Review of: "Disruption of left-right axis specification in Ciona induces molecular, cellular, and functional defects in asymmetric brain structures"

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Left−right asymmetries of animals

The L−R asymmetric features of animals are easily recognized by the positions of the organs. Many organs, such as the gut, liver, and heart, are asymmetrically located in a determinative manner. L−R asymmetries are a shared feature of metazoans; various aspects of animal characteristics are indeed asymmetric. For example, the winding direction of snail shells is usually clockwise, with a few examples of counterclockwise. Male grasshoppers have asymmetric wing shapes that create sounds to attract females. In echinoderm sea urchins, whose adult morphology is basically radially symmetric, the adult rudiment forms on one side near the gut of Pluteus larvae.

L−R asymmetry is not limited to morphology. The left and right sides of the cerebellum are known to have different roles (reviewed in [2]). This is associated with the different features of neurons, such as neuronal subtypes, gene expression, and axon trajectories. The L−R asymmetries of animals are also reflected in behavior. A famous example is the handedness of humans [3-4]. Behavioral asymmetries may be associated with the cellular and structural asymmetries of our bodies, including the nervous systems, because these factors determine behaviors. Thus, studies of L−R axis formation include molecular, cellular, and behavioral backgrounds to elucidate animal asymmetries, as the title of this manuscript suggests.

To this end, it’s necessary to consider that certain left- or right-biased characteristics, particularly behavioral ones, could be predominantly acquired features more than a result of genetic background. these acquired features must be distinguished from those determined by the genetic system. Because animals that have simple behavioral mechanisms are less influenced by acquired modifications, they will be valuable for thorough studies of the L−R axis.

Ascidians and their embryogenesis

Phylogenetic studies have shown that ascidians are the closest living relatives of vertebrates [5]. In this chordate group, it is not easy to recognize the phylogenetic position at the sessile adult stage; however, ascidians have a tadpole-shaped larval body, and the tadpole larva swims actively by swinging or beating its tail. The overall larval morphology and the tissue organization in the larval body, having a notochord

and a dorsally located, hollow CNS, are features shared among chordates [6].

In contrast to the phylogenetic position of ascidians as the sister group of vertebrates, the mechanisms underlying early embryogenesis in ascidians are very different from those in vertebrates. Ascidian eggs are called mosaic eggs, in which the determinants of cell fate specification are prelocalized [7-8]. A blastomere that receives a determinant is destined to differentiate into the cell type specified by the determinant. This mosaic mode of embryogenesis is in contrast to the regulative mode of vertebrate embryogenesis. Another characteristic of ascidian embryogenesis is its simplicity. In the model ascidian *Ciona*, gastrulation starts as early as the 110-cell stage, and a larva is constituted by ~3,000 cells. The CNS of *Ciona* includes 177 neurons whose synaptic connections have been well documented through electron microscopy-based studies [9-10].

The embryogenesis of ascidians progresses in the chorion. The chorion forms a strong barrier that limits the exchange of molecules between perivitelline space and outside seawater. The presence of the chorion is important for the completion of embryogenesis; removal of the chorion results in low fertilization efficiency, failure of morphogenesis (as seen in the Japanese ascidian *Halocynthia roretzi*), and improper sizing of the tunic (*Ciona*) [8, 11, 12]. Embryos developed from dechorionated eggs usually have lower rates of healthy development compared to control groups developed with the chorion. Dechorionation is an important technique in ascidians because it allows the introduction of exogeneous molecules such as DNA/RNA into embryos by means of electroporation or microinjection. However, its deleterious effect is annoying to researchers.

The L−R asymmetry of ascidian larva

Ascidian larvae have L−R asymmetric features [13-14]. In *Ciona*, an easy example is the location of the pigmented ocellus at the brain-corresponding region of the CNS; this structure is clearly visible only from the right side. This bias occurs because the larval brain of *Ciona* is twisted and the ocellus is located on the right side [reviewed in 15]. The otolith is the other pigmented sphere near the ocellus. The midline of the otolith is somewhat biased to the right. In larvae with the twisted CNS, many brain regions, which can be specified by the distribution of neuronal subtypes, are also asymmetrically located.

The L−R asymmetric features of ascidian larvae are established during embryogenesis. During cleavage stages, ascidian embryos are perfectly symmetric. For example, the left and right blastomeres of two-cell embryos respectively form the left and right sides of the body [16]. Therefore, the bilateral symmetry is disrupted at a certain developmental period. The earliest sign of L−R asymmetry is observed at the two-cell stage, in which asymmetric inositol triphosphate (IP3)-mediated calcium ion signaling occurs [17]. Then, the famous signature of L−R asymmetry is seen at the neurula stage. At this stage, the gene encoding Nodal, the key signaling molecule responsible for L−R asymmetry formation, is expressed only in the left side of the ectoderm [18-19]. These relays between molecules are suggested to create the L−R axis at the larval stage.

Removal of the chorion disrupts L−R asymmetries of larvae. This is because the chorion is necessary to

specify theL−R axis. In *Halocynthia*, the physical contact between the chorion and the left side of the embryo induces the left-side expression of *nodal* [20]. A similar phenomenon is observed in *Ciona*, suggesting that a conserved mechanism underlies the breakage of L−R symmetry. Larvae developed from dechorionated eggs exhibit randomization of the L−R axis; larvae with normal asymmetry, complete reversal, partial reversal, and the absence of some asymmetric features are all seen in a single experiment.

L−R disruption by dechorionation persists after metamorphosis, which converts tadpole-like swimming larvae into immotile adults. At the larval stage, many organs are at the premature state and are not functional in solitary ascidians such as *Ciona* [12; 21]. After metamorphosis, however, ascidian juveniles have developed organs such as the heart and digestive tube. The location and structure of these adult organs are asymmetric, like those in vertebrates. The asymmetric features are disrupted in juveniles that developed from dechorionated eggs, suggesting that the mechanism that specifies the L−R axis during embryogenesis also determines this axis after metamorphosis. The rudiments of the adult organs are specified during embryogenesis, and some organs start to form at the larval stage [22-23]. In this way, the effect of L−R axis disruption by dechorionation could persist after metamorphosis.

How does the disruption of the L−R axis influence *Ciona*?

Kourakis and colleagues observed detailed changes in L−R disrupted larvae by dechorionation. They employed a new way of removing the chorion after the L−R axis is established, and they compared larvae subjected to dechorionation at this later stage (late-dechorionated larvae) with larvae developed from dechorionated eggs (early-dechorionated larvae). The early-dechorionated larvae exhibited various ranges of morphological and cellular abnormalities that seemed to be beyond the mere morphological disruption of the characteristics along the L−R axis.

In normal and late-dechorionated larvae, for example, the ocellus is cup-shaped while the otolith looks like a complete sphere. In early-dechorionated larvae, on the other hand, two cup-shaped or two sphere-shaped pigments, or a single large pigment, is frequently observed, and the pigment or pigments are misoriented. These results suggest that L−R axis disruption is not merely a simple impairment of the arrangement of properly specified cells, tissues, or organs; rather, this disruption interferes with the specification processes. Accordingly, those authors showed that several sensory organs were absent in ~50% of the early-dechorionated larvae. The rate of loss of a neural structure in early-dechorionated larvae differs among the structures. This difference is probably caused by the different strengths of the association between the mechanisms of specification of structures and L−R axis formation.

In addition, those authors found that the bilateral structures along the L−R axis are also affected by dechorionation at the early stage. Pitx is the key transcription factor responsible for the specification of the L−R axis as a downstream factor of Nodal [24, 18-19]. *Pitx* is expressed only on the left side of the motor ganglion, where motor neurons are aligned bilaterally [25]. Moreover, the gene encoding AMPA-type glutamate receptor (AMPAR) is also expressed only on the left side of the motor ganglion. Therefore, left

and right motor neurons could have different functions by the left-side-specific reception of the neurotransmitter L-glutamate. Left-side-specific *pitx* and *AMPAR* expressions were impaired by early dechorionation, suggesting that the effects of dechorionation are not limited to the morphologically recognizable asymmetric characteristics but also influence the hidden asymmetric features of cells and tissues at the molecular level.

Behavioral changes in dechorionated larvae

The disruption of the L−R axis by dechorionation affects larval behavior. *Ciona* larvae exhibit negative gravitaxis at the initial stage after hatching [26-27]. By this activity, larvae swim toward the surface of the ocean; this movement is suggested to enhance the diffusion of siblings by the tidal movement. This gravitaxis is reproducibly observed in normally developed larvae. In addition to the gravitaxis, larvae start tail beating by responding to the dim conditions from a certain period after hatching [28]. Therefore, by using dimness to induce swimming, the authors observed swimming trajectories in relation to gravity. They found that, in contrast to the control groups, early-dechorionated larvae exhibited a much lower frequency of negative gravitaxis; many early-dechorionated larvae showed downward or sideways swimming in addition to upward. As mentioned above, L−R axis disruption causes various changes in the nervous systems beyond the positioning of structures along the L−R axis. Therefore, the variety of changes in swimming behavior is suggested to reflect the sum of the complexity of the effects caused by dechorionation.

The authors of the cited manuscript found that a portion of larvae from the early-dechorionation experiment had an axis that was a perfectly mirror image reversal of the normal axis. Using these mirrored larvae, the authors observed behavioral asymmetry in gravitaxis. When *Ciona* larvae with a normal L−R axis swam upward, they exhibited counterclockwise trajectories when viewed from above. In contrast, the L−R reversed larvae swam clockwise, suggesting that the mirroring of the body also causes the perfect reversal of swimming behavior with respect to gravity. This reversal means that the asymmetry in swimming behavior is based on the L−R asymmetric features in the body and could not be adjusted in an acquired manner.

Perspectives

L−R axis disruptions influence molecular, cellular, morphological, and behavioral aspects of ascidian larvae. Owing to this study and previous studies, we have accumulated knowledge about L−R asymmetric characteristics in ascidian larvae, the mechanisms underlying how the asymmetries are formed, and how the asymmetries influence animals. After reading the manuscript cited in this review, I have realized that there remains a big question: What is the significance of the rigid selection of L−R asymmetry? As the mirrored larvae suggest, the reversed construction of the L−R axis seems not to cause a deleterious effect on animals. Despite that, the L−R axis is rigidly determined. The molecules underlying L−R asymmetry construction, such as Nodal and Pitx, show conserved patterns of left-biased expressions and functions

throughout animals, with the exceptions seen in echinoderms [29, 30]. The simplicity of the *Ciona* larva, in both morphological and behavioral aspects, provides an ideal system with which to uncover this question by addressing how *Ciona* benefits by selecting, for example, a counterclockwise way of swimming in the negative gravitaxis.

Technically, chemical dechorionation is useful and widely used among *Ciona* researchers to introduce exogenous molecules, to simultaneously remove obstructive chorions before fixation for sampling of *in situ* hybridization, and so on. We have empirically learned the deleterious effects of this treatment, particularly on L−R axis formation. This study shows many concrete examples of how the L−R axis is disrupted by dechorionation. This information is helpful for judging whether early dechorionation will be appropriate or not in an experiment a researcher is planning to do. Moreover, this manuscript shows that dechorionation after embryogenesis is possible without impairing the L−R axis. The hesitation toward chemical dechorionation at a later stage exists because the dechorionation of ascidians uses a protease that breaks cell−cell adhesions [31-33]. Kourakis and colleagues showed that this negative effect does not matter if we follow their method appropriately. Now we have gained another option, that of later dechorionation, if this timing is suitable for specific research; for example, sampling for *in situ* hybridization at a later developmental stage before hatching.

References

1. Kourakis MJ, Bostwick M, Zabriskie A, Smith WC: **Disruption of left-right axis specification in Ciona induces molecular, cellular, and functional defects in asymmetric brain structures**. *BMC Biol* 2021, **19**(1):141.
2. Halpern ME, Gunturkun O, Hopkins WD, Rogers LJ: **Lateralization of the vertebrate brain: taking the side of model systems**. *J Neurosci* 2005, **25**(45):10351-10357.
3. Schmitz J, Gunturkun O, Ocklenburg S: **Building an Asymmetrical Brain: The Molecular Perspective**. *Front Psychol* 2019, **10**:982.
4. Brandler WM, Paracchini S: **The genetic relationship between handedness and neurodevelopmental disorders**. *Trends Mol Med* 2014, **20**(2):83-90.
5. Delsuc F, Brinkmann H, Chourrout D, Philippe H: **Tunicates and not cephalochordates are the closest living relatives of vertebrates.** *Nature* 2006, **439**(7079):965-968.
6. Satoh N: **The ascidian tadpole larva: comparative molecular development and genomics.** *Nat Rev Genet* 2003, **4**:285-295.
7. Nishida H: **Specification of embryonic axis and mosaic development in ascidians** . *Dev Dyn* 2005, **233**:1177-1193.
8. Nishida H, Sawada K: ***macho-1* encodes a localized mRNA in ascidian eggs that specifies muscle fate during embryogenesis**. *Nature* 2001, **409**(6821):679-680.
9. Ryan K, Lu Z, Meinertzhagen I: **The CNS connectome of a tadpole larva of *Ciona intestinalis* (L.) highlights sidedness in the brain of a chordate sibling.***Elife* 2016, **5**:e16962.

10. Ryan K, Lu Z, Meinertzhagen IA: **The peripheral nervous system of the ascidian tadpole larva: Types of neurons and their synaptic networks**. *J Comp Neurol* 2018, **526**(4):583-608.
11. Nishida H, Satoh N: **Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme II. The 16- and 32-cell stages**. *Dev Biol* 1985, **110**(2):440-454.
12. Cloney RA: **Ascidian larvae and the events of metamorphosis.** *Amer Zool* 1982, **22**:817-826.
13. Hirano T, Nishida H: **Developmental fates of larval tissues after metamorphosis in the ascidian, *Halocynthia roretzi* II. Origin of endodermal tissues of the juvenile** . *Dev Genes Evol* 2000, **210**:55-63.
14. Boorman CJ, Shimeld SM: **The evolution of left-right asymmetry in chordates** . *Bioessays* 2002, **24**(11):1004-1011.
15. Sasakura Y, Mita K, Ogura Y, Horie T: **Ascidians as excellent chordate models for studying the development of the nervous system during embryogenesis and metamorphosis**. *Dev Growth Differ* 2012, **54**(3):420-437.
16. Nishida H, Satoh N: **Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme I. Up to the eight-cell stage**. *Dev Biol* 1983, **99**(2):382-394.
17. Albrieux M, Villaz M: **Bilateral asymmetry of the inositol trisphosphate-mediated calcium signaling in two-cell ascidian embryos**. *Biol Cell* 2000, **92**(3-4):277-284.
18. Morokuma J, Ueno M, Kawanishi H, Saiga H, Nishida H: **HrNodal, the ascidian nodal-related gene, is expressed in the left side of the epidermis, and lies upstream of HrPitx**. *Dev Genes Evol* 2002, **212**(9):439-446.
19. Yoshida K, Saiga H: **Left-right asymmetric expression of Pitx is regulated by the asymmetric Nodal signaling through an intronic enhancer in Ciona intestinalis**. *Dev Genes Evol* 2008, **218**(7):353-360.
20. Nishide K, Mugitani M, Kumano G, Nishida H: **Neurula rotation determines left-right asymmetry in ascidian tadpole larvae**. *Development* 2012, **139**(8):1467-1475.
21. Sasakura Y, Hozumi A: **Formation of adult organs through metamorphosis in ascidians** . *WIREs Dev Biol* 2018, **7**:e304.
22. Nakazawa K, Yamazawa T, Moriyama Y, Ogura Y, Kawai N, Sasakura Y, Saiga H: **Formation of the digestive tract in *Ciona intestinalis* includes two distinct morphogenic processes between its anterior and posterior parts**. *Dev Dyn* 2013, **242**(10):1172-1183.
23. Kawai N, Ogura Y, Ikuta T, Saiga H, Hamada M, Sakuma T, Yamamoto T, Satoh N, Sasakura Y: ***Hox10*-regulated endodermal strand cell migration is essential for development of the ascidian intestine.** *Dev Biol* 2015, **403**(1):43-56.
24. Levin M: **Left-right asymmetry in embryonic development: a comprehensive review** . *Mech Dev* 2005, **122**(1):3-25.
25. Stolfi A, Levine M: **Neuronal subtype specification in the spinal cord of a protovertebrate** . *Development* 2011, **138**(5):995-1004.

26. Tsuda M, Sakurai D, Goda M: **Direct evidence for the role of pigment cells in the brain of ascidian larvae by laser ablation**. *J Exp Biol* 2003, **206**:1409-1417.
27. Bostwick M, Smith EL, Borba C, Newman-Smith E, Guleria I, Kourakis MJ, Smith WC: **Antagonistic Inhibitory Circuits Integrate Visual and Gravitactic Behaviors**. *Curr Biol* 2020, **30**(4):600-609 e602.
28. Nakagawa M, Miyamoto T, Ohkuma M, Tsuda M: **Action spectrum for the photophobic response of Ciona intestinalis (Ascidieacea, Urochordata) larvae implicates retinal protein**. *Photochem Photobiol* 1999, **70**(3):359-362.
29. Duboc V, Rottinger E, Lapraz F, Besnardeau L, Lepage T: **Left-right asymmetry in the sea urchin embryo is regulated by nodal signaling on the right side**. *Dev Cell* 2005, **9**(1):147-158.
30. Hibino T, Nishino A, Amemiya S: **Phylogenetic correspondence of the body axes in bilaterians is revealed by the right-sided expression of Pitx genes in echinoderm larvae**. *Dev Growth Differ* 2006, **48**(9):587-595.
31. Kobayashi K, Satou Y: **Microinjection of Exogenous Nucleic Acids into Eggs: Ciona Species** . *Adv Exp Med Biol* 2018, **1029**:5-13.
32. Yasuo H, McDougall A: **Practical Guide for Ascidian Microinjection: Phallusia mammillata** . *Adv Exp Med Biol* 2018, **1029**:15-24.
33. Kumano G: **Microinjection of Exogenous DNA into Eggs of Halocynthia roretzi** . *Adv Exp Med Biol* 2018, **1029**:25-35.