction, but they are most notable during the third to fifth cleavages (Mescher, 1975; Verdronk and van den Biggelaar, 1983; Lambert, 2010). Embryos manually forced to rotate clockwise way at the third cleavage opposite to the wild type, underwent anti-clockwise rotation first during the macromere division and then during the micromere division as well, in the subsequent fourth cleavage. We only pushed the forming micromeres (1q), during the third cleavage to create mirror-image 8-cell embryos, and did not touch the vegetal hemisphere of macromeres (Q = A, B, C and D). In the subsequent fourth cleavage, both 1Q and 1q (q = a, b, c and d) rotated opposite sense to the third cleavage, indicating it is not the result of intra-cellular event caused by cell pushing but of the relative placement of 1Q and 1q, i.e. micromere – macromere contact that determines the direction of the next spiral cleavage.

Our results of manipulation indicate that l-r symmetry is not yet established before the third cleavage. Difference between the sinistral and dextral snails is distinguishable as early as at the second or even at the first cleavages (Mescher, 1975). However, when we altered, by manipulation, the directions of blastomere rotations at the first or at the second cleavage to produce reversed blastomere configuration at the 4-cell stage, the manipulated embryos all developed to perform original-sense third cleavage and resulted in normal 8-cell stage in both the sinistral and the dextral L. stagnalis embryos (Kuroda et al., 2009). We also observed that sinistral embryos occasionally showed dextral-type blastomere arrangement at the 4-cell stage even inside the egg capsules.
but they all showed normal counterclockwise cleavage at the third division (Kuroda et al., 2009). These results support our conclusion that characteristic macromere - micromere cell contact at the 8-cell stage embryos is the first determining step for asymmetric development of snails, although chirality is indicated before then. The single handedness-determining gene dictates SD and SI at the third cleavage. This agrees well with our previous finding that the handedness-determining gene locus is genetically closely linked to SD and SI, using the congenic F4 (Shibazaki et al., 2004) and F7 (Kuroda et al., 2009) strains as well as F10 strains (Kuroda, 2014) of L. stagnalis we constructed.

The genetically determined critical third cleavage step was overridden by the mechanical micromanipulation, but once chirality is established at this stage, the following development proceeded normally except for the handedness. Body handedness of offspring which were born from the situs inversus mother reverted to the original genetically-determined chirality.

Still we do not know why and how the spiral cleavages occur in alternating manner. It is plausible that this is the way to transfer chirality information from the third cleavage (which is dictated by the single handedness-determining gene locus) to the later developmental stage. In the fourth cleavage, after the recurrent blastomere compaction during post-mitotic phase in which the morphological chirality of embryos was seemingly lost, both the artificially reversed and control embryos exhibited rotation in the opposite sense to the previous third cleavage. Thus, spindle orientation at spiral cleavage stages is controlled by spatial arrangement of blastomeres that is determined by the previous cell cleavage event. How the rotation, especially alternating rotation is achieved is a fascinating topic of research.

**Nodal-Pitx pathway operates in P. acuta**

Nodal-Pitx pathway is critically important in the determination of the asymmetric body plan in deuterostomes (Grande and Patel, 2009). The Nodal pathway was not found in Ecdysozoa such as flies and nematodes, but Grande and Patel for the first time showed the asymmetric expression of nodal and its target Pitx genes in the sinistral snail Biomphalaria glabrata and the dextral snail Lottia gigantea (Grande and Patel, 2009). We ourselves reported that recessive sinistral and dominant dextral snails of the same species, L. stagnalis, express nodal and Pitx genes in mirror image patterns (Kuroda et al., 2009). We have shown that when chirality at the 8-cell stage of P. acuta embryos was reversed by micromanipulation, the nodal and Pitx expression patterns at the late trochophore stage were completely reversed (Fig. 6), as in the case of L. stagnalis.

Nodal is a member of the transforming growth factor-β superfamily (Kingsley, 1994; Hogan, 1994). All members of the nodal subfamily of the TGF-β superfamily are synthesized as large prepro precursors, which are cleaved at an RXRR site to release a C terminal peptide of 110-140 amino acids. Nodal orthologue of P. acuta (PaNodal) has RKKR cleavage sequence and the amino acid sequence of the mature domain, particularly after the first of seven cysteine residues is well conserved with that of other members of Lophotrochozoa as well as zebrafish (Ndr2) and Xenopus (Xnr1) (Fig. 5A). This suggests that P. acuta’s nodal orthologue is also released from the cell and act as a morphogen. Pitx, the target of Nodal, is a homeobox gene that directs the formation of many body structures during early embryonic development (Hamada et al., 2002). Pitx orthologue of P. acuta (PaPitx) has a highly conserved homeobox region with mouse, ascidian, sea urchin and other gastropods (Fig. 5B) (Gage and Camper, 1997).

These results may suggest that similar Nodal-Pitx pathway to vertebrates operates in P. acuta as well. In fact, similar Nodal pathway has been found in sea urchins (Duboc and Lepage, 2008; Bessodes et al., 2012). Nodal acts over a distance to elicit dose-dependent responses, and the pathway is tightly regulated by diverse mechanisms including the control of ligand processing, utilization of co-receptors, expression of soluble antagonists, Lefty, as well as positive- and negative-feedback activities (Hamada et al., 2002; Shen, 2007; Müller et al., 2012). Lefty orthologue was reported not found in snails (Grande and Patel, 2009a) and if this were the case, different feedback mechanisms must operate to inhibit the diffusion of Nodal to the other side of midline.

In vertebrates, asymmetric nodal expression occurs in the left side of the lateral plate mesoderm for a short period of time. In contrast, snails express nodal for a longer period of time asymmetrically starting from the 33-64 cell stage (Grande and Patel, 2009a; Kuroda et al., 2009a; Kuroda, 2014). Thus, it is quite interesting to find out how the role and regulation mechanisms have changed during the course of evolution. We plan to carry out detailed analysis on nodal expression patterns during the course of development and the contribution toward the chiro genesis using snails which provide simpler model system for study. The mechanisms by which graded signals are generated and interpreted to organismal chiromorphology are of particular interest because they are fundamental for embryonic tissue patterning.

**Snails are ideal model animals for the study of l-r symmetry breaking**

Nodal and Pitx genes are not yet expressed by the end of spiral cleavage (fifth cleavage to form from 16 to 24 cell stage), and started to be expressed at 33-64 cell stage (Kuroda, 2014). Here, nodal is already expressed asymmetrically. Thus, spiral cleavage’s role may be to transfer chirality information from the third to the fifth cleavage, and then the transferred information at the 24-cell stage is used for the asymmetric expression of nodal. The mechanisms for the spiral cleavages as well as those for leading to the asymmetric nodal gene expression seem to be common for L. stagnalis and P. acuta, and further, these may be the fundamental mechanisms for the l-r determination in spiralia.

We have shown that the blastomere geometry at the 8-cell stage as a result of the third cleavage is the first step for the handedness determination. It is intriguing to think if the handedness determining gene is the same or similar for P. acuta and L. stagnalis, how it can act in a completely mirror-imaged manner. There may be some other unknown factors which determine the direction of third cleavage rotation. To find answers to these questions, comparative work on P. acuta with L. stagnalis or with other members of spiralia is underway.

In the case of L. stagnalis, l-r asymmetry is determined already only 3 hours after the first blastomere cleavage, which is determined by a maternal factor before the initiation of zygotic gene expression (Morrill, 1982; Meshcheryakov, 1990). Nodal is expressed asymmetrically at the 33-64 cell stage, about 12 hours after the first cleavage (Kuroda et al., 2009; Kuroda, 2014). These events occur much later in vertebrates. For example, in the case of mouse, nodal flow and asymmetric nodal expression were observed...
at E7.5 and E8.25 days, respectively, which means that it is a long step from the first symmetry breaking to the nodal flow/asymmetric nodal expression, which involves many cells and molecules. In contrast, snails’ body handedness is determined during the early development period where only a relatively small number of cells and molecules are acting. Thus, snails present an excellent model system for studying l-r symmetry breaking.

In conclusion, we have shown a handedness determining pathway starting from the single handedness-determining gene to chiral blastomere arrangement at the 8-cell stage, which then leads to the asymmetric expression of the zygotic genes relevant to asymmetric body formation. During the early developmental stage, consecutive spiral cleavages have important roles, establishing chirality at the spiral third cleavage, and transferring chirality information via spiral fourth and fifth cleavages. That process seems common in sinistral-dominant and dextral-dominant snail species but it leaves questions about the mechanism by which opposite rotation direction is determined. Further studies are required towards an understanding of the symmetry-breaking event in snails.

Materials and Methods

Snails
Adult freshwater snails of P. acuta were collected at a pond in the Risoukai Nature Park of Tokyo University of Science, or rice field in Noda, Chiba prefecture, Japan. The snails were reared at 25°C and given artificial food for tropical fish. In our laboratory condition, the snails laid eggs almost every day.

Micromanipulation
Manipulation of P. acuta embryos at third cleavage was performed as described previously for L. stagnalis embryos (Kuroda et al., 2009). Manipulated and non-manipulated embryos were enclosed in a glass capillary tube together with the natural capsular fluid, and cultured for about 2 weeks until they developed into juvenile snails. Time lapse images for the fourth cleavage of manipulated and non-manipulated embryos were obtained using a Zeiss Axioskop2 microscope equipped with an Axioscam MRc colour digital camera, using AxioVision 3.1 software.

Identification of PaNodal and PaPitx and their sequence alignment
Degenerate primers for isolation of partial sequences of P. acuta nodal-related gene (PaNodal) and Pitx-related gene (PaPitx) were designed from a comparison of nodal and Pitx-related proteins in other snails: nodal deg-Fw1 primer, 5’-ATHGGNTGGGGNCARTGGAT-3’, corresponding to the amino acid sequence H(D/E)(D/E)MIA; and Pitx deg-Rv1 primer, 5’-TTCATRTTNARNCCCCANGGRAA-3’, corresponding to the amino acid sequence FPWGLNMN. RT-PCR was performed using deg-Rv1 primer, 5’-TTCATRTTNARNCCCCANGGRAA-3’, corresponding to the amino acid sequence YPDMATR; and deg-Fw1 primer, 5’-ATHGGNTGGGGNCARTGGAT-3’, corresponding to the amino acid sequence YPDMATR.

Whole mount in situ hybridization and immunohistochemistry
Embryos developed to the late trophophore stage in capillary were fixed in 4% paraformaldehyde in MTSTr buffer overnight at 4°C. Digoxigenin-labeled RNA probes were synthesized from the coding regions sequences of cDNA in both antisense and sense strands. Whole mount in situ hybridization was performed as described previously (Kuroda et al., 2009). Stained embryo images were acquired using a Zeiss Axioskop2 microscope. Immunohistochemistry was carried out as described previously (Shibazaki et al., 2004). Images were obtained using a Zeiss LSM5 pascal confocal microscope with a 20×objective lens.

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