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LETTERS

Chiral blastomere arrangement dictates zygotic left–right asymmetry pathway in snails

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Most animals display internal and/or external left–right asymmetry. Several mechanisms for left–right asymmetry determination have been proposed for vertebrates[1–10] and invertebrates[1,2,4,9,11–14] but they are still not well characterized, particularly at the early developmental stage. The gastropods *Lymnaea stagnalis* and the closely related *Lymnaea peregra* have both the sinistral (recessive) and the dextral (dominant) snails within a species and the chirality is hereditary, determined by a single locus that functions maternally[15–18]. Intriguingly, the handedness-determining gene(s) and the mechanisms are not yet identified. Here we show that in *L. stagnalis*, the chiral blastomere arrangement at the eight-cell stage (but not the two- or four-cell stage) determines the left–right asymmetry throughout the developmental programme, and acts upstream of the Nodal signalling pathway. Thus, we could demonstrate that mechanical micromanipulation of the third cleavage chirality (from the four- to the eight-cell stage) leads to reversal of embryonic handedness. These manipulated embryos grew to 'dextralized' sinistral and 'sinistralized' dextral snails—that is, normal healthy fertile organisms with all the usual left–right asymmetries reversed to that encoded by the mothers' genetic information. Moreover, manipulation reversed the embryonic *nodal* expression patterns. Using backcrossed F_7 congenic animals, we could demonstrate a strong genetic linkage between the handedness-determining gene(s) and the chiral cytoskeletal dynamics at the third cleavage that promotes the dominant-type blastomere arrangement. These results establish the crucial importance of the maternally determined blastomere arrangement at the eight-cell stage in dictating zygotic signalling pathways in the organismal chiromorphogenesis. Similar chiral blastomere configuration mechanisms may also operate upstream of the Nodal pathway in left–right patterning of deuterostomes/vertebrates.

Embryonic morphogenesis along the anterior–posterior and dorsal–ventral axes has been well characterized, but that of the left–right axis has only recently begun to be elucidated[1–14]. In some vertebrates, directional nodal flow appears to be important for left–right asymmetry determination[5–8], and in invertebrates such as *Drosophila* and *Caenorhabditis elegans*, the actin cytoskeleton and an associated type I myosin[11,12], and a Gα protein regulating spindle orientation[14], seem to be involved, respectively. However, the initial symmetry-breaking steps are not yet known. An intracellular model of early left–right patterning has been highlighted, where asymmetric gene expression is initiated by oriented cytoskeletal elements[9] and ion flux[10].

We have focused on the snail *L. stagnalis* as a system with unique advantages for studying chiromorphogenesis from the molecular to the organismal level. The correlation of the handedness of the spiral blastomere cleavage with the directions of shell coiling was first proposed based on the observation of sinistral *Physa heterostropha* and dextral *Lymnaea columella*[19]. Indeed clockwise and anticlockwise third cleavage have been observed for the dextral and the sinistral snails within a species of *L. peregra* and *L. stagnalis*, respectively[17,20].

To examine directly the role and timing of blastomere arrangements on shell coiling, we have used micromanipulation to reverse the genetically specified third-cleavage directions in both sinistral and dextral embryos of *L. stagnalis*. At metaphase-anaphase (for dextral snails) or telophase (for sinistral snails) of the third cleavage, we used two glass rods to push the animal surface of each blastomere in the directions opposite to the normal third cleavage (Fig. 1a, d). A constant mechanical force was applied to each cell during furrow ingression until contact between newly formed adjacent pairs of a micromere and a macromere was established (Fig. 1b, e). The manipulation reversed the spindle orientations, shifted the cleavage planes towards the opposite directions, and created the chirality-inverted embryos (Fig. 1c, f and Supplementary Fig. 1). Judging from the blastomere configurations and intercellular contacts, nearly 78% (71/91) of sinistral embryos were successfully reversed to 'dextralized' eight-cell stage embryos and 78% (67/86) of dextral embryos to 'sinistralized' eight-cell stage ones (Supplementary Table 1).

Spiral cleavage is characterized by the alternating clockwise and anticlockwise cleavages during the third to fifth cycles. At 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 dextral (48/67) and sinistral (38/71) embryos exhibited rotation in the opposite sense to their mothers' genetic information, keeping the alternative rotation direction in successive cleavages (Supplementary Fig. 1, Supplementary Table 1). The reversed fourth cleavage direction was clearly seen in the fluorescence imaged cell lineage tracing (Fig. 1k–q, with their bright field image in Fig. 1g–j). 72% (48/67) of the dextral embryos and 54% (38/71) of the sinistral embryos displayed totally inverted blastomere arrangements at the 16-cell stage (Supplementary Table 1). Thus, spindle orientation at spiral cleavage stages is controlled by spatial arrangement of blastomeres that is determined by the previous cell cleavage event.

We further investigated whether the effect of totally inverted blastomere arrangements at the third cleavage continues throughout the whole developmental programme of the snail. We incubated the manipulated embryos in a glass capillary tube with their natural capsular fluid. After about 17 days, 31% (13/42) of the inverted dextral embryos developed into juvenile snails, and remarkably, all of them had the sinistrally coiled shells with completely reversed features (Fig. 2a–c, Supplementary Table 1). 46% (10/22) of the reversed sinistral embryos also developed into juvenile snails, and again they all showed the dextrally coiled shells (Fig. 2g–i, Supplementary Table 1). They can be compared with the normal sinistral (Fig. 2m–o) and the normal dextral (Fig. 2s–u) snails. The juvenile snails were reared to adults (Fig. 2d, j; compare their respective normal snails,

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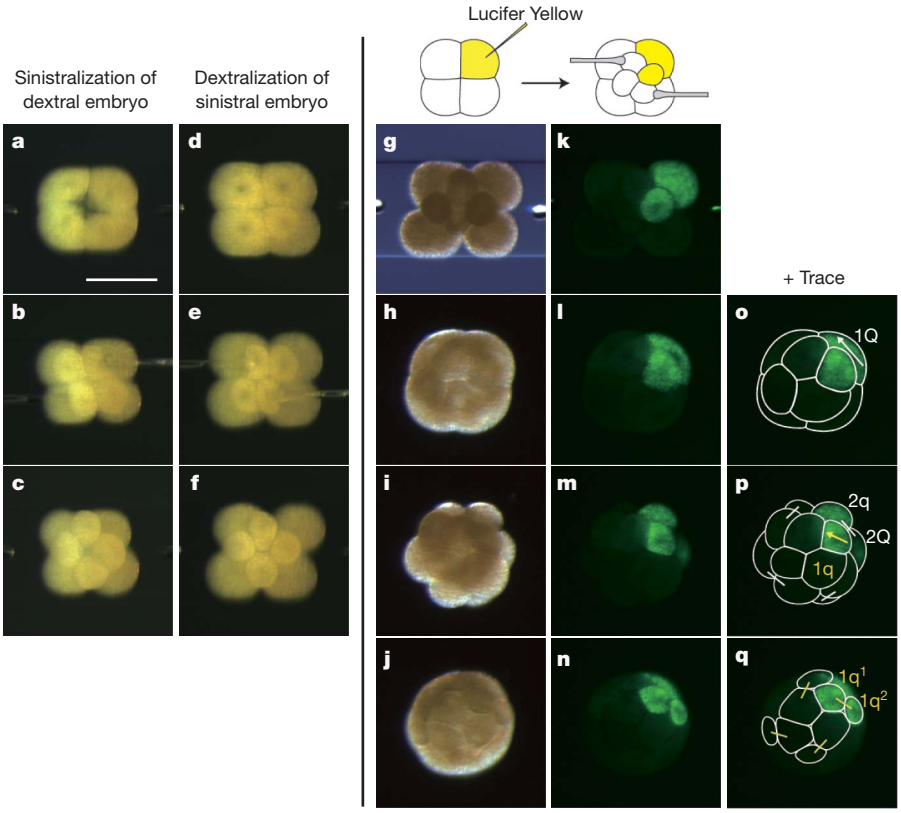

Figure 1 | Reversal of the third cleavage directions by micromanipulation and the resultant 8-, 12- and 16-cell stage embryos. Dextral embryos at the metaphase-anaphase (**a**) and sinistral embryos at the telophase (**d**) of the third cleavage were manipulated. The first quartet of micromeres getting generated was continuously pushed towards the direction opposite to normal by glass rods (sinistrally for the dextral embryo (**b**) and dextrally for the sinistral embryo (**e**)), which resulted in chirality-inverted sinistral-type (**c**) and dextral-type (**f**) eight-cell embryos, respectively. Fluorescence-imaged cell-lineage tracing was carried out by injecting Lucifer Yellow dye into one quadrant of the four-cell stage sinistral embryo, then reversing the chirality by manipulating as in **d**–**f** and culturing them. The resultant dextral-type eight-cell stage sinistral embryo (**g**, **k**) was compacted (**h**, **l**, **o**) and then cleaved into 12- (**i**, **m**, **p**) and 16-cell (**j**, **n**, **q**) embryos, which arose from the typical non-synchronous division of macromeres (1Q) and micromeres (1q). Each blastomere of 1Q (**o**) and 1q (**p**) divided in the dextral-type anticlockwise direction and produced their descendants 2q-2Q (**p**) and $1q^1$-$1q^2$(**q**), respectively. **a**–**j**, Bright field image; **k**–**q**, fluorescence image with outline of blastomeres (**o**–**q**). Arrows (**o**, **p**) indicate the spindle orientation. Scale bar, 100 μm.

Fig. 2p, v) and their internal organ asymmetry was examined in detail. Fully grown 'sinistralized' and 'dextralized' snails had pulmonary sac, anus, male and female genital pores open at the left or right side of the body (Fig. 2e, k), just like the normal sinistral (Fig. 2q) and the dextral (Fig. 2w) snails, respectively, and internal organs, such as heart, stomach, liver coiling and gut looping, with the shape and positions (Fig. 2f, l) just like the normal sinistral (Fig. 2r) and dextral (Fig. 2x) snails, respectively. Thus, the chirality-reversed embryos at the eight-cell stage developed to situs inversus. We did not observe situs solitus or situs ambiguus. The reversed-coiled snails were fertile, and produced sinistral or dextral progenies dictated by their genotype and not the reversed body handedness (Supplementary Table 1).

Although chirality is the most prominent at the third cleavage, it can be traced back to the first and second cleavages[21]. We altered, by manipulation, the directions of blastomere rotations of both the sinistral and dextral embryos at the first or the second cleavage to produce reversed blastomere configuration at the four-cell stage. However, the manipulated embryos all reverted to the original-type third cleavage (Supplementary Fig. 2). We also observed that sinistral embryos occasionally showed dextral-type blastomere arrangement at the four-cell stage even in the egg capsules, but they all showed normal anticlockwise cleavage at the third division. Thus, macromere–micromere cell contacts at the eight-cell stage embryo appear to be the first determining step for asymmetric development of snails.

We have previously reported that dextral and sinistral snail embryos are not mirror images of each other at the third cleavage (refs 20, 22). The dominant dextral snails exhibit spiral deformation (SD) and spindle inclination (SI), while the recessive sinistral snails do not show them[20] (see below). SD is a helical deformation of the blastomeres at the metaphase–anaphase, and SI is a spiral orientation of the four spindles, as a consequence of SD, before the cleavage furrow ingression[20]. We have succeeded in making F_7 congenic animals, which inherit 99.2% of sinistral strain-derived and 0.8% of the dextral strain-derived genome. Remarkably, SD and SI were observed in all the dextral embryos oviposited by F_7 animals that inherit the dextrality gene(s), but not in any of the sinistral embryos oviposited by F_7 snails devoid of the dextrality gene(s). Thus, the organismal handedness-determining gene(s) is strongly linked to, or is, the gene that induces or activates SD and/or SI. We made dextral snails by pushing the micromeres of sinistral embryos from the telophase without SD.

These results suggest that chiral blastomere configuration is the key factor in handedness determination, which is achieved by SD and SI genetically in the wild, and by micromanipulation in our experiments. The epigenetic manipulation reprograms the left–right asymmetry determination most probably by altering blastomere arrangement around the 3D organizer which is specified at the 24-cell stage[23]. In the case of *C. elegans*, it has been reported[13] that mechanical treatment at the six-cell stage produced chirality-reversed animals, similar to the case of *L. stagnalis*. Although spindle orientation is important in both species, *L. stagnalis* appears to adopt a different chirality determining pathway (see below). We have studied the orthologues of Gα and several cell polarity-related proteins (for example, Par6, atypical PKC) for the sinistral and the dextral *L. stagnalis*, but no chirality-dependent difference was observed in their expression (T. Homma, M.S. and R.K., unpublished results).

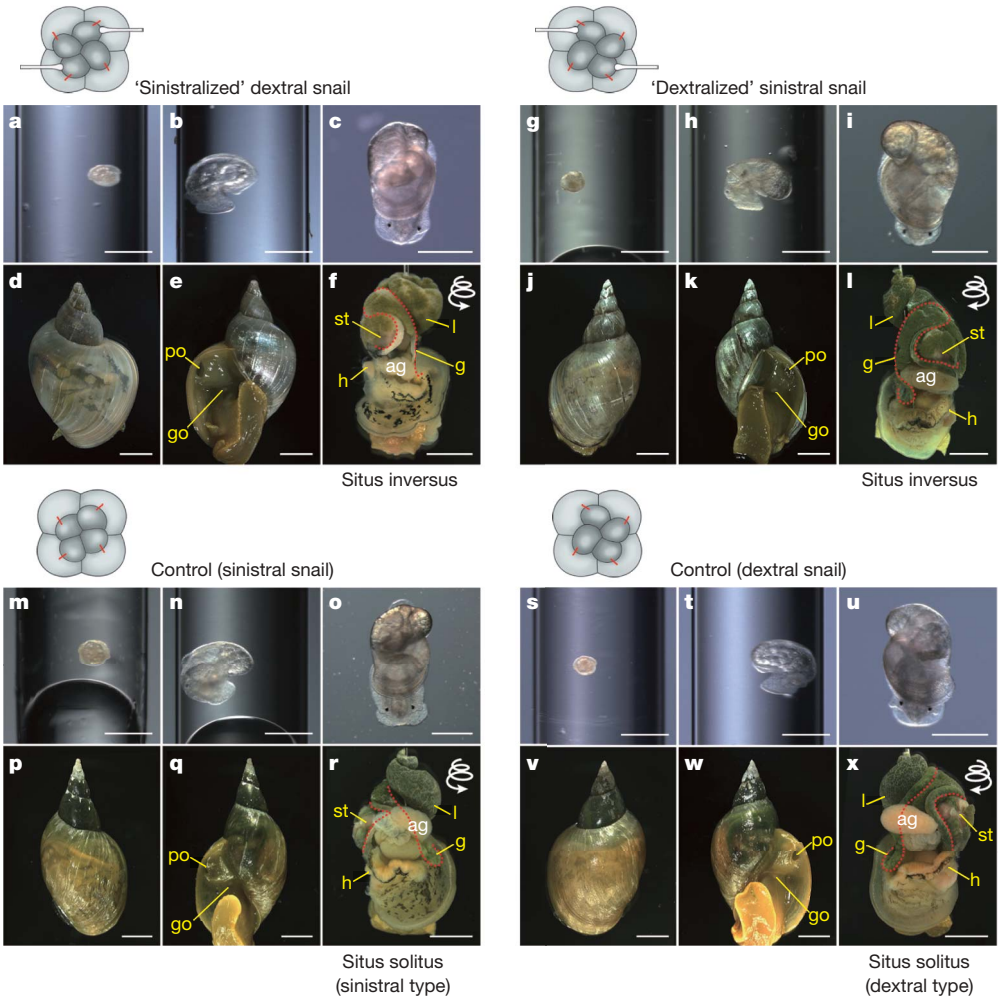

Figure 2 | Chirality-reversed eight-cell stage embryos developed to snails with an oppositely-coiled shell and visceral situs inversus. After the third-cleavage manipulations, both sinistralized dextral embryos (**a**) and dextralized sinistral embryos (**g**) were raised to adult snails. Non-manipulated sinistral (**m**) and dextral (**s**) embryos were also cultured in parallel as control. Development was observed at trochophore (**a**, **g**, **m**, **s**), veliger (**b**, **h**, **n**, **t**) and juvenile snail (**c**, **i**, **o**, **u**) stages. Adult snails were pictured dorsally (**d**, **j**, **p**, **v**) and ventrally (**e**, **k**, **q**, **w**). The shell was removed to observe the position of internal organs (**f**, **l**, **r**, **x**, dorsal view). The sinistralized dextral snails (**a**–**f**) and control sinistral snails (**m**–**r**) are morphologically identical, so are the dextralized sinistral snails (**g**–**l**) and control dextral snails (**s**–**x**). ag, albumen gland; g with dotted red line, gut; go, female genital opening; h, heart; l with white coil, liver; st, stomach; po, pulmonary sac opening. Scale bars: **a**–**c**, **g**–**i**, **m**–**o**, **s**–**u**, 0.5 mm; **d**–**f**, **j**–**l**, **p**–**r**, **v**–**x**, 5 mm.

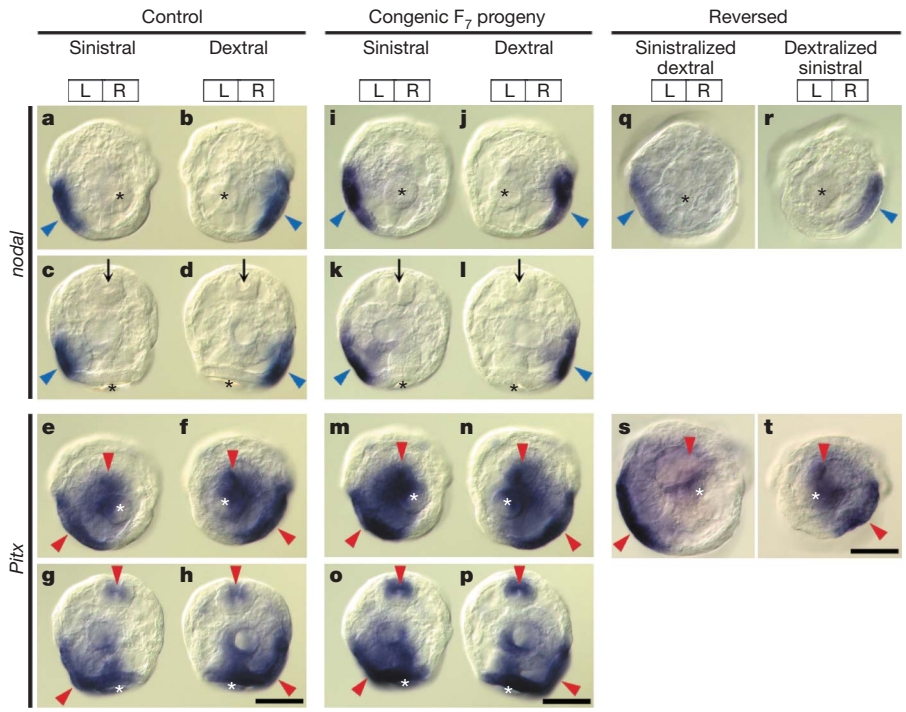

Figure 3 | *nodal* and *Pitx* expression in control, congenic F_7 progeny, and chirality-inverted *L. stagnalis* embryos. L and R indicate left and right sides of late trochophore stage embryos. Pairs of sinistral and dextral embryos were placed side-by-side and pictured in the same field. **a**, **b**, **e**, **f**, **i**, **j**, **m**, **n**, **q**, **r**, **s** and **t** are posterior views, while **c**, **d**, **g**, **h**, **k**, **l**, **o** and **p** are dorsal views. Position of the shell gland is marked by an asterisk, and that of the stomodeum by arrows in dorsal view **c**, **d**, **k** and **l**. *nodal* (blue arrowhead) is expressed in the left lateral ectoderm, near the left side of the developing shell for the sinistral embryos (**a**, **c**), but in the right lateral ectoderm, near the right side of the developing shell for the dextral embryos (**b**, **d**). *Pitx* (red arrowhead) is expressed in the stomodeum, visceral mass, and left or right side of posterolateral ectoderm for the sinistral (**e**, **g**) and dextral (**f**, **h**) *L. stagnalis* embryos, respectively. *nodal* and *Pitx* expressions of embryos of F_7 congenic progeny exhibited similar patterns (**i**–**l** and **m**–**p**) to those of control (parental inbred strain) (**a**–**d** and **e**–**h**). Chirality reversed embryos, that is, sinistralized dextral (**q**, **s**) and dextralized sinistral (**r**, **t**) embryos, showed identical expression patterns to the control (**a**, **e**) and progeny of F_7 (**i**, **m**) sinistral, and the control (**b**, **f**) and progeny of F_7 (**j**, **n**) dextral embryos, respectively. Scale bars, 100 μm.

Asymmetric activation of the Nodal pathway is a conserved feature of deuterostomes for the determination of the asymmetric body plan[24]. The Nodal pathway does not appear to be involved in Ecdysozoa such as flies and nematodes. However, a recent report[25] revealed that the Nodal pathway does operate in gastropods (Lophotrochozoa), as evidenced by the contrasting asymmetric expression of *nodal* and its downstream *Pitx* genes in the sinistral snail *Biomphalaria glabrata* and compared with the dextral snail *Lottia gigantea*. To determine whether left–right asymmetry at the eight-cell stage affects the *nodal* expression pattern, we cloned the orthologues of *nodal* and *Pitx* genes and investigated their expression patterns in non-manipulated and manipulated *L. stagnalis* by whole mount *in situ* hybridization at the late trochophore stage. For the sinistral *L. stagnalis*, *nodal* is expressed in the left lateral ectoderm, near the left side of the developing shell (Fig. 3a, c), whereas it is found in the right lateral ectoderm, near the right side of the developing shell, for the dextral *L. stagnalis* (Fig. 3b, d). Nodal expression was detected first during the 32–64 cell stages (data not shown). Similarly, *Pitx* is expressed in the stomodeum, and visceral mass, and asymmetrically in the left or right side regions of posterior and lateral ectoderm for the sinistral and dextral *L. stagnalis*, respectively (Fig. 3e–h), the sinistral case being similar to the results for *B. glabrata*[25]. Embryos of progenies of sinistral and dextral F_7 congenic snails exhibited asymmetric *nodal* and *Pitx* patterns (Fig. 3i–p) exactly the same as control strains described above (Fig. 3a–h), indicating that the Nodal pathway acts downstream of the handedness-determining gene product(s). Remarkably, when chirality at the eight-cell stage was reversed by micromanipulation, the *nodal* and *Pitx* expression patterns at the late trochophore stage were completely reversed (2/2 for *nodal* and 6/6 for *Pitx*), as is clearly seen in Fig. 3q–t. Thus, the maternally-determined blastomere arrangement at the eight-cell stage dictates the zygotic Nodal signalling pathway.

The features of maternal inheritance of chirality in *Lymnaea*[15,16] and the correlation of shell coiling handedness with the spiral blastomere cleavage are long established[19]. However, the nature of the link between them has remained obscure. In this paper, we show for the first time that the chiral blastomere arrangement at the eight-cell stage, whose cytoskeletal dynamics is directly controlled by the handedness-determining gene(s), dictates the Nodal pathway at the late trochophore stage, leading to the left–right body asymmetries (summarized in Fig. 4). These results indicate that the role of genetically important SD and SI is to achieve the correct micromere–macromere arrangement of dominantly handed snails at the eight-cell stage. Precisely how a particular blastomere arrangement affects the fate of blastomeres and engagement of the Nodal pathway is unknown, as is the mechanism by which chiral memory is transferred through subsequent spiral cleavage cycles. Studies in *Lymnaea* should provide a tractable system in which to answer such intriguing and fundamental questions that have relevance for left–right asymmetry not only for spiralians but also more widely in other more complex organisms.

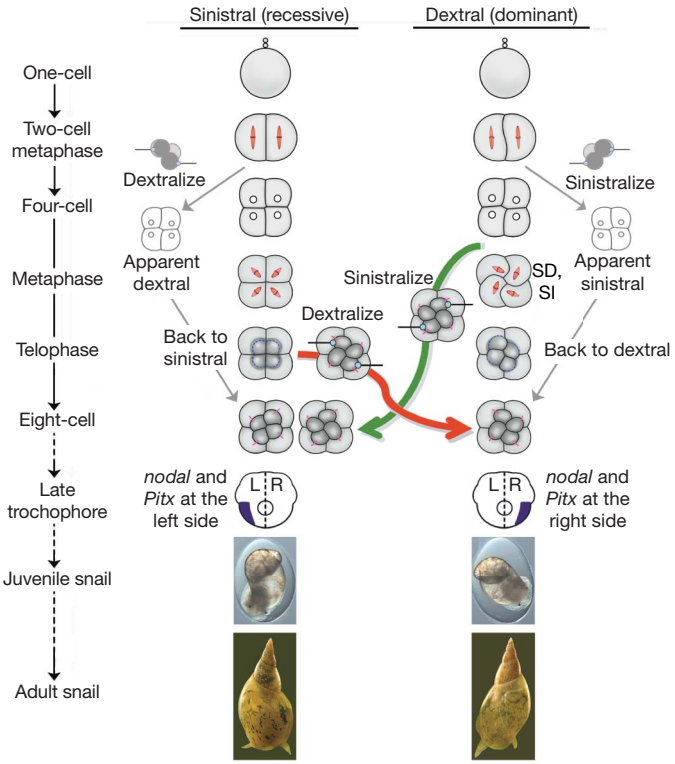

Figure 4 | Determinants of chirality in the snail *L. stagnalis*. This scheme summarizes the development of left- and right-handed snails from the one-cell stage to mature adults. In *L. stagnalis*, reversing the chirality by micromanipulation at the first or second cleavage stage does not alter the organismal chirality, as the manipulated embryos revert to form eight-cell embryos of original handedness (thin arrow). In contrast, embryos whose chirality is reversed by micromanipulation at the third cleavage grow to chirality inverted juvenile and then to healthy and fertile adult snails, with oppositely-coiled shell and situs inversus viscerum (thick arrow). *nodal* and *Pitx* expressions are also reversed by this manipulation. Dextralized snails are produced from sinistral snails without SD (spiral deformation), a unique feature observed only at the third-cleavage metaphase-anaphase of dominant dextral snails, and directly linked to the handedness-determining gene(s).

METHODS SUMMARY

Our original stocks of both the sinistral and the dextral *L. stagnalis* were kindly supplied by G. Smit (Vrijie Universiteit) and have been reared in our laboratory over many years, essentially as described earlier[18]. Micromanipulation of cleavage directions was performed under a stereomicroscope (Leica) with two handmade glass rods controlled by hydraulic micromanipulators (Narishige) while holding embryos in a groove on an agarose slab. Manipulated and non-manipulated embryos were transferred into glass capillary tubes containing natural capsular fluid and were cultured for 2–3 weeks until they had developed into juvenile snails. Juveniles were transferred to small aquaria and reared to adult. For the fluorescence imaging, four-cell stage embryos, after 5 mM DTT treatment, were injected with Lucifer Yellow by using a conventional hydraulic injection system, and the embryos were observed under a fluoro-stereomicroscope (Leica). For double staining of F-actin and microtubules in embryos, Alexa 488-phalloidin (Molecular Probes) and Cy3-conjugated monoclonal anti-β-tubulin antibody (Sigma) were used. DAPI was used for DNA staining. Images were obtained by fluorescence microscopy (Axio Imager M1, Zeiss). Three-dimensional-reconstruction images were made from *z*-series of optical sections acquired every 1.0 μm. Whole mount *in situ* hybridization was performed as described[26] except for the following conditions: snail embryos were fixed with 3.2% formaldehyde in MTSTr (50 mM PIPES-KOH, pH 6.9, 25 mM EGTA, 150 mM KCl, 25 mM $MgCl_2$, 0.1% Triton X-100) overnight at 4 °C. We used BM purple (Roche) instead of NBT-BCIP for the detection of signals.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions R.K. conceived the study, designed/coordinated the experiments and wrote the manuscript. B.E. performed the reversal experiments and whole mount *in situ* hybridization (WISH) on reversed embryos. M.A. performed WISH on control and F_7 congenic snails. M.S. cloned and characterized *nodal* and *Pitx* from *L. stagnalis* to make template vector for the WISH probes. B.E. and M.S. provided comments on the manuscript.

Author Information Sequences of *L. stagnalis nodal* and *Pitx* are deposited at GenBank, with accession numbers respectively GU073383 and GU073384. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to R.K. (ckuroda@mail.ecc.u-tokyo.ac.jp).

METHODS

PCR amplification of Nodal sequence from *L. stagnalis* genomic DNA was accomplished using degenerate primers which were designed using the consensus-degenerate hybrid oligonucleotide (CODEHOP) program available at http://blocks.fhcrc.org/codehop.html. Degenerate PCR primers for amplifying Pitx sequence from *L. stagnalis* trochophore stage cDNA were designed as described[27]. To clone the full-length cDNA for the above genes, 5′ and 3′ RACE PCR was performed using the SMART-RACE kit from Clontech. Probes for *in situ* hybridization were designed using the Probe Search System available at http://probe-search.ccr.tokushima-u.ac.jp.

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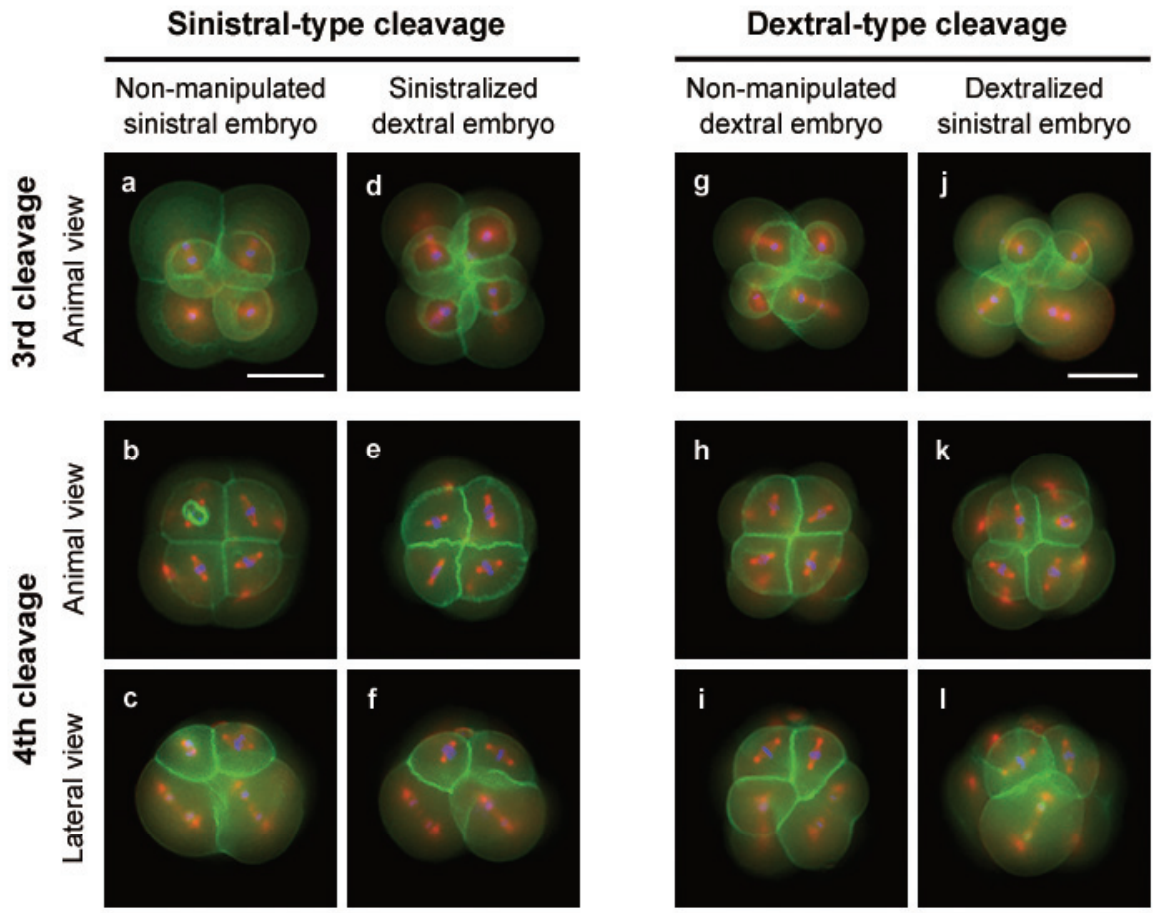
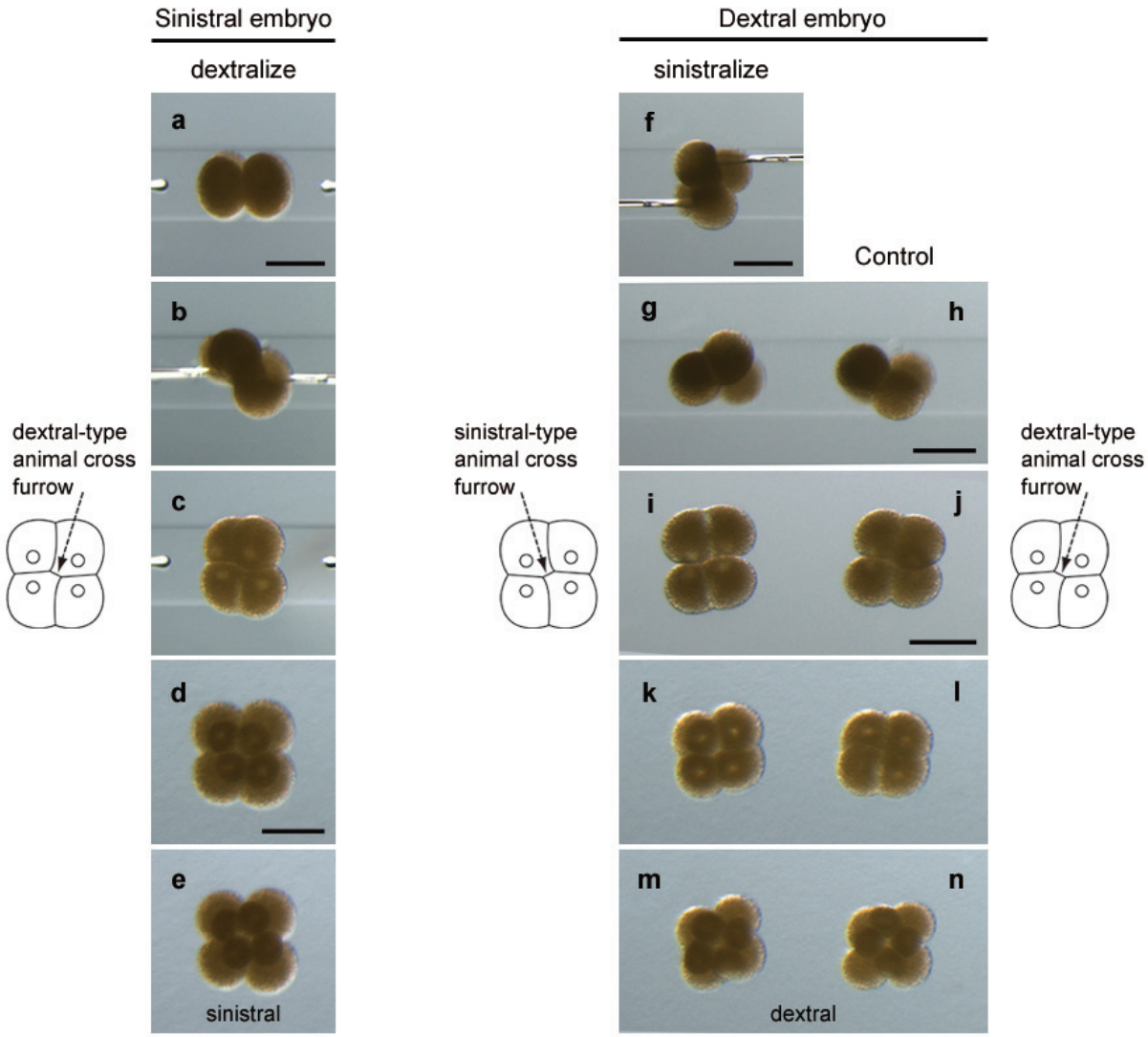
Supplementary Figure



Supplementary Figure 1. Blastomere arrangement and spindle orientation at the third and fourth cleavages for the manipulated and non-manipulated (control) embryos as studied by whole-mount immuno-fluorescence and Alexa 488-phalloidin staining. Microtubules (red), F-actin (green), DNA (blue). Both sinistralized dextral (**d**) and dextralized sinistral (**j**) third cleavage embryos exhibit reversed chirality at the successive 4th cleavage (**e**, **f** and **k**, **l**), exhibiting exactly the same pattern as for the non-manipulated sinistral (**b**, **c**) and dextral embryos (**h**, **i**), respectively. Stages of individual embryos were defined by DAPI staining of DNA: **a**, the onset of telophase of the third cleavage, **d**, **g**, **j**, telophase of the third cleavage, **b**, **e**, **h**, **k**, metaphase of the fourth cleavage of the first micromere quartet, **c**, **f**, **i**, **l**, late anaphase–the onset of telophase of the fourth cleavage of the first macromere quartet. Scale bars denote 50 μm.



Supplementary Figure 2. Manipulation at the second cleavage. Alteration of the animal cross-furrow type at the 4-cell stage by the manipulation at the 2nd cleavage does not change the embryonic handedness permanently and the embryos regain their original chirality during the successive 3rd cleavage. **a**-**e**, dextralized sinistral embryo, **f**, **g**, **i**, **k**, **m**, sinistralized dextral embryos and **h**, **j**, **l**, **n**, control, non-manipulated dextral embryos. Scale bars are 100 μm.

Supplementary Table

Types of embryos	Third cleavage 4-cell to 8-cell				Subsequent fourth cleavage* 8-cell to 16-cell				Subsequent development of cultured embryos**						
	Number of embryos	Manipulation	Direction	Reversed 8-cell	Clockwise (Sinistral-type)		Counterclockwise (Dextral-type)		Number of embryos	Arrested			Survived juvenile snails direction of shell coiling		
					Complete	Partial	Complete	Partial		~Gastrula	Trochophore	Veliger	Dextral	Sinistral	Non-coiled
Dextral	86	reversed	Pushed to counterclockwise	67	48	19	0	0	42	23	1	5	0	13	0
	84	none	Clockwise	none	0	0	78	6	54	33	1	3	15	0	2
Sinistral	91	reversed	Pushed to clockwise	71	0	0	38	33	22	8	1	1	10	0	2
	117	none	Counterclockwise	none	65	52	0	0	45	17	9	2	0	17	0

Supplementary Table 1. Summary of embryo development after manipulation at the third spiral cleavage

*'Complete cleavage' is a normal cleavage where both macromere and micromere quartet divided in proper orientation. On the other hand, some blastomere abnormally divided along or perpendicular to AV axis in 'Partial cleavage' embryos. The ratio of complete cleavage for the non-manipulated sinistral embryos was as low as 55.6% (65/117), suggesting the effect of decapsulation of eggs.

**Embryos were selected for further cultivation from the population which showed complete fourth cleavage. The manipulated embryos were difficult to survive beyond gastrula stage, similar to the non-manipulated snails reared in a similar manner in capillary tubes. From two out of 10 dextralized snails produced sinistral progenies by self-fertilization. From one out of 13 sinistralized snails produced dextral progenies by self-fertilization.