Evidence that gastropod torsion is driven by asymmetric cell proliferation activated by TGF-β signalling

Yoshihisa Kurita, Hiroshi Wada

Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan

Yoshihisa Kurita: katachiya@gmail.com

Summary

Gastropods are characterized by their asymmetric bodyplan, which develops through a unique ontogenetic process called “torsion.” Despite several intensive studies, the driving force of torsion remains to be determined. Although torsion was traditionally believed to be driven by contraction of the retractor muscle connecting the foot and the shell, some recent reports cast doubt on that idea. Here, we report that torsion is accompanied by left-right asymmetric cell proliferation in the mantle epithelium in the limpet *Nipponacmea fuscoviridis*. Furthermore, we found that pharmacological inhibition of the TGF-β signalling pathway, including that of Nodal, blocked torsion. We confirmed that the blocking was brought about through failure of the activation of cell proliferation in the right-hand side of the mantle epithelium, while the retractor muscle apparently developed normally. These results suggest that limpet torsion is driven by left-right asymmetric cell proliferation in the mantle epithelium, induced by the TGF-β pathway.

Keywords: Mollusca; Torsion; Left-right asymmetry

1. INTRODUCTION

Triploblastic animals have a mostly bilaterally symmetric bodyplan; however, the symmetry is not always complete and most show greater or lesser degrees of left-right asymmetry. Gastropods transform the symmetric bodyplan into a highly asymmetric one through a unique process called torsion.

Torsion involves the 180˚ rotation of the velum and the foot of the developing larval body relative to the larval shell and visceral mass (electronic supplementary material 1). This unique developmental process is a characteristic feature of gastropods, and has intrigued many developmental and evolutionary biologists for over a century as a model case of transition to a novel bodyplan [1-6]. Although the onset of torsion and its completion are readily observed in several species, the underlying mechanism of morphological movement remains to be determined [5-7].

Traditionally, it was widely accepted that torsion was driven by contraction of the larval retractor muscle connecting the foot and shell plate [2, 3, 6]. However, some recent studies have questioned this idea, because torsion occurred even in larvae where the muscle failed to attach to the shell [7, 8]. As an alternative hypothesis, it was proposed that torsion is caused by asymmetric growth of the mantle epithelium which is the surface region of trunk ectoderm posterior to the foot [5, 9]. To further investigate

asymmetric growth during gastropod torsion, we investigated cell proliferation activity during development in a limpet species *Nipponacmea fuscoviridis*.

Gastropods possess another distinct aspect of left-right asymmetry in the handedness of shell coiling. The handedness of shell coiling is known to correlate with the modes of the spiral cleavage pattern (i.e., if the third cleavage micromere is displaced clockwise, as observed in most gastropods, shell coiling is dextral, and *vice versa*) in most species, with the possible exception of hyperstrophic gastropods [10]. Additionally, shell coiling correlates with the direction of torsion. While dextral species undergo torsion in a counter-clockwise manner, torsion in sinistral species occurs in the opposite direction [4]. A recent study by Grande and Patel found that the asymmetric activation of the Nodal signal is involved in the left-right specification and the formation of shell coiling in gastropods [11]. Interestingly, they reported that the asymmetric expression of Nodal is also observed in limpets, which do not show an obvious handedness in shell coiling even in early larval stages [12]. Thus, we reasoned that the Nodal pathway may also be involved in the direction of torsion.

In this study, we report that in *N. fuscoviridis* cell proliferation in the larval mantle epithelium is significantly higher on the right-hand side. Additionally, this asymmetric proliferation was sensitive to pharmacological inhibition of TGF-β signalling,

including Nodal. Thus, we conclude that asymmetric cell proliferation, regulated by TGF-β signalling, is a major driving force of gastropod torsion.

2. MATERIALS AND METHODS

Sexually mature individuals of *N. fuscoviridis* were collected in Yoshidahama harbor, Miyagi Prefecture, Japan, during the breeding season (March to July, and October to November). In vitro fertilization was performed following Deguchi [13]. Embryos were cultured in filtered-seawater (FSW) at 22˚C. Inhibition of TGF-β signalling was performed using SB-431542 (TOCRIS Bioscience) dissolved in DMSO and cell proliferation was analyzed using 5-bromo-2-deoxyuridine (BrdU; Roche). Statistical analysis of cell proliferation was performed using a Mann-Whitney U-test or two-way ANOVA followed by Tukey's test [14].

Nfnodal and *Nfpitx* were amplified by PCR using the primers are shown in the electronic supplementary material 2. In situ hybridization was performed as described in Kin *et al*. [15]. More detailed method is shown in electronic supplementary material 3.

3. RESULTS

First, we briefly describe the time course of the torsion of *N. fuscoviridis* cultured

at 22°C. In this study, we describe the orientation of the larval body axis by referring to the axis of the velum and foot (i.e., lateral view from the right-hand side indicates that that image is viewed from the right-hand side of the velum and foot). Overall, torsion occurred more-or-less synchronously and was completed within 5 h. Until about 22 hpf, we did not observe any signs of torsion (electronic supplementary material 1*a*). At 23 hpf, the torsion process had begun and showed an approximately 30-45° rotation of the visceral mass and larval shell relative to the velum and foot. At 25 hpf, the rotation was approximately 90° (electronic supplementary material 1*b*). Larvae completed the 180° rotation by 27 hpf (electronic supplementary material figure 1*c*).

To examine whether asymmetric cell proliferation occurs during torsion, we measured cell proliferation activity separately in the mantle epithelium and the mantle edge (refer to electronic supplementary material 1*a*), and compared the cell proliferation activity between the left and right sides. We examined cell proliferation activity during the first 90° of rotation (from 22 to 25 hpf) because at that stage it is easy to align the degree of rotation, and thus, the number of BrdU-positive cells was counted accurately. We found asymmetric growth in the mantle epithelium, but not in the mantle edge. While an average of about 8.8 BrdU-positive cells (n=10) were detected on the left-hand side of the mantle epithelium, there was a significantly larger number

(approximately 43.2; *n*=10) of BrdU-positive cells in the right half of the mantle epithelium (figure 1*a*). The mantle edge possessed BrdU-positive cells at similar levels to those in the left side of the mantle epithelium (approximately 7.4: left and 7.9: right; *n*=10 each), and there was no significant difference between left and right sides (figure 1*b*).

We wanted to know whether the Nodal signalling pathway was involved in the asymmetric cell proliferation. As reported for other gastropod species [11], asymmetric expression of *nodal* and *pitx* was observed in *N. fuscoviridis.* The earliest expressions were detected at 8 hpf for both of them, and no expressions detected before 7 hpf (figure 2*a*-*b*). The expressions were retained until 11 hpf, and down regulated by 12 hpf. Thus, asymmetric cell proliferation may not be directly controlled by Nodal signalling. We used the chemical inhibitor SB-431542, a known inhibitor of the TGF-β type I receptor and reported to inhibit the Nodal signalling pathway during early development in the snail [11]. When early trochophore larvae of *N. fuscoviridis* (8 hpf) were treated with SB-431542 until fixation at 27 hpf, torsion was completely suppressed at a concentration of 5 μM (table 1*,* figure 2*c*-*d*). Thus, we examined whether TGF-β signalling contributed to asymmetric cell proliferation in the mantle epithelium of the limpet; we measured cell proliferation activity during torsion (22-25 hpf) in 5 μM

SB-431542 treated larvae from 8 to 22 hpf. We found that the 5 μM SB-431542 treatment in the pre-torsion stage (22hpf) was sufficient to suppress torsion (100%; *n*=28) and resulted in symmetric growth of the mantle epithelium. No activation of cell proliferation was observed in the right-hand side, and cell proliferation occurred at a level comparable to the left-hand side of the control larvae (figure 1*c-i*). No effect was observed on the left-hand side in drug treated larvae (figure 1*e,h*). It is also notable that the retractor muscle apparently developed normally in SB-431542-treated larvae (figure 2*d*).

4. DISUCUSSION

Since the first description of gastropod torsion by Lankester [1], the driving force behind it has been intensively discussed [2, 3, 5-7]. Traditionally, it was widely accepted that torsion is driven by contraction of the larval retractor muscle that rigidly attached to the calcified shell. Wanninger *et al*. reported that muscle contraction indeed occurred during torsion [6]. However, this idea was questioned by recent studies that reported that torsion occurred before the larval shell becomes rigidly calcified [7]. Furthermore, Page reported that torsion still occurred in drug-treated larvae that did not form muscle attachments to the shell [8]. We found that asymmetric cell proliferation

occurred during torsion in the mantle epithelium. High cell proliferation activity on the right-hand side of the mantle epithelium may be a driving force of torsion, by rotating the shell and visceral mass to the left-hand side relative to the velum and foot (figure 2*e*).

We found that torsion was prevented by the TGF-β signalling inhibitor SB-431542, and these drug-treated larvae did not show a high cell proliferation rate on the right-hand side of the mantle epithelium. In a previous report, *nodal* expression of limpet and snail was detected at 2c descant cells [11] which develop into the ectoderm of the right side of the foot and the mantle fold in veliger larval stage [16]. Thus, this expression matches with the region that shows asymmetric cell proliferation. Our report provides evidence that TGF-β signalling (perhaps the Nodal pathway among them) is not only involved in the asymmetry of shell coiling [11], but also in torsion, another aspect of asymmetry, by means of activating asymmetric cell proliferation in the mantle.

Acknowledgements

We thank Ibaraki Prefectural Oarai Aquarium for providing us natural sea water.

References

1 Lankester, E. R.1883 *Mollusca*. In Encyclopedia Britannica, 9th edn. Vol.16. pp. 632-695. Chicago: Werner.

2 Garstang, W. 1928 The origin and evolution of larval forms. *Rep. Brit. Assoc. Adv. Sci. Section D*, 77–98.

3 Crofts, D. R. 1937 The development of *Haliotis tuberculata*, with special reference to the organogenesis during torsion. *Philos. Trans. R. Soc. Lond. B* **228**, 219–268.

4 Verdonk, N. H. 1979 *Symmetry and asymmetry in the embryonic development of molluscs*. In Pathway in Malacology (ed. Van Den Spoel, S., Van Bruggen, A. C. & Lever. J.), pp. 28-54. Utrecht-Deventer: Scheltema & Holkema.

5 Page, L. R. 1997 Ontogenetic Torsion and Protoconch Form in the Archaeogastropod *Haliotis kamtschatkana*: Evolutionary Implications. *Acta zoologica* **78**, 227-245. (doi: 10.1111/j.1463-6395.1997.tb01009.x)

6 Wanninger, A., Ruthensteiner, B., & Haszprunar, G. 2000. Torsion in Patella caerulea (Mollusca, Patellogastropoda): ontogenetic process, timing, and mechanisms. *Invert. Biol.* **119**, 177–187. (doi: 10.1111/j.1744-7410.2000.tb00006.x)

7 Hickman, C. S. & Hadfield, M. G. 2001 Larval muscle contraction fails to produce

torsion in a trochoidean gastropod. *Biol. Bull* **200**, 257–260

8 Page, L. R. 2002 Ontogenetic torsion in two basal gastropods occurs without shell attachments for larval retractor muscles. *Evol. Dev.* **4**, 212-222. (doi: 10.1046/j.1525-142X.2002.02005.x)

9 Bandel, K. 1982 Morphologie und Bildung der frühontogenetischen Gehäuse bei conchiferen Mollusken. *Facies (Erlangen)* **7**, 1-198.

10 Robertson, R. 1993 Snail handness. *Nat. Geo. Res. & Expl.* **9**, 104-119.

11 Grande, C & Patel, N. H. 2009 Nodal signalling is involved in left–right asymmetry in snails. *Nature* **462**, 790-794 (doi:10.1038/nature07603)

12 Kay, C. K. & Emlet, R. B. 2002 Laboratory spawning, larval development, and metamorphosis of the limpets *Lottia digitalis* and *Lottia asmi* (Patellogastropoda, Lottiidae). *Invertebrate Biology* **121**, 11-24. (doi: 10.1111/j.1744-7410.2002.tb00125.x)

13 Deguchi, R. 2007 Fertilization causes a single Ca2+ increase that fully depends on Ca2+ influx in oocytes of limpets (Phylum Mollusca, Class Gastropoda). *Dev. Biol* **304**, 652-663 (doi:10.1016/j.ydbio.2007.01.017)

14 Chen, J. F. *et al.* 2001 The role of the D_2 dopamine receptor (D_2R) in A_{2A} adenosine receptor ($A_{2A}R$)-mediated behavioral and cellular responses as revealed

by A_{2A} and D_2 receptor knockout mice. *Proc. Natl. Acad. Sci. USA* **98**, 1970-1975. (doi: 10.1073/pnas.041542498)

15 Kin, K., Kakoi, S. & Wada, H. 2009 A novel role for dpp in the shaping of bivalve shells revealed in a conserved molluscan developmental program. *Dev. Biol* **329**, 152-166 (doi:10.1016/j.ydbio.2009.01.021)

16 Dictus, W. J. A. G. & Damen, P. 1997 Cell lineage and clonal-contribution map of the trochophore larva of Patella vulgata (Mollusca). *Mech. Dev.* **62**, 213–226. (doi:10.1016/S0925-4773(97)00666-7)

Figures and Tables

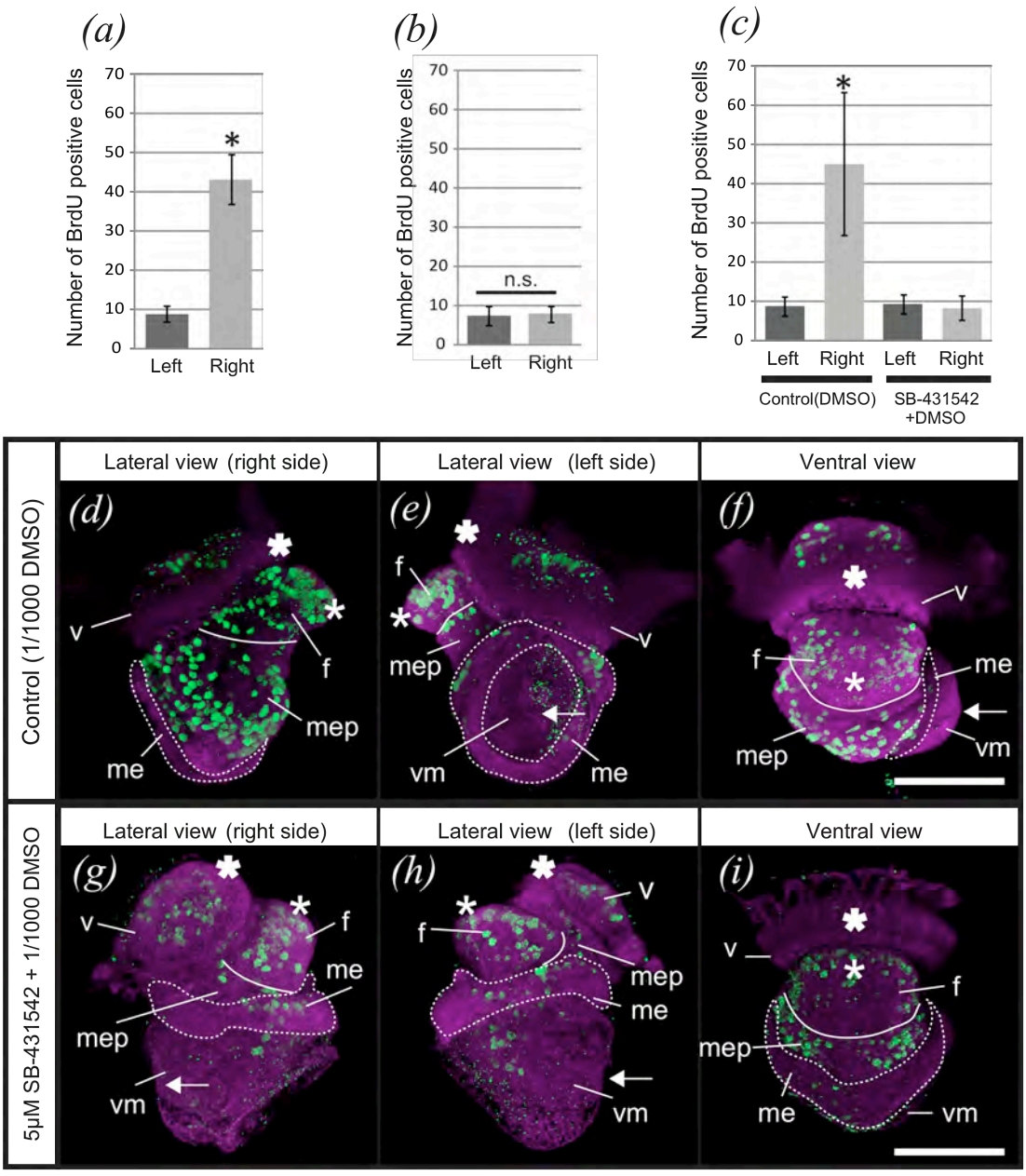


Figure 1. Cell proliferation activity during the first 90° of torsion in *N. fuscoviridis*.

(*a*-*b*) Comparison of the number of proliferating cells during normal development in the mantle epithelium (*a*), and mantle edge (*b*). Mann-Whitney U-test, * P<0.01, n.s.: not

significant (*n*=10). (*c*) Comparison of the number of proliferating cells in 5μM SB-431542 or the same amount (1/1000) of DMSO-treated larval mantle epithelia. Two-way ANOVA followed by Tukey's test; *p<0.01 (*n*=10). (*d-i*) Confocal 3D projection images of BrdU incorporated larva (25 hpf) cultured in DMSO-FSW (*d-f*) and in 5μM SB-431542 in FSW. Anterior towards the top. BrdU-positive cells were shown as green signals and the filamentous actin underlying plasma membrane was shown as magenta. Broken lines indicate the boundary of the mantle edge. The solid lines indicate the boundary between the foot and the mantle epithelium. Asterisks indicate the ventral surface of the foot. Bold asterisks indicate the ventral surface of the velum. Arrows indicate the dorsal midline of the visceral mass. v, velum; f, foot; mep, mantle epithelium; me, mantle edge; vm, visceral mass. Scale bars: 50μm.

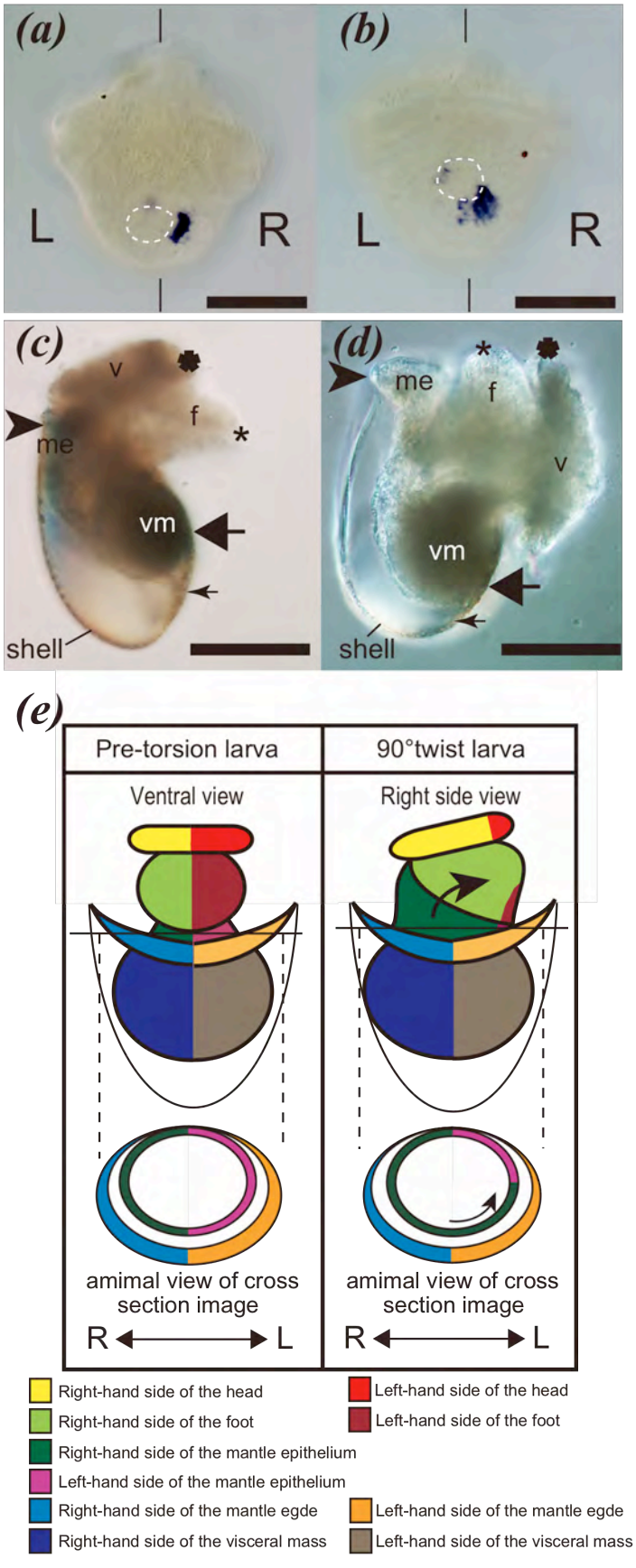
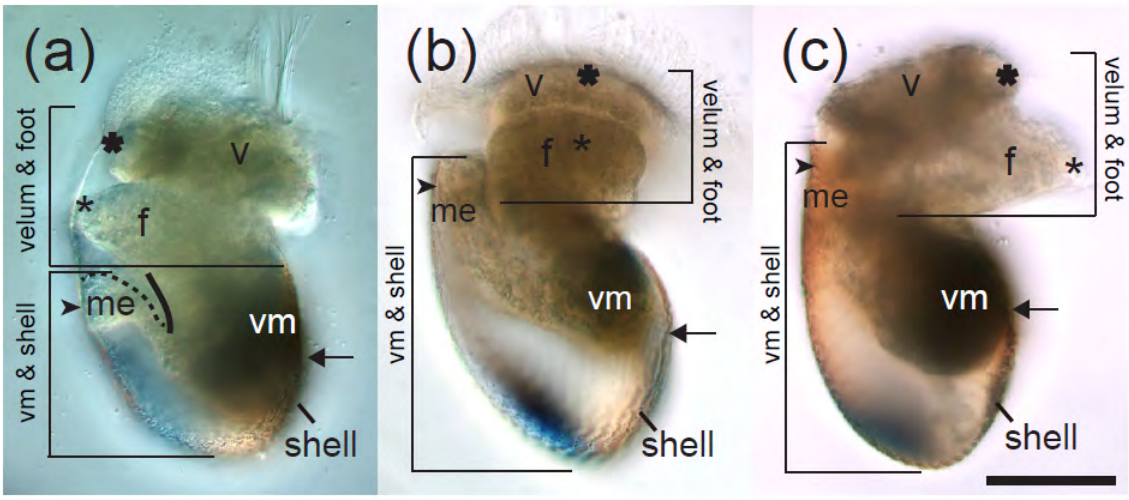

(a)
L
R
(b)
L
R
(c)
v
f
me
*
vm
shell
(d)
me
*
f
v
vm
shell
(e)
Pre-torsion larva
90°twist larva
Ventral view
Right side view
amimal view of cross section image
R
L
amimal view of cross section image
R
L
Right-hand side of the head
Left-hand side of the head
Right-hand side of the foot
Left-hand side of the foot
Right-hand side of the mantle epithelium
Left-hand side of the mantle epithelium
Right-hand side of the mantle egde
Left-hand side of the mantle egde
Right-hand side of the visceral mass
Left-hand side of the visceral mass

Figure 2. *nodal* and *pitx* expression in *N. fuscoviridis* and effect of SB-431542 on torsion, and schematic illustration of asymmetric cell proliferation. Anterior toward the top. (*a*-*b*) Dorsal view of 8 hpf trochophore larvae. L and R indicate left and right sides. (*a*) *nodal* and (*b*) *pitx* were expressed in the right lateral ectoderm adjacent to the shell field. White broken line indicates shell field. (*c*) Control (1/1000) DMSO-treated larva at 27 hpf. Lateral view from the right side. (*d*) 5μM SB-431542-treated larva at 27 hpf. Lateral view from the left side. Small asterisks indicate the ventral midline of the foot, and large asterisks indicate the ventral midline of the velum. Large arrows indicate the dorsal midline of the visceral mass. Small arrows indicate the attachment site of the larval retractor muscle. v, velum; f, foot; me, mantle edge; vm, visceral mass. Scale bars, 50μm. (*e*) Schematic illustration of asymmetric cell proliferation during torsion. Only the right-hand side of the mantle epithelium shows active cell proliferation, which may be the driving force of torsion. The arrow indicates the direction of expansion of the epithelium.

Table 1. Phenotype of *N. fuscoviridis* larva after several concentration SB-431542 treatments

Results of treatment (8-27 hpf)	Control n=52	1μM n=60	2.5μM n=18	5μM n=51	10μM n=26
Normal torsion (%)	100	100	17	0	0
No torsion (%)	0	0	83	100	100



Electronic supplementary material 1. Ontogeny of the torsion in *N. fuscoviridis*.

(a) Lateral view of the left side of pre-torsion veliger larva (22 hpf). Broken line indicates the mantle edge, and bold solid line indicates the mantle epithelium. (*b*) Ventral view of 90° rotation veliger larva (25 hpf). Velum and foot twisted 90° relative to the visceral mass and shell. (*c*) Lateral view of the right side view of post-torsion veliger larva at 27 hpf when 180° torsion completed. Anterior toward the top. Small aterisks indicate the ventral midline of the foot epithelium, and large asterisks indicates the ventral midline of the velum epithelium. Arrow heads point the ventral midline of shell. Arrows indicate the dorsal midline of the visceral mass. v, velum. f, foot. me, mantle edge. vm, visceral mass. Scale bar, 50μm.

Electronic supplementary material 2. The primer sequences for cloning of *Nfnodal* and *Nfpitx*.

*nodal*_F	5'- GTGAAGTTCTGACGAGGAATATCA -3'
*nodal*_R	5'- ATCTACAACCACATTCTGAGGCTAT -3'
*pitx*_F	5'- ACCCATTTTACTAGTCAACAACTGC -3'
*pitx*_R	5'- CTGTAGCATACTGACAAGCTGATA -3'

Electronic supplementary material 3. Detailed method description.

Inhibition of TGF-β signaling was performed using SB-431542 (TOCRIS Bioscience) dissolved in DMSO. Early trochophore larvae were transferred to FSW containing SB-431542 at final concentrations of 1, 2.5, 5, and 10 μM or the same amount of DMSO (diluting 1/500 with FSW) at 8 h post-fertilization (8 hpf) and cultured until fixation at 27 hpf. Treated larvae were collected by pipetting, then were fixed in 4% paraformaldehyde, 0.1 M MOPS (3- [N-morpholino] propanesulfonic acid; pH 7.5), 2 mM EGTA, and 0.5M NaCl for overnight at 4°C and stored in 70% ethanol at -20°C.

Cell proliferation was analyzed using 5-bromo-2-deoxyuridine (BrdU; Roche Applied Science). Larvae cultured in FSW, 5 μM SB-431542, or the same amount of DMSO (diluting 1/1000 with FSW) from 8 to 22 hpf, were transferred to FSW containing 1 μM BrdU (without SB-431542) until fixation at 25 hpf. Treated larvae were collected by pipetting, then ware fixed in 50 mM glycin-HCl, 70% ethanol, pH 2.0 for 40 min at -20˚C. Fixed larvae were processed for immunochemical staining with 1/50 anti-BrdU antibody (Roche Applied Science) diluted with PBT for 1h at 37 ˚C and then incubated overnight at 4˚C in a 1/250 anti-mouse Alexa488-conjugated IgG

(Molecular Probes) diluted with PBT. Specimens were observed after visualizing the filamentous actin underlying plasma membrane using Alexa543-conjugated phalloidin under a confocal laser scanning microscope, as described previously (Kin *et al*. 2009). Because the larvae were fixed in acid and ethanol that damaged filamentous actin component, Alexa543 signal appeared slightly defusing.

Short title: TGF-β signalling in gastropod torsion