Molluscan dorsal-ventral patterning relying on BMP2/4 and Chordin provides insights into spiralian development and bilaterian body plan evolution

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Abstract

The molecular mechanisms of dorsal-ventral (DV) patterning in Spiralia are poorly understood. The few available studies indicate that derived DV patterning mechanisms occurred in particular spiralian lineages and likely were related to the loss of Chordin gene. Here, a functional study of the first spiralian Chordin showed that BMP2/4 and Chordin regulate DV patterning in the mollusk Lottia goshimai, thus revealing the first spiralian case that retains this conserved mechanism. We then showed that Chordin but not BMP2/4 transferred the positional information of the D-quadrant organizer to establish the BMP signaling gradient along the presumed DV axis. Further investigations on the molluscan embryos with influenced DV patterning suggested a role of BMP signaling in regulating the organization of the larval nervous system and indicated that the blastopore localization is correlated with the BMP signaling gradient. These findings provide insights into the evolution of animal DV patterning, the unique development mode of spiralians driven by the D-quadrant organizer, and the evolution of bilaterian body plans.

Keywords: Dorsal-ventral, mollusk, organizer, BMP, Chordin
The existence of a dorsal-ventral (DV) axis is a key character in Bilateria, the animals show a bilateral body plan and account for the majority of the animal kingdom (e.g., insects, mollusks and vertebrates). Generally, a conserved molecular logic, namely the BMP ligand BMP2/4 and its antagonist Chordin, patterns the DV axis of bilaterians (1-6). It is suggested that these two genes even pattern a body axis in non-bilaterian animal lineages (Cnidaria (7) and Placozoa (8)), indicating broad conservation. However, despite such conservation, the DV patterning mechanism exhibits a considerable degree of variations (9). In extreme cases, the DV patterning no longer depends on BMP2/4 and Chordin. In two of the three major bilaterian clades, Ecdysozoa and Deuterostomia, such exceptional cases (e.g., nematodes and ascidians (10, 11)) are considered lineage-specific characters since extensive evidence reveals BMP2/4-Chordin-dependent mechanisms in their relatives (e.g., insects and vertebrates (9, 12)).

The situation in the other bilaterian clade, Spiralia, is very different. Unlike those in ecdysozoans and deuterostomes, the molecular mechanisms of spiralian DV patterning remain largely elusive. The study in a leech annelid (Helobdella robusta) reveals that its DV patterning does not use BMP2/4 and Chordin (but rather BMP5-8 and Gremlin) (13). Researchers propose that Helobdella DV patterning may be a derived character due to the lack of Chordin gene (13). In another spiralian, the platyhelminth Dugesia japonica, although BMP2/4 is revealed to regulated DV patterning, it is unknown whether Chordin is involved (14). In fact, Chordin may also have been lost in platyhelminthes since a subsequent study retrieves no Chordin homolog from several platyhelminth transcriptomes (15). Together, current studies using two spiralian representatives reveal quite unusual characters of DV patterning mechanisms in this animal clade, which likely involve the lack of the Chordin gene (Fig. 1a).

Although the ancestral BMP2/4-Chordin-dependent DV patterning mechanism has been generally accepted for bilaterians (16), the unusual characters in spiralian
DV patterning raises a caveat that some spiralian lineages, or perhaps all of them, may have lost this character (indeed, the Chordin gene was also not retrieved from some rotifers (15)). Nevertheless, Chordin does exist in some spiralian lineages (e.g., mollusks) (15). Molluscan Chordin is further revealed to be expressed on the opposite side to BMP2/4 expression along the DV axis (17), indicating a function in DV patterning. While the roles of BMP2/4 in molluscan DV patterning has been revealed (18), those of Chordin remain unknown. Thus, mollusks provide an ideal opportunity to explore whether the conserved BMP2/4-Chordin-dependent DV patterning mechanism has been retained in any spiralian (Fig. 1).

The study on molluscan DV patterning is also essential to decipher the unique development mode of spiralian that relies on the D-quadrant organizer. The spiralian organizer refers to a special blastomere that regulates the development of the whole embryo (e.g., 3D or 4d, according to the nomenclature for describing the spiral cleavage; see Fig. 1b) (19-21). Broadly observed in various spiralian but not in other animal lineages and showing high conservation in cell lineages and developmental functions, the organizer is considered a key to understanding spiralian development and evolution (22). With the deepening of the knowledge of the spiralian organizer (23-26), a link between organizer and BMP2/4 has been established by proving that BMP2/4 mediates the organizer signaling (18), which explains the conserved organizer function to induce DV patterning. Nevertheless, it is notable that the crucial step in DV patterning (i.e., the organizer function) is not the BMP ligand itself but the gradient of BMP signaling along the presumed DV axis (12). Given that the spatial distributions of BMP signaling are largely determined by various extracellular BMP regulators (27, 28), despite the involvement of a BMP ligand in organizer function, it is still to be elucidated how the BMP signaling gradient forms under the regulation of organizer (which would involve BMP regulators). In DV patterning, the primary extracellular BMP regulator is Chordin, which can determine the BMP signaling gradient even irrespective of BMP2/4 expression patterns (9). Given the conserved
roles of spiralian organizer in inducing DV patterning, it is reasonable to hypothesize that Chordin would be an essential missing node to understand organizer function. Thus, it is necessary to explore whether Chordin also participates in mediating organizer signaling and conversely whether Chordin function is regulated by the organizer.

Together, molluscan Chordin represents a key molecule to the understanding of two essential questions: the conservation and plasticity of animal DV patterning (Fig. 1a) and the molecular network underlying the unique spiralian D-quadrant organizer (Fig. 1b). Here, we investigated the roles of BMP2/4 and Chordin in a mollusk (the limpet *Lottia goshimai*) and confirmed that the two genes both regulated DV patterning and participated in organizer function. By further examining the embryos with influenced DV patterning, we revealed evidence suggesting the profound effects of the stereotype spiral cleavage, the roles of BMP signaling in regulating the organization of nervous system, and the correlation between blastopore localization and BMP signaling gradient. These findings provide insights into the unique developmental mode of spiralian and the evolution of bilaterian body plans.

![Fig. 1 Chordin represents a key missing node to understand the evolution of animal DV patterning and spiralian organizer function.](image)

*a. Conservation of animal DV patterning mechanisms.* In most major animal clades, BMP2/4 and Chordin determine a body axis (it is the DV axis for bilaterians). For spiralian, however, the knowledge is elusive and some results argue...
against this conserved mechanism (e.g., in the leech and platyhelminth, highlighted by the blue and gray letters). In this context, mollusks provide an ideal opportunity to explore the conservation of spiralian DV patterning since a Chordin gene is retained in their genome (the red arrow).

b. **Unique spiralian developmental mode relying on a D-quadrant organizer.** The upper diagram shows a representative molluscan embryo (lateral view with animal pole to the top), in which the organizer (org.) is formed at the 32-cell stage that regulated the subsequent development of many tissues. After its formation, the organizer is suggested to activate BMP signaling by regulating BMP2/4 (green letters). However, whether Chordin is involved in this process remains unknown (red letters). The lower diagram shows a veliger larva of gastropod mollusks, and the processes regulated by the organizer are highlighted: DV patterning (generally indicated by the dashed line) and organogenesis (purple letters).

**Results**

**BMP2/4 and Chordin regulated DV patterning in L. goshimai**

Sequences of BMP2/4 and Chordin were retrieved from the developmental transcriptome of *L. goshimai*. We found that as in a bivalve mollusk (17), BMP2/4 and Chordin were expressed dorsally and ventrally, respectively, when the DV axis was established in early embryos of *L. goshimai* (Fig. 2a-e). We then used specific morpholinos (MOs) to inhibit gene translation and explore the roles of the two genes. We found that inhibiting either one of the genes caused the loss of DV axis and radialized early development. As shown in Fig. 2f-i, in normal embryos at 6 hour post fertilization (hpf), though the gastrulation was still ongoing, a dorsal marker gene (GATA2/3, expressed in the shell field) and a ventral marker gene (SoxB, expressed in the ventral plate (29)), as well as BMP2/4 and Chordin themselves, showed asymmetrically dorsal or ventral expression. In contrast, in the embryos with BMP2/4 or Chordin knockdown, despite significant differences between the two phenotypes (supplemental Fig. S2), expression of these genes generally exhibited a radial pattern (Fig. 2f''-i'', f'''-i''). Treatment of early embryos using a recombinant human BMP4 protein (rhBMP4) also generated a similar radialized phenotype (supplemental Fig.
The radial gene expression resembles the phenotypes seen in various animals when inhibiting DV patterning genes (3, 6, 30), suggesting that *L. goshimai* uses BMP2/4 and Chordin to pattern the DV axis.

**Fig. 2** BMP2/4 and Chordin expression and gene knockdown phenotypes. **a.** DV axis of *L. goshimai* could be traced back to the 32-cell stage when a particular blastomere, the organizer (3D), was formed (see supplemental Fig. S1 for details). At 8 hpf, with the development of dorsal (shell field, sf) and ventral tissues (ventral plate, vp), the DV axis is well established, which is related to the orientation of the 3Q blastomeres. **b-e.** Normal expression of BMP2/4 and Chordin at 8 hpf, which are on the dorsal and ventral side, respectively. **f-i.** Gene expression in early embryos (6 hpf) after knockdown of BMP2/4 or Chordin, including the two genes themselves and two other genes used as dorsal (GATA2/3) or ventral (SoxB) markers. All panels are posterior views. After knockdown of BMP2/4 or Chordin, these genes show radial expression in general. The inserts in panels **f’’** and **h’** show anterior views since the gene expression was mainly detected in the pretrochal region. See more details in supplemental Fig. S2. bp, blastopore; pt, prototoch; sf, shell field; vp, ventral plate. Bars represent 50 μm.
**Chordin transferred the breakdown-of-symmetry signal from the organizer**

By revealing the roles of *L. goshimai* BMP2/4 and Chordin in DV patterning, we next asked their roles in organizer function (especially for Chordin). For *L. goshimai*, we determined that its organizer was 3D that formed at the 32-cell stage (supplemental text and Fig. S1a). We then found that the Chordin mRNA expression and the distributions of BMP signaling showed a strong correlation with the organizer (Fig. 3a-h and supplemental Fig. S4). In brief, we detected sequential developmental events from 32- to 64-cell stage: organizer formation at the 32-cell stage, activation of BMP signaling throughout the embryo at the late 32-cell stage (Fig. 3f), transition of Chordin expression into an asymmetrical manner since the 32-to-40-cell stage (Fig. 3c), and formation of a gradient of BMP signaling since the 52-cell stage (marking a molecular DV axis) (Fig. 3h). Notably, the asymmetrical Chordin expression was the first sign of symmetry breakdown along the presumed DV axis at the molecular level (the arrow in Fig. 3c, while the formation of organizer is the first sign of asymmetry at the cellular level). For BMP2/4, its mRNA expression generally showed a radial pattern and did not change significantly during the period investigated (supplemental Fig. S4a-d).

The correlation between organizer formation and the dynamics of Chordin mRNA and BMP signaling suggests regulatory relationships. We first confirmed that the organizer formation triggered BMP signaling. When the organizer formation was inhibited by the MAPK inhibitor U0126 (as described previously (23)) (Fig. 3j), the activation of BMP signaling was prevented (Fig. 3o). We then demonstrated that the activation of BMP signaling after organizer formation was mostly mediated by BMP2/4 because injecting an antisense BMP2/4 MO largely eliminated pSmad1/5/8 staining (Fig. 3q), while it did not influence with the organizer formation (Fig. 3l). Notably, the regulation of BMP2/4 by the organizer should be at the post-transcriptional level since no significant change in BMP2/4 mRNA expression was detected before and after organizer formation (supplemental Fig. S4a-d) or after
U0126 treatment (supplemental Fig. S5h). On the other hand, the BMP signaling that was activated by organizer formation (through regulating BMP2/4) only showed an universal distribution initially. We proved that Chordin was required to transit this universal distribution to a gradient manner. When Chordin was inhibited by injecting an antisense MO, no gradient formed, and the universal BMP signaling sustained in subsequent developmental stages (till at least 64-cell stage, Fig. 3r), despite the normally formed organizer (Fig. 3m).

These results suggest that the key to form the BMP signaling gradient is the asymmetrical expressed Chordin. We found that the formation of such asymmetrical Chordin expression pattern was regulated by the organizer: when the organizer formation was inhibited, symmetrical Chordin expression was no longer interrupted (supplemental Fig. S5). On the other hand, since inhibiting organizer formation did not eliminate Chordin expression, the regulation of Chordin expression by the organizer seems to restrict to generating an asymmetrical pattern, but not maintaining the basic expression.

Based on these results, we conclude the regulatory relationships among organizer, BMP2/4, Chordin, and BMP signaling (Fig. 3s-v). When the 32-cell embryo initially forms, the four macromeres (3M) are equivalent (Fig. 3s). One of them is subsequently induced to be the organizer (3D) due to the establishment of direct contacts with micromeres at the animal pole (31), MAPK signaling is then activated in this blastomere (23) (highlighted with purple in Fig. 3t). The organizer then triggers BMP2/4 (green arrows in Fig. 3t) that induces universal BMP signaling activities (Fig. 3u), which should be on the post-transcription level. Meanwhile, still under the regulation of organizer, Chordin expression becomes asymmetrical and is distributed on the opposite side to the organizer. This asymmetry expressed Chordin modulates the BMP signaling to form a gradient along the presumptive DV axis (Fig. 3v). In summary, under the regulation of the organizer, BMP2/4 and Chordin coordinate to generate the correct BMP signaling gradient to regulate DV patterning:
BMP2/4 activates the signaling and Chordin determines the spatial distribution (gradient) of the signaling. From this point of view, Chordin is the key molecule to transfer the breakdown-of-symmetry signal from the organizer to form the secondary body axis.
**Fig. 3 Regulatory relationships among the organizer, BMP2/4 and Chordin.** a-h. Vegetal views, Chordin expression (a-d) and the state of BMP signaling that is indicated by phosphorylated Smad1/5/8 (pSmad1/5/8) staining (e-h, confocal projections) from 16- to 60-cell stage. The arrow in c indicates the weakened Chordin expression in the organizer (3D) at the late 32-cell stage. More details are provided in supplemental Fig. S4. i-r. the states of organizer (i-m) and BMP signaling (n-r) under different manipulations, all samples at the 60- or 63-cell stage. In i-m, the organizer is identifiable based on the characteristic 4-cell arrangement at the vegetal pole (see supplemental Fig. S1a), and whether an organizer was formed is indicated by “yes” or “no” in the panels. n-r. Diagrams showing pSmad1/5/8 staining along the 3B-3D axis (lateral views with the animal pole to the top). Both animal (ani., n'-r') and vegetal (veg., n''-r'') views are shown. s-v. A hypothesis assuming regulatory relationships among organizer, BMP2/4 and Chordin (see details in the text).

**Asymmetrical development after BMP2/4 or Chordin knockdown: the profound effect of the stereotype spiral cleavage**

Due to organizer formation, the divisions of 3rd-quartet micromeres (3q) were different: larger 3a/b on the 3B side and larger 3c/d on the 3D side, establishing a 3B-3D polarity at the 60-cell stage (Fig. 4e). We found that such 3D-3B polarity was not altered after BMP2/4 or Chordin knockdown (Fig. 3l, m). This stereotype cleavage pattern indicates that even the BMP signaling was influenced, the 3D-3B polarity may still affect the embryonic development. This idea was supported by the result that although inhibition of BMP2/4 or Chordin both caused radial development in early embryos (Fig. 2), the radial development was broken down in subsequent development (see below). For clarity, in the following text, we will describe the orientation of the manipulated embryos based on the locations of 3Q (e.g., the 3B or 3D side) since the normal DV patterning was influenced. In normal embryos, the 3D and 3B sides correspond to the dorsal and ventral sides, respectively (Fig. 2a).

For the BMP2/4 morphants, although the radial development largely sustained (supplemental Fig. S6e-h), minor asymmetry was detected (e.g., polarized Chordin
expression, see supplemental Fig. S6g); we could not determine the direction of the asymmetry. For the Chordin morphants, the asymmetrical development in late developmental stages was much more evident (Fig. 4a-d and supplemental Fig. S6j-l). Such asymmetrical development occurred along the 3B-3D axis (Fig. 4a-d), which was consistent with the 3B-3D polarity of early embryos (see supplemental Fig. S7 for the details to determine the orientation of the manipulated embryos). In particular, in the posterior part of the embryo, the 3D side showed much greater development than the 3B side, which showed expression of ventral marker genes (red arrows in Fig. 4a-d).

The 3D-side tissues were further divided into two bilateral lobes in the Chordin morphants (arrowheads in Fig. 4a’-d’), exhibiting a pseudotwin phenotype. Most of these embryos did not develop discriminable posttrochal organs (Fig. 4g and supplemental Fig. S8, contrasting with the normal veliger larva shown in Fig. 4f), except that a shell was developed occasionally. However, in a brood among over hundreds of replicates that we performed, duplicated shells were developed in two of 41 recorded larvae (highlighted by arrows in Fig. 4h), indicating that the pseudotwin phenotype have the potential to develop duplicated structures (at least duplicated shell fields). The development of duplicated shells, however, never reemerged when we repeated the experiments using varied MO concentrations or a different MO, indicating a very special genetic background of that brood. Nevertheless, despite the poor reproducibility, we think it is necessary to describe this duplicate-shell phenotype (probably the first report of gastropod larvae with duplicated shells, each shell of which even showing a spiral shape; see Fig. 4h) since it may provide insights into DV patterning, shell formation and their relationships.
Fig. 4 Late embryos of Chordin morphant showed asymmetric development along the 3B-3D axis and a pseudotwin phenotype. The orientation of the embryos is described based on the 3rd-quartets of early embryos (3A-3D, see Fig. 2a). a-d show 3A-side views and a'-d' show 3B-side views of the Chordin morphants. Due to the differential development on 3B and 3D sides, the expanded 3D-side tissues constitute the majority of the posterior embryo (red arrows in a-d), in which the expression of ventral marker gene Chordin (c) and SoxB (d) was detected. These tissues are further divided into two bilateral lobes (arrowheads in a'-d') and exhibit a pseudotwin phenotype. The diagram in e shows the asymmetry on 3B and 3D sides at the cellular level recognizable in early embryos (3B-3D polarity; highlighted by green and blue colors). Panel f shows a normal veliger larva. The Chordin morphants developed no discriminable posttrochal structures (g), while duplicated shells were observed in rare cases (arrows in h). The bars represent 50 μm.
Effects of BMP signaling on neurogenesis in *L. goshimai*

There has been extensive evidence indicating the roles of the molluscan organizer in eye development, with some inconsistency among studies (24, 32-34). A recent study placed eye development into the context of neurogenesis and suggested a positive role of BMP signaling in neurogenesis of a mollusk (the gastropod *Ilyanassa*) (18). This effect, however, is opposite to nearly all other animals (35). We therefore investigated the relationship between BMP signaling and eye development/neurogenesis in *L. goshimai*. We found that, consistent with previous reports (18, 32, 33), BMP signaling (i.e., the inductive signals from the organizer) promoted eye development in *L. goshimai*: no eye formed after BMP2/4 knockdown, and extra eyes formed under hyperactive BMP signaling when Chordin was inhibited (Fig. 5a-c and supplemental Fig. S9). rhBMP4 treatment also generated similar phenotypes (although a dose-dependent effect was indicated; see details in supplemental Fig. S3), which are comparable to that of a previous study (18).

However, subsequent analyses of neural marker genes did not support the proposal (18) that BMP signaling promotes neurogenesis. Although Chordin inhibition expanded the expression of the neural patterning gene Pax6 in a portion of larvae (the arrow in Fig. 5f), the Pax6 expression in the Chordin-knockdown larvae exhibited considerable heterogeneity (Fig. 5f, i); thus, it was difficult to conclude a general pattern. Moreover, after BMP2/4 knockdown, Pax6 expression did not show the expected downregulation (Fig. 5e, h). Therefore, Pax6 expression in the manipulated larvae did not suggest whether neurogenesis is promoted or inhibited by BMP signaling. Although the expanded Pax6 expression in BMP4-treated *Ilyanassa* larvae is considered an indicator of promoted neurogenesis (18), this result can also be interpreted to reflect the development of extra eyes given that the *Ilyanassa* Pax6 expression was mostly detected in the pretrochal region at the stage examined (18) and that we found that the Pax6 expression in the pretrochal region showed an apparent
correlation with the distributions of larval eyes (when BMP signaling was activated, compare Fig. 5c and f).

Given that Pax6 might be not an appropriate marker for the overall neurogenesis (it might only contribute to the development of subpopulations of neural tissues) and that the conserved roles of BMP signaling in neurogenesis may be detectable only in the early phase of neurogenesis (18), we analyzed two additional marker genes: Elav, a universal neuron marker (36), and SoxB, which plays essential roles in the early phase of neurogenesis (37). Elav expression was similar to that of Pax6 (Fig. 5j-l), and it is difficult to conclude whether neurogenesis is inhibited or promoted in any group. For SoxB, we focused on its expression at the early gastrula stage (4 hpf) when gastrulation is just beginning. Neurogenesis should be in the early phase at this stage (featuring processes, such as definition of the neuroectoderm and commitment of neural stem cells). The results (Fig. 5m-o) showed that although SoxB expression indeed changed after inhibition of BMP2/4 or Chordin (e.g., it evidently expands in the pretrochal region after BMP knockdown, as shown in Fig. 5n’), it was not highest after BMP2/4 knockdown or lowest after Chordin knockdown, which also did not indicate whether BMP signaling inhibits or promotes neurogenesis in L. goshimai.

Instead of indicating positive or inhibitory effects, our results suggest that BMP signaling seems to be irrelevant to neurogenesis per se but affects the organization of nervous system. As revealed by of both Pax6 and Elav expression, a common phenotype after BMP2/4 or Chordin knockdown was the loss of featured bilaterally distributed neural tissues (Fig. 5g-l). Indeed, we found that although SoxB expression showed a fully radial pattern under the high-dose rhBMP4 treatment (Fig. 5p), a bilateral pattern was restored when the treatment was weaker (Fig. 5q).
Fig. 5 Effects of BMP signaling on eye formation and neurogenesis. a-c. Anterior views showing the eyes of 48-hpf larvae; a summary is shown in supplemental Fig. S9. d-o. Expression of the neural markers Pax6 and Elav in larvae (15-16 hpf) and SoxB expression in early gastrulae (4 hpf). For SoxB expression, animal (ani., \( m'\)-o') and vegetal (veg., \( m''\)-\( o''\)) views are also provided. Since Pax6 and Elav expression after Chordin knockdown shows relatively high levels of heterogeneity, representative larval phenotypes are provided (f, i and l), and it is not possible to provide the number of individuals. The pretrchal Pax6 expression shows apparent correlations with eye distribution (compare c and f), however, there is not a one-to-one relationship between
the two panels. pt, prototroch. p and q show SoxB expression after different doses of rhBMP4 treatment (posterior view, see more information in supplemental Fig. S3). The bars represent 50 μm.

*Posteriorized blastopore: correlation between BMP signaling and the localization of digestive opening*

After knockdown of either BMP2/4 or Chordin, we found that the blastopore location of *L. goshimai* embryo became posteriorized (despite minor polarity caused by the asymmetrical development) (Fig. 6b-c), showing sharp contrast with the normal blastopore formed ventrally (Fig. 6a). The posteriorized blastopore was confirmed by the expression of the blastopore marker gene FoxA (Fig. 6d-f). For *L. goshimai*, the blastopore forms as a consequence of extensive cell movements during gastrulation (mainly epiboly, similarly to *Patella* (38, 39)). The changes in blastopore location in these manipulated embryos indicate altered cell movement during gastrulation. We did not obtain sufficient information to elucidate how gastrulation changed after gene knockdown. Nevertheless, despite these uncertain details, it seems safe to conclude that a correlation occurred between the BMP signaling gradient and the ventral localization of the blastopore. We found that the elimination of the BMP signaling gradient, either by inhibition of signaling (through BMP2/4 knockdown) or generation of a universal distribution (through Chordin knockdown), caused posteriorization of the blastopore (Fig. 6g-i). The blastopore localization in one side (ventral) of the larval body is likely determined by the direction of the BMP signaling gradient.
**Fig. 6 Changes in blastopore location after manipulation of BMP signaling.** Locations of the blastopore (white arrows in a-c) are very different in a normal embryo (a, ventral) and manipulated embryos (b and c, posterior), which show consistency in the expression of the blastopore marker FoxA (d-f). The diagrams in g-i show the correlations between the BMP signaling gradients and the blastopore locations (blue stars): when the BMP signaling gradient is eliminated, the blastopore forms posteriorly (h and i). The bars represent 50 μm.

**Discussion**

As a key process of early embryonic development and a crucial innovation at the origin of bilaterian evolution, DV patterning affects many aspects of animal development and is essential to understanding animal evolution. Moreover, the spiralians evolve a unique developmental mode, in which the DV patterning is deeply integrated into an organizer-driving ontogenesis, which further emphasizes the importance of DV patterning in these animals. Using a representative spiralian mollusk, we revealed evidence essential to understanding spiralian development and animal evolution. Fig. 7 summarizes the major findings of the present study.
Fig. 7 Schematic diagram showing the major findings of the current study. Focusing on the roles of BMP2/4 and Chordin in the mollusk *L. goshimai*, we revealed the roles of the two genes (i.e., BMP signaling) in DV patterning and organizer function. A close look in the manipulated embryos revealed evidence regarding the effects of stereotype spiral cleavage (asymmetrical development after knockdown of BMP2/4 or Chordin), the roles of BMP signaling in regulating neurogenesis and the correlation between blastopore localization and the BMP signaling gradient. There results provide insights into the conservation and plasticity of animal DV patterning (a), the unique developmental mode of spiralian (b) and the evolution of bilaterian body plans (c). The diagram in panel c is based on previous reports (40, 41). NS, nervous system; DO, digestive opening; LCA, last common ancestor.

*Conservation and plasticity of spiralian DV patterning*

The poor knowledge of the molecular mechanisms of spiralian DV patterning, as well as the unusual characters observed in a few representative species (13, 14), raise two essential questions: whether the conserved DV patterning mechanism dependent on BMP-Chordin system is retained in spiralian (i.e., the conservation), and if it is retained in some lineages, whether other lineages (and how many of them) show derived mechanisms (i.e., the plasticity). Our results show that the mollusk *L. goshimai* uses BMP2/4 and Chordin in DV patterning, a spiralian case retaining this conserved DV patterning mechanisms that has been suggested but not revealed for a long time. This result also favors the previous speculation that the unusual DV patterning mechanism in the leech annelid should be a derived character (13).
Nevertheless, it should not simply assume that the unusual Chordin-independent DV patterning is restricted to annelids (and probably platyhelminthes (14, 15)). In fact, Chordin was indicated to be lost from another spiralian lineage (rotifers) in a previous study (15). Through an extended investigation, we revealed the possible loss of Chordin from additional spiralian lineages (e.g., entoprocts; see supplemental table S1 and Fig. S12). Interestingly, we noticed an apparent correlation between the lack of Chordin and the absence of a coelom in spiralians (the coelom is absent when no Chordin is revealed; see supplemental Fig. S11), vaguely indicating a correlation between the modification of DV patterning and the secondary simplification of body plans. Given that the secondary simplification is suggested to be a fundamental trend in spiralian evolution (42), despite the potential limits of our result (supplemental Fig. S11), this apparent correlation is worthy of further investigation.

*Insights into the development mode of spiralian*

The D-quadrant organizer has been a central issue in the research field of spiralian development and evolution (22). The underlying molecular mechanisms of organizer function received much attention (21, 23-26, 31, 43-45) but still remain largely unknown. Although MAPK signaling proved essential in organizer function (23, 24), the involved molecules are poorly understood. The demonstration of the involvement of Ilyanassa BMP2/4 in organizer function represents a key progress toward an in-depth understanding of spiralian organizer (18). In the present study, we confirmed that BMP2/4 played similar roles in L. goshimai, an equal-cleaver, as its orthologue in the unequal-cleaver Ilyanassa (although the manners of organizer activation and MAPK signaling dynamics would differ significantly between the two types of embryos (23, 24)). More importantly, we showed that after activation by the organizer, BMP2/4 itself was not sufficient to establish the BMP signaling gradient, and that the formation of such a gradient required (the asymmetrically expressed) Chordin. This result supports our speculation suggesting the indispensable role of Chordin in organizer function. Our hypothesis regarding the regulatory relationships
among the organizer, BMP2/4 and Chordin suggests that the canonical DV patterning molecular network has been deeply integrated into organizer function in spiralian development, thus consolidating the link between a highly clade-specific character (a D-quadrant organizer) and a conserved biological process (DV patterning) (18). This hypothesis can be tested in more spiralian lineages, which would be important to understand the unique developmental mode in spiralian (22, 46, 47).

The asymmetric development in the manipulated L. goshimai embryos (Fig. 4 & supplemental Fig. S6) is very different from the knockdown phenotypes of other animals, in which BMP2/4 or Chordin knockdown causes relatively stable radial development (3, 6, 30). These results should not be caused by technical problems (e.g., the degradation of MOs) since 1) the asymmetrical development emerged relatively quickly (earlier than 8 hpf), 2) similar phenotypes were obtained when using higher concentrations of MO, and 3) there were significant differences between the phenotypes after Chordin and BMP2/4 knockdown. We propose that such 3B-3D asymmetric development should be related to the 3B-3D polarity that emerges soon after organizer formation (mainly referring to the asymmetrical divisions of 3q, see Fig. 4e), although the BMP signaling gradient is eliminated in these influenced embryos. At the molecular level, the early 3B-3D polarity may cause lineage-specific specifications (e.g., different fates of 3a/b2 and 3c/d2, see Fig. 4e), or generate an asymmetrical expression of other DV patterning genes (e.g., ADMP, tolloid, etc.) on 3B and 3D sides (9, 12). Further investigations are required to clarify which factor is at work or whether the two factors act in combination. In any case, the asymmetrical development after BMP2/4 or Chordin knockdown indicate the roles of the organizer independent of BMP2/4 or Chordin (and thus the correct BMP signaling gradient), suggesting the profound effects of the stereotype cleavage pattern on early embryonic development of spiralian.

DV patterning and bilaterian body plan evolution: neurogenesis and blastopore localization
The inhibitory effect of BMP signaling on neurogenesis has been proposed to be conserved in all bilaterians (35) and even in a cnidarian (7) (but see (48) for a different viewpoint). However, researchers proposed that BMP signaling may promote neurogenesis in mollusks based on the fact that BMP4 treatment caused extra eye formation and expanded Pax6 expression (18). Such positive effects are quite unusual in animals (35). Our results firstly reveal differential effects of BMP signaling on eye formation and neurogenesis in L. goshimai. While strong evidence suggests that BMP signaling promotes eye formation, no such effect was observed for the whole neurogenesis (despite the dose-dependent effect, supplemental Fig. S3). Furthermore, by investigating the expression of three marker genes related to neurogenesis (Pax6, Elav and SoxB), our results do not support the recent proposal (positive) (18) or the overwhelming viewpoint (inhibitory) (35) regarding the effects of BMP signaling on neurogenesis. Alternatively, our results suggest a potential role of BMP signaling in the formation of correct (bilateral) organization of the nervous system (ONS) in L. goshimai (Fig. 5). This suspected role of BMP signaling is reminiscent of a recent finding revealing the effects of BMP signaling in regulating ONS in a hemichordate (Ptychodera flava) (49). These results seem to indicate a (ancient) role of BMP signaling to regulate ONS (though may be lost from many animal lineages). Indeed, the ONS regulated by the DV patterning signals (BMP signaling) supports the scenario in which ONS transforms with the innovation of a DV axis in the common ancestor of bilaterians (40) (Fig. 7c). It would be intriguing to explore whether there are other animal lineages in which the BMP signaling would regulate ONS to infer the ancient roles of BMP signaling in bilaterian neurogenesis.

The localization of blastopores/digestive openings is an essential research topic because it represents a key node during the evolution from an assumed cnidarian-like ancestor to bilaterians (41) (Fig. 7c) and is also essential to understand the splitting of two major bilaterian clades (protostomes and deuterostomes) (50). In L. goshimai, as a typical protostome bilaterian, a ventral mouth is derived from the ventral blastopore,
indicating the blastopore’s ventral localization is essential for the correct development of the larval mouth. Our results that the BMP signaling gradient along the DV axis regulates the ventral localization of the blastopore are in line with a previous study suggesting the involvement of BMP signaling in the localization of blastopores/mouths in brachiopod larvae (51). Theoretically, it is still difficult to determine whether the posteriorized blastopore in manipulated L. goshimai embryos could be interpreted to be similar to those of cnidarians or deuterostomes, in which the blastopore both forms along the anterior-posterior axis (despite the differences in the blastopore locations at the animal or vegetal pole, see supplemental Fig. S10). Nevertheless, we tend to consider it a cnidarian-type blastopore given that a DV axis is largely disrupted in these embryos. A thorough investigation on the marker genes of the whole digestive tract (e.g., the parahox genes (41, 52)) is required to certify this proposal. In summary, by revealing the correlation between the blastopore localization and BMP signaling gradient (DV patterning), these results provide insight into the transition of blastopores/digestive openings during animal evolution.

**Material and Methods**

**Animals**

Adults of *L. goshimai* Nakayama, Sasaki & Nakano, 2017, were collected from intertidal rocks in Qingdao China. Spawning occurred after collection during the reproductive season (from June to August). During other seasons, algae were scraped from the surfaces of rocks inhabited by the limpets and cultured on plastic sheets under constant light. At 18-22 °C, the limpets fed these cultured algae could become sexually mature in several weeks. On some occasions, spawning was induced through elevated temperature, drying, rigorous water flow or sperm suspensions. The adult limpets were allowed to spawn in separate 100-mL cups, and the gametes were collected. Artificial fertilization was performed by mixing sperm and oocyte suspensions.

Fertilized eggs were incubated in filtered seawater (FSW) containing antibiotics (100 unit/mL benzylpenicillin and 200 μg/mL streptomycin sulfate) in an incubator at
25°C. The units of all developmental stages are in hpf except for the very early developmental stages before the 64-cell stage. For in situ hybridization (ISH), samples at the desired developmental stages were fixed in 4% paraformaldehyde (1× PBS, 100 mM EDTA, and 0.1% Tween-20, pH 7.4), transferred to methanol and stored at -20°C until use. Older larvae (after 15 hpf) were anesthetized with 0.1% sodium azide or 125 mM magnesium chloride before fixation. Analyses of the samples were performed as previously described, including ISH (29), pSmad1/5/8 staining (30), phalloidin staining (53) and scanning electron microscopy (SEM)(17).

**Genes and MOs**

*L. goshimai* gene sequences were first retrieved from a developmental transcriptome that we developed previously (29), and the orthologies were verified through subsequent phylogenetic analyses. Translation-blocking MOs targeting BMP2/4 (BMP2/4 MO) and Chordin (Chordin MO1), as well as two negative control MOs (a muted Chordin MO (control MO1) and a standard MO (control MO2)), were synthesized (supplemental text). In preliminary experiments, we confirmed that the two negative control MOs did not generate any detectable effects on the development of *L. goshimai* at the concentrations we used. Therefore, the muted Chordin MO (control MO1) was used as the negative control MO in most experiments. We also used another nonoverlapping MO to inhibit Chordin gene (Chordin MO2), and confirmed that it generated a similar phenotype to that when using Chordin MO1.

**MO microinjection**

Microinjection was performed using a micromanipulator. The injection solutions contained 0.05% phenol red, 500 ng/μL FITC-conjugated dextran and 0.25 mM MO. No more than 1.5% of the oocyte volume of injection solution was injected into the unfertilized oocytes (estimated by the diameter of the injected solution). After fertilization, successful injections were confirmed by the presence of green fluorescence inside the cells; embryos that exhibited no fluorescence were removed. In the trials aiming to explore pSmad1/5/8 distribution, FITC-conjugated dextran was
excluded from the injection solution to avoid causing relatively high background values in subsequent immunostaining. On these occasions, the injections were performed slowly and carefully to ensure that every injection was successful.

_Treatments with rhBMP4 or U0126_

rhBMP4 (R&D Systems, USA; Cat. No. 314-BP) was resuspended in the suspending solution (0.2% BSA containing 4mM HCl) at a concentration of 50 μg/mL and stored at -80°C according to the manufacturer’s instruction. Two doses of treatments were conducted, as determined by preliminary experiments testing a series of treatment parameters. In the high-dose treatment, the rhBMP4 was added at a final concentration of 0.5 μg/mL right after fertilization and the protein was eliminated from the culture system by three FSW washing at 6 hpf. In the low-dose treatment, the rhBMP4 was added at a final concentration of 0.125 μg/mL at the beginning of 32-cell stage (around 2 hpf) and also eliminated at 6 hpf (supplemental Fig. S3). In the control groups, the same volume of suspending solution was added and the same treatment time windows were used. The samples were collected at 6 hpf (for ISH) and 48 hpf (for investigations of eye development) before fixation.

U0126 (Beyotime, China; Cat. No. S1901) was dissolved in DMSO at a concentration of 25 mM and stored at -20°C. At the 16-cell stage, the U0126 storage solution was added to the seawater to a final concentration of 75 μM. In the control group, the same volume of DMSO was added. The embryos at the 60- to 64-cell stage were transferred to FSW followed by three FSW washes to terminate the treatment, and were then collected and fixed.

Oocytes from at least three females were used in every assay involving rhBMP4/U0126 treatment or MO injection (ISH, immunostaining, eye number investigation, and SEM), and we confirmed that maternal effects did not evidently influence the outcomes.

_Imaging_
Images were recorded using a Nikon 80i microscope or an LSM 710 laser-scanning confocal microscopy system (ZEISS, Germany). The contrast and brightness of the images were adjusted using Photoshop software; when performed, such adjustments were applied to the whole image rather than to any particular regions.

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