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Vitelline membrane proteins promote left-sided *nodal* expression after neurula rotation in the ascidian, *Halocynthia roretzi*

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ABSTRACT

Stereotyped left–right asymmetry both in external and internal organization is found in various animals. Left-right symmetry is broken by the neurula rotation in the ascidian, *Halocynthia roretzi*. Neurula embryos rotate along the anterior–posterior axis in a counterclockwise direction, and the rotation stops when the left side of the embryo is oriented downwards, resulting in contact of the left-side epidermis with the vitelline membrane at the bottom of perivitelline space. Then, such contact induces the expression of *nodal* and its downstream *Pitx2* gene in the left-side epidermis. Vitelline membrane is required for the promotion of *nodal* expression. Here, we showed that a chemical signal from the vitelline membrane promotes *nodal* gene expression, but mechanical stimulus at the point of contact is unnecessary since the treatment of devitellinated neurulae with an extract of the vitelline membrane promoted *nodal* expression on both sides. The signal molecules are already present in the vitelline membranes of unfertilized eggs. These signal molecules are proteins but not sugars. Specific fractions in gel filtration chromatography had the *nodal* promoting activity. By mass spectrometry, we selected 48 candidate proteins. Proteins that contain both a zona pellucida (ZP) domain and epidermal growth factor (EGF) repeats were enriched in the candidates of the *nodal* inducing molecules. Six of the ZP proteins had multiple EGF repeats that are only found in ascidian ZP proteins. These were considered to be the most viable candidates of the *nodal*-inducing molecules. Signal molecules are anchored to the entire vitelline membrane, and contact sites of signal-receiving cells are spatially and mechanically controlled by the neurula rotation. In this context, ascidians are unusual with respect to mechanisms for specification of the left-right axis. By suppressing formation of epidermis monocilia, we also showed that epidermal cilia drive the neurula rotation but are dispensable for sensing the signal from the vitelline membrane.

1. Introduction

Stereotyped left–right asymmetry (L-R asymmetry) both in external and internal organization is found in various animals. At a certain stage of embryogenesis, the L-R symmetry is broken by key events (Spéder et al., 2007; Vandenberg and Levin, 2013; Namigai et al., 2014; Blum et al., 2014). Vertebrates, such as mouse, frog, and fish, break L-R symmetry using motile cilia within the L-R organizer (Nonaka et al., 1998; Okada et al., 2005). Cilia in the node of mouse generate leftward nodal fluid flow, and eventually, promote left-side-specific *nodal* gene expression in the lateral plate mesoderm (Nonaka et al., 2002; Takaoka et al., 2007).

Tunicates including ascidians are phylogenetically the closest relatives of vertebrates. Ascidian embryos develop into tadpole larvae. The tadpole larvae show morphological L-R asymmetry in tail bending within the vitelline membrane and brain structures (Taniguchi and Nishida, 2004; Morokuma et al., 2002). They express *nodal* and *Pitx2* genes in the left side epidermis from the neurula to tailbud stages (Morokuma et al., 2002; Yoshida and Saiga, 2008, 2011). Ascidian embryos do not have a cavity corresponding to the vertebrate L-R organizer. Instead, entire embryos rotate along the anterior-posterior axis at the neurula stage (Neurula rotation) (Nishide et al., 2012; Yamada et al., 2019). Neurulae of *Halocynthia roretzi* rotate in a counterclockwise direction when viewed from the posterior pole and they rotate less than 360 degree in ten minutes at 15 h after fertilization, and this rotation stops when the left side of the embryo is oriented downwards (Fig. 1A). Protrusion of the neural fold at the dorsal side

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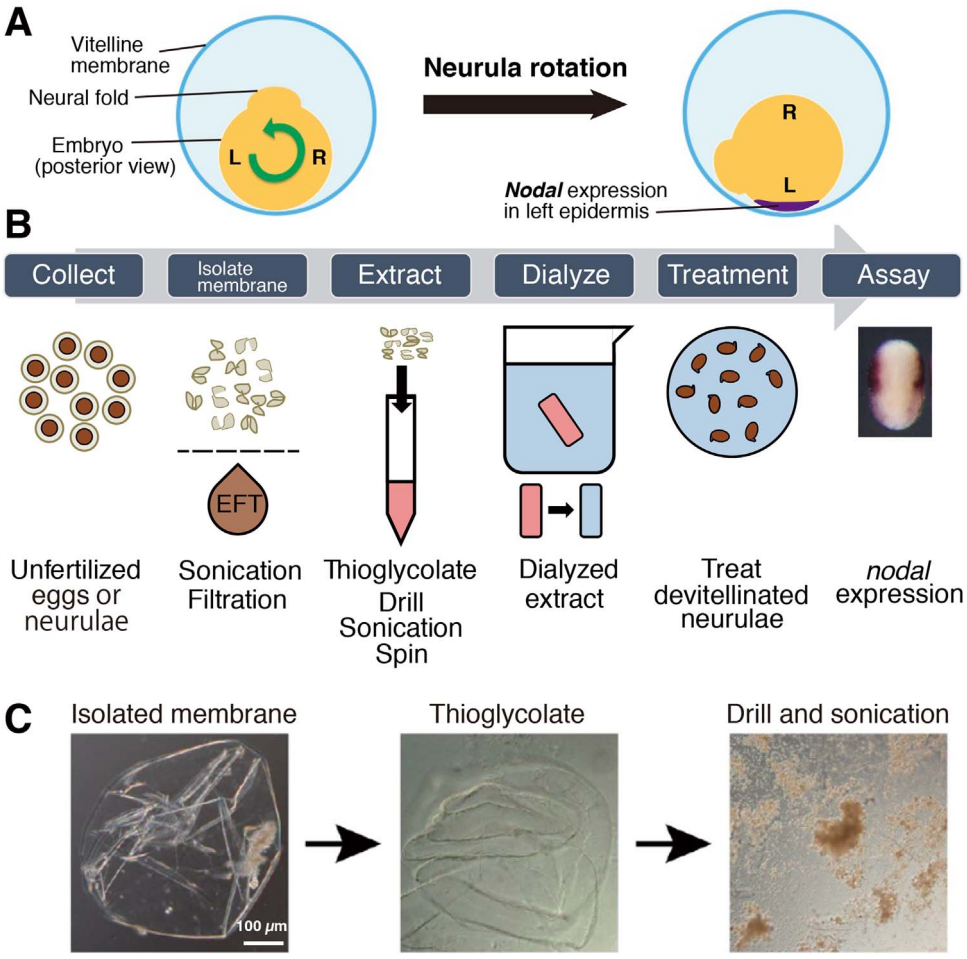

Fig. 1. Vitelline membrane extract and the *nodal* expression assay. (**A**) Schematic diagram of neurula rotation. The neurula is viewed from the posterior pole. The neurula embryo rotates in a counterclockwise direction (arrow) at 15 h of development, and this rotation stops when the left side of the embryo is oriented downwards. Protrusion of the neural fold physically prevents embryos from further rotating. Contact of epidermis and the vitelline membrane promotes *nodal* expression (purple) in the left-side epidermis. L, left. R, right. (**B**) Schematic illustrations of preparation of the vitelline membrane extract and the *nodal* expression assay involving treatment of devitellinated embryos with the extract. See details in the Material and methods. (**C**) Isolated egg membranes were swollen and softened by treatment with sodium thioglycolate, and then, were broken into very small pieces by drilling and sonication. Supernatant was dialyzed and used as the vitelline membrane extract. EFT, embryonic flow-through.

physically prevents embryos from rotating further. Thus, the left side epidermis contacts with the vitelline membrane after the rotation. The rotation is driven by wavy and slow movements of the monocilia on the epidermis (Yamada et al., 2019).

Most non-chordate deuterostomes develop embryos and larvae that swim with cilia, such as the blastula and pluteus of sea urchins, the tornaria of hemichordates, and *Amphioxus* embryos (Satoh, 2009). They swim by rotating in a counterclockwise direction, which is the same direction as the ascidian neurula rotation. In contrast, ascidian larva swims with their tail. Ascidians might co-opt their ancestral epidermal cilia for neurula rotation, but not for swimming (Nishide et al., 2012).

Eventually, the contact of the left epidermis and the vitelline membrane promotes *nodal* expression in the left-side epidermis two hours after the neurula rotation in *H. roretzi* (Fig. 1A) (Nishide et al., 2012). Removal of the vitelline membrane before and just after neurula rotation results in failure of *nodal* expression. More than 30 min of contact between the epidermis and the vitelline membrane is required for *nodal* expression. When the right-side epidermis is pushed towards the vitelline membrane by centrifugal force, morphological L-R asymmetries are reversed, and *nodal* is expressed on the right side. When embryos are sandwiched between coverslips such that the embryo is in contact with the vitelline membrane on both left and right sides, the L-R axis is randomized, and expression of *nodal* is observed on both sides (Nishide et al., 2012). The dorsal and ventral epidermis are not competent to express *nodal*. These observations suggested that contact of the lateral epidermis with the vitelline membrane, but not rotational movement itself, is required for induction of *nodal* expression (Nishide et al., 2012). Follicle cells attached outside of the membrane and test cells within perivitelline space are dispensable for promotion of *nodal* expression. Naked embryos sandwiched between coverslips do not express *nodal*. However, it is still not known whether mechanical stimulation at the point of contact or chemical stimulation from the vitelline membrane induces *nodal* expression.

In the present study, we showed that treatment of naked neurulae with an extract of the vitelline membrane promoted *nodal* expression on both sides, indicating that chemical signals from the vitelline membrane are involved in *nodal* expression. The responsible signal molecules are proteins and not sugars. Specific fractions showed the *nodal*-promoting activity after gel filtration chromatography. After mass spectrometry, we selected potential candidate proteins. We also showed that epidermal cilia are not required for sensing the signal from the vitelline membrane.

2. Materials and methods

2.1. Embryos

Naturally spawned eggs of the ascidian *H. roretzi* were fertilized with a suspension of non-self sperm and raised in Millipore-filtered

seawater or artificial sea water (Rei-Sea Marine, REI-SEA IWAKI, Tokyo) containing 50 μg/ml streptomycin sulfate and 50 μg/ml kanamycin sulfate at 11–13 °C. Early neurula stage was attained 15 h after fertilization at 13 °C and used for the *nodal* expression assay.

2.2. Vitelline membrane extract

Unfertilized eggs or neurula embryos in seawater were sonicated for 1 min (Branson Sonifier 250, at the output control 5, Danbury, CT). Eggs and embryos were completely destroyed while vitelline membranes were partially broken (Fig. 1B, C). The samples were filtered through Cell Strainers (Falcon, mesh size 70 μm). Flow-through of embryo homogenate was kept as embryonic flow-through (EFT). Vitelline membranes on the mesh (Fig. 1C) were thoroughly washed with seawater, collected in spin tubes, and centrifuged at 4 °C and 15,000 rpm for 1 min to pack them. Approximately 0.8 ml of purified vitelline membrane (obtained from 60,000 eggs) was used for a single experiment. Samples were kept at – 80 °C until further use.

Packed vitelline membrane were suspended in 1 ml of 1% sodium thioglycolate in seawater (pH 10) and mixed thoroughly for 1 h. Membranes swelled and softened as the disulfide bonds were cleaved (Fig. 1C). After centrifugation (4 °C, 15,000 rpm, 2 min), the supernatant was transferred into another spin tube and stored. Swelled membranes were then drilled using a pestle with a flat-bottom (Watson Bio Lab., Kobe, Japan) twice for 1 min each. After adding some amount of the previously stored supernatant back to the drilled sample, membranes were sonicated (1 min at the output control 1) (Fig. 1C). Then, all the previously stored supernatant was added back to sonicated sample. They were mixed well and centrifuged (4 °C, 15,000 rpm, 20 min), and the supernatant collected was the vitelline membrane extract. A large amount of debris precipitated, but we only used the supernatant. The solution was then dialyzed against seawater using a cellulose tube (8/32, Sekisui Medical, Tokyo, Japan) overnight at 4 °C; seawater was exchanged twice to remove sodium thioglycolate. The extract was then centrifuged (4 °C, 15,000 rpm, 10 min), and the supernatant was used for the *nodal* expression assay. Approximately 0.8 ml of purified vitelline membrane (obtained from 60,000 eggs) yielded 1 ml of the extract containing 1 mg/ml proteins.

2.3. Nodal expression assay

Embryos from which vitelline membranes were removed before the neurula-rotation stage (15 h after fertilization) do not express the *nodal* gene (Nishide et al., 2012). During the assay of the vitelline membrane extract for detection of the activity to promote *nodal* expression, embryos were manually devitellinated 13–15 h after fertilization using tungsten needles. More than 10 embryos were transferred into solutions in gelatin-coated wells or in caps of spin tubes (at least 100 μl solution was required). They were fixed for *in situ* hybridization at 18 h when *nodal* is expressed. *In situ* hybridization of *nodal* was performed as described previously (Nishide et al., 2012).

2.4. Sugar degradation and protein digestion

The membrane extract (2.72 mg/ml protein) was processed for sugar degradation and protein digestion. The volume of the yield was finally adjusted to approximately twice the volume of the original extract using seawater. For sugar degradation, periodate oxidation was used. Seawater (117 μl) containing 10 mM sodium periodate was mixed with 350 μl of the extract and incubated at 4 °C overnight. Reaction was stopped by adding 250 μl of ethylene glycol and incubation for 1 h at 4 °C, and then, the mixture was dialyzed against seawater. The samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Sugars were detected using HRP labeled-concanavalin A (Cosmo Bio) and DAB staining kit (Muto Pure Chemicals, Tokyo, Japan).

For protein digestion, 150 μl of immobilized TPCK Trypsin (Thermo Fisher Scientific) was thoroughly washed with seawater. Then, 150 μl extract was added and the samples were incubated at 37 °C for 1 h. Samples were then centrifuged (4 °C, 15,000 rpm, 20 min) and the supernatants were filtered with Centrifugation Filter Units (Millipore, 0.22 μm) to remove trypsin, which is immobilized on beaded agarose.

2.5. Gel filtration chromatography

Ten milliliters of the vitelline membrane extract were concentrated ten times with Amicon Ultra (30 K, Merck) and subjected to gel filtration chromatography in one experiment. The experiment was repeated twice. One milliliter of the concentrated extract was applied to an Econo-column (Bio-Rad, size 20 ml) of Sephacryl S-300 (GE Healthcare) equilibrated with seawater containing 0.2 mg/ml BSA to protect nonspecific interaction; then, BSA was washed out. Gel filtration chromatography was carried out at 4 °C and fractions (1.3 ml each) were collected. Protein concentration was measured with the BCA Protein Assay Kit (Thermo Fisher Scientific). SDS-PAGE was carried out using 10% poly acrylamide gel and proteins were detected using the Silver Stain Regent kit (Cosmo Bio).

2.6. Protein identification by liquid chromatography/mass spectrometry

Protein sample (5.3 μg) was carboxyamidomethylated in 360 μl of 25 mM ammonium bicarbonate solution containing 7 mM iodoacetamide. The solution was incubated with 4 μg of trypsin (Trypsin Gold, Mass Spec Grade, Promega, WI, USA) at 37 °C for 16 h. Tryptic digest was dried by SpeedVac and reconstituted in 0.1% formic acid, and the sample solution was analyzed by LC/MS using an Orbitrap Elite Mass Spectrometer (Thermo Fisher Scientific, CA, USA) connected to a UltiMate 3000 Nano LC system (Thermo Fisher Scientific). The analytical column was a reversed-phase column (MonoCap C18 HighResolution 2000, 0.1 × 2000 mm, GL sciences, Tokyo, Japan). The mobile phase was composed of 0.1% formic acid (FA) solution (A buffer) and acetonitrile containing 0.1% FA (B buffer). The peptides were eluted at a flow rate of 500 nl/min with a gradient of 10–50% of B buffer in 180 min. Full mass spectra and MS/MS spectra were acquired using Orbitrap and linear ion trap, respectively. The spectral data obtained by MS/MS were used for database search analysis with the SEQUEST algorithm (Proteome Discoverer 2.1, Thermo Fisher Scientific) using protein data encoded from *H. roretzi* genome (HR20141210.fasta, ANISEED database, Brozovic et al., 2018).

2.7. Ciliobrevin D treatment

To inhibit cilia formation, embryos were treated with 10–30 μM Ciliobrevin D (Merck) as previously described (Yamada et. al, 2019). Ciliobrevin D inhibits dynein ATPase activity, which is required for cilia formation (Firestone et al., 2012). The treatment was initiated just before epidermal cilia formation at the neurula stage. Control embryos were treated with DMSO.

3. Results

3.1. Extract of the vitelline membrane promotes nodal expression

Vitelline membranes of unfertilized eggs and of neurula embryos were collected by sonication and filtration (Fig. 1B). Eggs and embryos were completely destroyed while vitelline membranes were collected after sonication. Flow-through that contained destroyed embryos and accessory cells (follicle and test cells) was kept as embryonic flow-

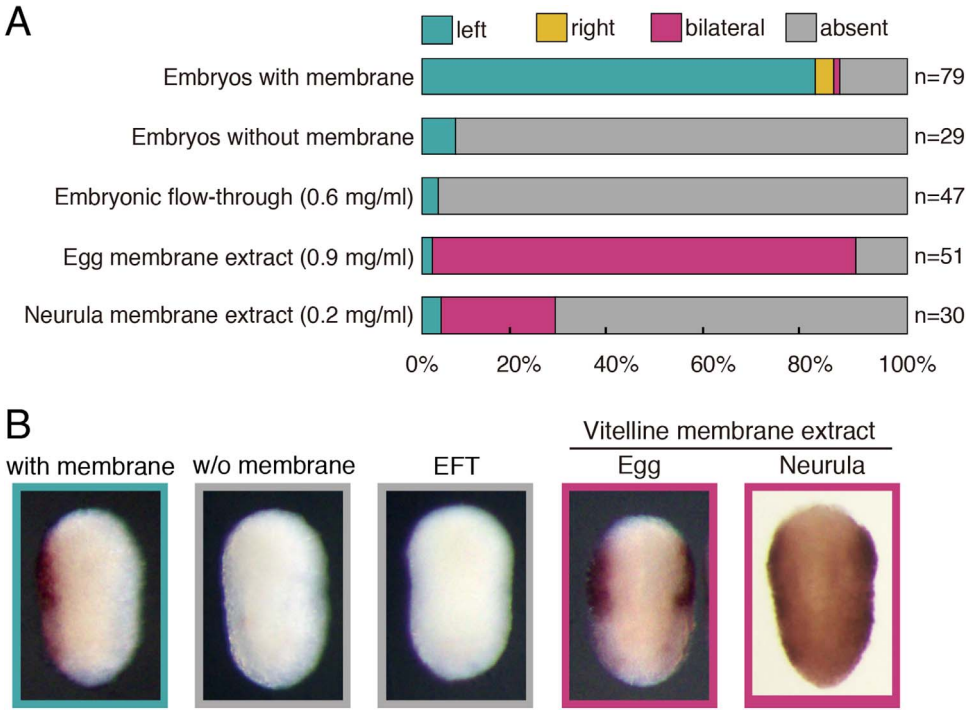

Fig. 2. *Nodal* expression assay. (A) Proportion of embryos that showed *nodal* expression in the left, right, and both lateral sides. From top to down: normal embryos with vitelline membrane; embryos from which vitelline membrane was removed; devitellinated embryos treated with the embryonic flow-through (EFT), which contained embryonic homogenate; embryos treated with the vitelline membrane extract of unfertilized eggs; and embryos treated with the vitelline membrane extract of neurulae. The protein concentration is indicated in parentheses. (B) Initial tailbud embryos probed for *nodal*. *nodal* was not expressed in naked embryos, but it was expressed on both sides in naked embryos when treated with the vitelline membrane extracts.

through (EFT). Vitelline membranes isolated on the mesh were processed by treatment with sodium thioglycolate, drilling, sonication, and dialysis against seawater (Fig. 1B, C). Approximately 0.8 ml of purified vitelline membrane (obtained from 60,000 eggs) yielded 1 ml of the extract containing 1 mg/ml proteins.

Embryos from which vitelline membranes were removed before the neurula-rotation stage do not express the *nodal* gene (Nishide et al., 2012). When assaying the vitelline membrane extract for detection of the activity to promote *nodal* expression (*nodal* expression assay), more than 10 naked early neurulae were treated with dialyzed vitelline membrane extract in one experiment. Embryos were then fixed for *in situ* hybridization at 18 h when *nodal* is expressed in normal embryos.

In positive controls, initial tailbud embryos with vitelline membrane expressed *nodal* on the left side in more than 80% of cases (Fig. 2A, B). Only 7% of embryos without the vitelline membranes expressed *nodal* among the negative controls. Embryos treated with EFT rarely showed *nodal* expression. In contrast, the vitelline membrane extract of unfertilized eggs showed remarkable activity (87%, n = 51). The embryos expressed *nodal* in epidermis on both left and right sides (Fig. 2B) because the extract interacted with entire surface of embryo and because both dorsal and ventral epidermis are incompetent to express *nodal* (Nishide et al., 2012). When these active extracts were filtrated through Millipore filter (pore size, 0.22 μm), the activity was retained in flow-through, indicating that large particles were not responsible for such activity. The vitelline membrane extract at the neurula stage showed moderate activity (24%, n = 30). This was probably because the extract of vitelline membrane at the neurula stage contained less amount of proteins compared to that of eggs (Fig. 2A, parentheses). Vitelline membranes expand and are hardened at fertilization, and thus, they became more difficult to be extracted. Even if the same amount of isolated vitelline membrane was used, membranes at the neurula stage yielded an extract with lower concentration of proteins. The speculation is supported by the evidence that when the vitelline membrane extract of unfertilized eggs was diluted three times, the activity was reduced to 36%, although this was done in another set of experiments. When it was diluted nine times, we did not detect the activity any more.

This result clearly showed that chemical signal from the vitelline membrane promotes *nodal* expression in epidermis cells and that the signal molecules are already present in vitelline membranes of unfertilized egg. Therefore, we used the vitelline membrane extract obtained from unfertilized eggs for further biochemical analyses.

3.2. Signal molecules are proteins and not sugars

To distinguish whether the active molecules were protein or sugar, the membrane extract (2.72 mg/ml protein) was processed for sugar degradation or protein digestion. The volume of the yield solution was finally adjusted to approximately twice the volume of the original extract with seawater. First, deglycosylation was carried out by periodate oxidation, which opens sugar rings. Sugars were significantly degraded during the reaction as shown by lectin blotting with Concanavalin A (Fig. 3B; ConA), although some amount of protein was lost (Fig. 3B; protein, extract vs. deglycosylation product). The extract still retained complete activity, suggesting that sugar chains were dispensable for the *nodal* inducing activity (Fig. 3A).

In contrast, protein digestion significantly reduced the extract's activity. Proteins were digested by trypsin at 37 °C for 1 h. SDS-PAGE showed that most of the proteins were digested except for a single band (Fig. 3D; protein digestion). In control, that is the extract sample incubated without trypsin, protein bands were almost intact (Figs. 3D; 37 °C, 1 h). The activity of the extract was reduced from 66% to 8% after trypsin digestion (Fig. 3C, 37 °C incubation vs. protein digestion, $p < 0.01$, Fisher's exact test). Deglycosylation and protein digestion experiments were repeated once more (in which proteins were digested for two hours), and essentially similar results were obtained. Thus,

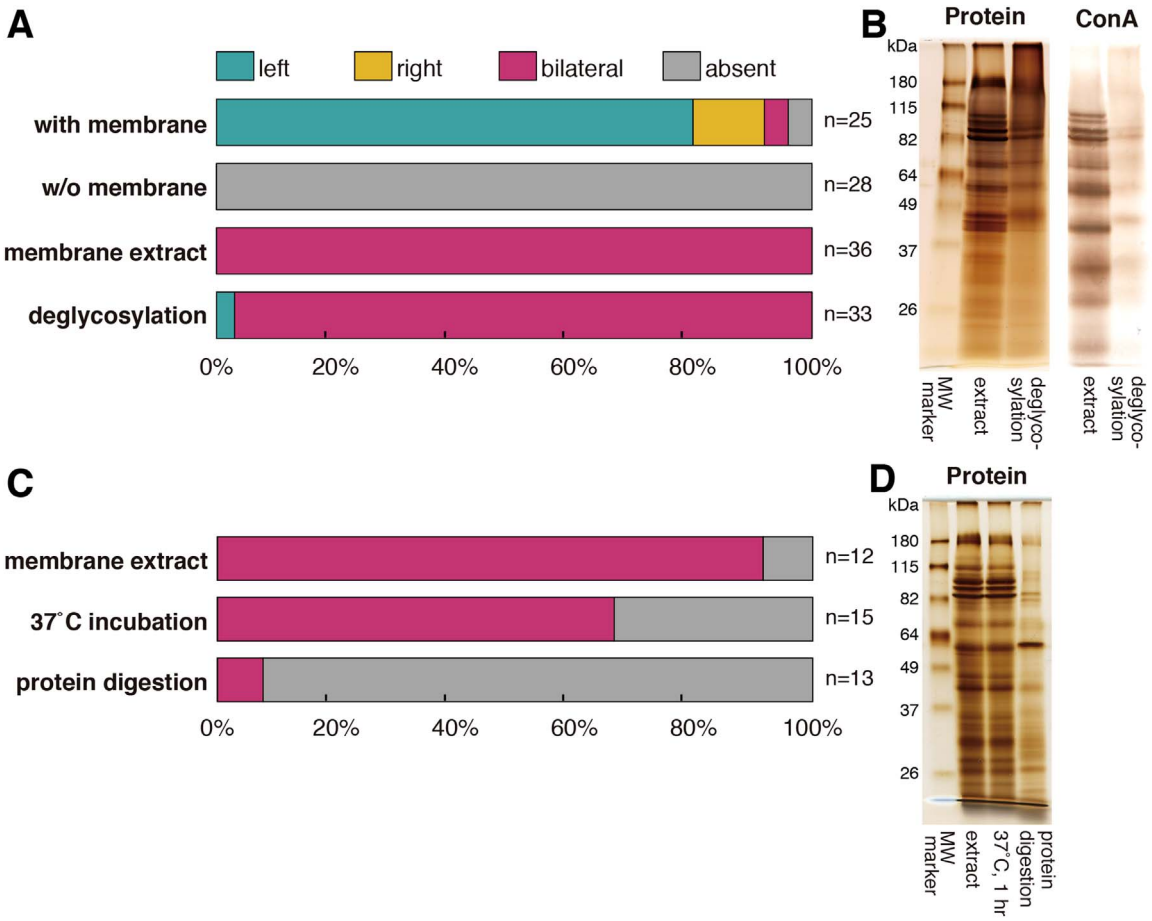

Fig. 3. Deglycosylation and protein digestion of the vitelline membrane extracts. (**A**) Deglycosylation was performed using periodic acid oxidation. Proportion of embryos that showed *nodal* expression in the left, right, and both lateral sides. (**B**) Deglycosylation. (Left) SDS-PAGE, in which proteins were detected by silver staining. (Right) Lectin-probed western blot analysis with concanavalin A. (**C**) Proteins were digested with trypsin-conjugated beads. From top to down; devitellinated embryos treated with the vitelline membrane extract of unfertilized eggs; embryos treated with the vitelline membrane extract that was kept at 37 °C without trypsin as control; embryos treated with the vitelline membrane extract that was digested with trypsin. The extract lost the activity upon protein digestion. (**D**) Protein digestion. SDS-PAGE, in which proteins were detected by silver staining.

these results indicated that the signal molecules in the vitelline membrane extract were most likely proteins.

3.3. Specific fractions after gel filtration chromatography showed the nodal promoting activity

Gel filtration chromatography using Sephacryl S-300 was carried out to isolate vitelline membrane proteins (Fig. 4). The extract was eluted with seawater so that the activity of each fraction could be checked by the *nodal* expression assay. The *nodal* inducing activity was observed for fractions 7 and 8 (Fig. 4B). Fraction 7 had higher activity. This was confirmed by two independent experiments, and the second experiment gave similar results in which the first and second fractions after the void volume had the activity. SDS-PAGE of each fraction showed that proteins were fractionated depending on molecular weights; however, fractions 7 and 8 contained small as well as large proteins (Fig. 4C). It seemed plausible that the small proteins were oligomeric proteins that may have aggregated with larger proteins at a molecular level in the extract. This could be because the vitelline membranes originally contained molecules that were immiscible in seawater, and hence, they could easily aggregate. The embryonic flow-through was also fractionated (Fig. 4D). Major embryonic proteins could contaminate the vitelline membrane extract because it seems that some major bands in the embryonic flow-through are also present in the extract. Embryonic debris was not thoroughly washed out or they would also have aggregated with vitelline membrane proteins during the first sonication done to destroy embryos.

3.4. Mass spectrometry to identify candidate proteins

To identify potential *nodal* inducing candidates, mass spectrometry was performed. Trypsin digestion products of the vitelline membrane fraction 7 (with activity) and fraction 9 (without activity) were analyzed. The products of the fraction 7 of embryonic flow-through were also analyzed to subtract embryonic contaminations from the vitelline membrane data. The resultant list is shown in Supplementary Table 1. The proteins were categorized into four groups (listed in each pages of Supplementary Table 1). The red category included proteins that were uniquely detected in the vitelline membrane fraction 7 (32 proteins). The yellow group included proteins found both in the vitelline membrane fractions 7 and 9. In this group, the amount of each protein was compared between fractions 7 and 9. The relative amounts of proteins were estimated using three approaches: PSM (the number of peptide spectrum matches; http://proteomics.princeton.edu/services/mass-spec-results-definitions), SEQUEST score (Martínez-Bartolomé et al., 2008), and MS Amanda score (Dorfer et al., 2014). If the fraction 7 (F7): fraction 9 (F9) values were higher than 1, they were highlighted in yellow in Supplementary Table 1 (16 proteins), and proteins that have F7:F9 values less than 1 (4 proteins) were excluded from the candidate list. The blue group included proteins that were detected both in the vitelline membrane F7 and embryonic flow-through F7 (19 proteins). These were likely contaminations from embryonic proteins, and thus, were excluded from the candidate list. White group included proteins that were detected in all three fractions (9 proteins). These were also

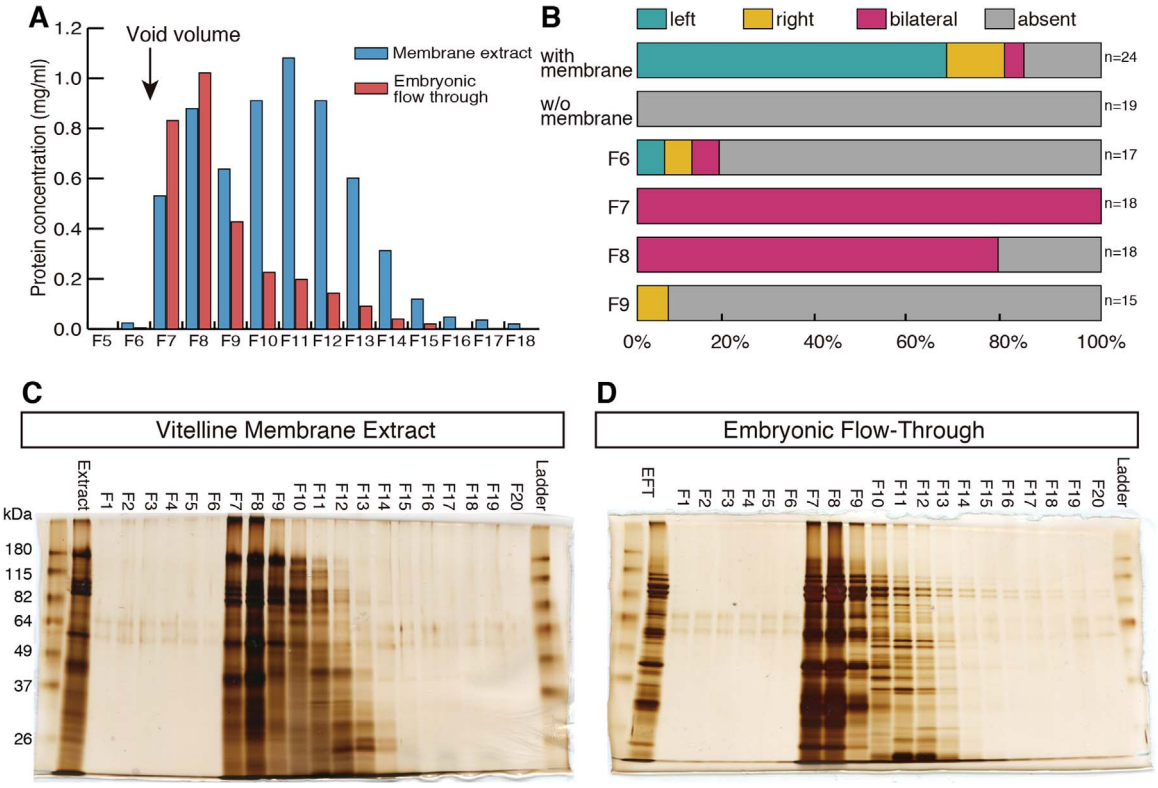

Fig. 4. Gel fractionation of the vitelline membrane extracts and the embryonic flow-through. (**A**) Protein concentration in each fraction. (**B**) Proportion of embryos that showed *nodal* expression in the left, right, and both lateral sides. One representative result of two repeated experiments is shown. The samples of the experiment shown here were used for mass spectrometry analysis. Embryos were treated with different fractions of the vitelline membrane extract. Fractions, F10–20 did not promote *nodal* expression. (**C**) SDS-PAGE, in which vitelline membrane proteins were detected by silver staining. (**D**) SDS-PAGE of embryonic proteins.

speculated to be contaminations from embryonic proteins. Thus, 48 proteins were finally selected as the potential candidates for *nodal* induction.

However, BLAST search results suggested that some of the candidate proteins were apparently nuclear, cytoplasmic, and cell membrane proteins, such as histone H4, integrin alpha and beta, transformer 2 homolog, Pax2/5/8, cadherin-23, guanylate cyclase, and casein kinase II. These might be trapped by vitelline membrane during the first sonication done to destroy embryos.

3.5. Zona pellucida domain proteins

Multiple proteins harboring the zona pellucida (ZP) domain are frequently found in the egg coats of vertebrates (Jovine et al., 2002, 2005; Spargo, Hope, 2003; Okumura et al., 2004; Smith et al., 2005; Aagaard et al., 2006; Goudet et al., 2008; Wassarman et al., 2008). ZP domain is found in secreted eukaryotic glycoproteins. It consists of around 260 amino acids and is characterized by eight strictly conserved cysteines, which form disulfide bonds. It functions as protein polymerization module. In the ascidian, *Ciona robusta* (formerly *C. intestinalis*, Type A), 11 ZP proteins were found by proteomics analysis of the vitelline membrane (Yamada et al., 2009). Interestingly, eight of them had EGF repeats, which are not found in ZP proteins of vertebrate egg coat.

By conducting a BLAST search using the ZP domain amino acid sequence of HrVC120 vitelline coat protein (Sawada et al., 2002, 2004) as a query, we found nine proteins with a ZP domain in *H. roretzi* genome (Fig. 5A). All of them had a ZP domain and a transmembrane (TM) domain in their C-terminals. The TM domains are eventually removed by furin (Wassarman, 2008), as furin cleavage sites (Arg-X-Lys/Arg-Arg) are present between the TM domains and ZP domains in these ascidian ZP proteins, similar to other animals. Similar to *C. robusta*, seven of the proteins had EGF repeats (Fig. 5B). Eight out of nine ZP proteins were detected in our MS analysis of the vitelline membrane (ZP protein #1–8, Fig. 5A, Supplementary Table 1) and six of them (#1, #4–8) were listed as candidates for *nodal* induction. Staged RNA-seq results (ANISEED database, https://www.aniseed.cnrs.fr/, Brozovic et al., 2018) indicated that expression of the *ZP protein #9* gene is initiated at the tail bud stage. This was consistent with our data that ZP protein #9 was not found in the vitelline membrane extract. Transcripts of all other ZP proteins #1–8 were present in unfertilized eggs, suggesting that these proteins were synthesized in and secreted from oocytes. These findings suggested that proteins with a ZP domain and EGF repeats are indeed enriched in the candidates for *nodal* induction. Due to the presence of multiple EGF repeats that are specific to ascidian vitelline membrane ZP proteins, these proteins were considered to be most important candidates for *nodal* induction.

3.6. Epidermal cilia are not required for sensing the signal from the vitelline membrane

Monocilia are involved in sensing several kinds of inductive signals such as Shh, as well as PDGF, Wnt, and Delta (reviewed in Sasai and Briscoe, 2012; Pal and Mukhopadhyay, 2015). At the neurula stage, when ascidian embryos sense the *nodal* inducing signal from the vitelline membrane, epidermal cells have monocilia (Nishide et al., 2012; Thompson et al., 2012; Katsumoto et al., 2013; Negishi et al., 2016; Palmquist and Davidson, 2017). Movements of these cilia drive the neural rotation (Yamada et al., 2019). We wondered whether the epidermal monocilia are also involved in sensing the *nodal* inducing signal.

To test this, formation of epidermal cilia was inhibited, and then, *nodal* expression was monitored. Ciliobrevin D inhibits dynein ATPase activity that is required for cilia formation (Firestone et al., 2012). When the treatment was initiated just before epidermal cilia formation, embryos failed to form cilia that were detected by Arl13b-Venus

A **ZP (Zona pellucida) domain proteins of *H. roretzi***

	Gene	Score (Bits)	MS category	NCBI Protein
#1	Harore.CG.MTP2014.S4.g10388	168	yellow	VC120 (precursor of VC70) [*H. roretzi*]
#2	Harore.CG.MTP2014.S214.g10227	105	white	VC70-like protein-2 [*H. roretzi*]
#3	Harore.CG.MTP2014.S73.g06014	68.6	white	VC70-like protein-1 [*H. roretzi*]
#4	Harore.CG.MTP2014.S12.g12746	89	yellow	
#5	Harore.CG.MTP2014.S395.g06604	57	red	
#6	Harore.CG.MTP2014.S78.g07592	67	yellow	
#7	Harore.CG.MTP2014.S1111.g02890	65.1	yellow	
#8	Harore.CG.MTP2014.S827.g01563	48.5	yellow	
#9	Harore.CG.MTP2014.S149.g08446	37.4	N.D.	

Score (Bits) shows similarity to the ZP domain of VC-120 protein.

B

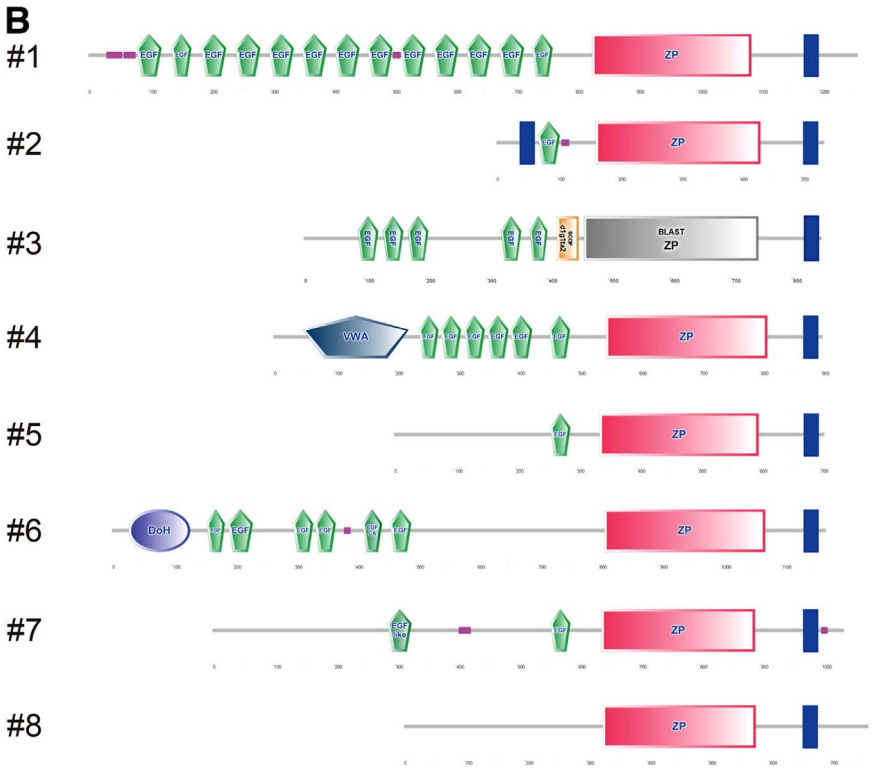

Fig. 5. Zona pellucida domain containing proteins of *H. roretzi*. (**A**) List of zona pellucida (ZP) domain containing proteins. First three proteins were already registered in the GenBank (BAD60812.1, BAF56475.1, and BAF56474.1). Gene names were used as mentioned in the Aniseed database. Score (Bits) shows amino acid similarity to the ZP domain of VC-120 protein (#1). Each protein was categorized according to the results of the mass spectrometry. N.D., not detected. (**B**) Domain compositions as analyzed using the SMART database (http://smart.embl-heidelberg.de/). Pink and gray, ZP domain. Green, EGF repeat. Blue rectangles, transmembrane domain.

protein in ascidians (Yamada et al., 2019). We reconfirmed this by observing the occurrence of the neurula rotation and presence of cilia with scanning electron microscopy. Embryos in which the dorsal side was upwards were selected, and they treated with ciliobrevin D after the last epidermis cell division at the neurula stage and just before cilia formation. Embryos which laid on their left side were scored after neurula rotation stage to evaluate occurrence of neurula rotation. Neurula rotation was inhibited by 10–30 μM ciliobrevin D in a dose-dependent manner (Fig. 6A). 10 μM Ciliobrevin D suppressed neurula rotation in 80% of embryos. Embryos treated with 10 μM ciliobrevin D exhibited cilia development only in 6% of cells (Fig. 6B, n = 100 cells of 5 embryos). In controls treated with DMSO, 96% of cells had cilia (n = 100 cells of 5 embryos).

After treatment with ciliobrevin D, 86% of embryos eventually expressed *nodal* in broader regions on both lateral sides (Fig. 7A). The shape of the treated embryos was rounder, which might be due to inhibition of cell division at the initial tailbud stage (Fig. 7B). These embryos failed to exhibit neurula rotation, and thus, they were laid on the ventral side. Epidermal cells on the both sides were in contact with vitelline membrane, resulting in *nodal* expression on both sides. Most of untreated and DMSO-treated embryos expressed *nodal* on the left side. Similarly, more than 70% of embryos, treated with 30 μM ciliobrevin D, expressed *nodal* on both sides (data not shown). Therefore, it was shown that epidermal cilia were not required for sensing the signal from the vitelline membrane.

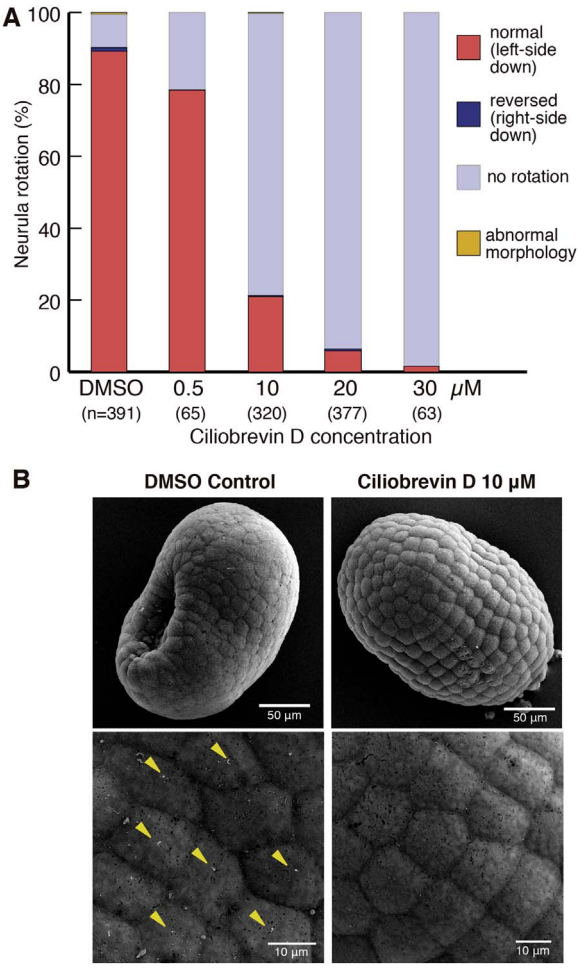

Fig. 6. Ciliobrevin D treatments. (A) Occurrence of neurula rotation after treatment with various concentrations of ciliobrevin D. Embryos with their ventral side down were selected before neurula rotation, and then, treated with ciliobrevin D. Orientation of embryos was monitored one hour after completion of neurula rotation in untreated embryos. (B) (Top) Scanning electron micrographs of neurula embryos treated with either ciliobrevin D or DMSO. (Bottom) Closer views of epidermal monocilia (yellow arrowheads), which look like dots because they stood vertically. Cilia were absent in embryos treated with ciliobrevin D.

4. Discussion

The results clearly indicated that chemical stimulus by the vitelline membrane proteins induces *nodal* expression on the left-side epidermis, which is in contact with the vitelline membrane; however, mechanical stimulus at the point of contact is unnecessary. Epidermal cilia are dispensable for sensing the signal. The relevant signal molecules are already present in the vitelline membrane of unfertilized eggs. However, cellular competence to sense the signal or to activate *nodal* expression would appear at the neurula stage after neurula rotation. Expression of the receptor or signal transducing proteins could be initiated at the neurula stage. The signal molecules are loaded into vitelline membrane during oogenesis or oocyte maturation within the ovary.

Ascidian unfertilized eggs are covered by two types of accessory cells, follicle cells and test cells. Follicle cells are attached to the outer surface of vitelline membrane, while test cells are present in perivitelline space. Results of the present study indicate that *nodal* inducing molecules are already present in the vitelline membrane of unfertilized eggs and that these accessory cells are dispensable. This is consistent with our previous observation that these accessory cells are dispensable for *nodal* expression when the induction occurs (Nishide et al., 2012). Vitelline membrane ultrastructurally appears as a network of interwoven microfibrils (De Santis et al., 1980). Formation of the vitelline membrane has been studied in some ascidian species (Kessel and Kemp, 1962; Sugino et al., 1987). At early oogenesis stages, precursor cells of both follicle cells and test cells are in contact with oocyte. Then vitelline membrane rudiments forms between follicle cells and oocyte as well as at outer surface of test cells, dividing these two accessory cells into outer and inner groups, respectively. These cells would contribute to secretion of vitelline membrane materials as they contain much rough endoplasmic reticulum. On the other hand, mRNAs of most of the ZP domain proteins (#1–8) in *Halocynthia* are abundantly found in the RNA-seq data of unfertilized eggs from which these accessory cells were removed (ANISEED database). This suggests vitelline membrane materials are also secreted from oocytes. It is still unknown whether oocytes, follicle cell precursors, test cells precursors, or any other cells load vitelline membrane with the signal molecule for *nodal* induction.

In this ascidian system, signals from outside of embryos are involved in the embryonic axis specification. In *Drosophila*, embryonic dorsoventral axis is determined by cues from vitelline membrane. *Pipe*, encoding a sulfotransferase, is specifically expressed in ventral follicle cells. It acts on eggshell proteins, which leads to local activation of the protease cascade, and eventually, cleavage of Spätzle into active Toll ligand on the ventral side (Stein and Stevens, 2014). Similarly, both anterior and posterior terminal regions of *Drosophila* are determined by a localized vitelline membrane protein, Torso-like, which locally activates the protease cascade, and eventually, leads to cleavage of Trunk protein into active ligand for Torso, a receptor tyrosine kinase, in the terminal regions (Li, 2005). Similar mechanisms could operate in

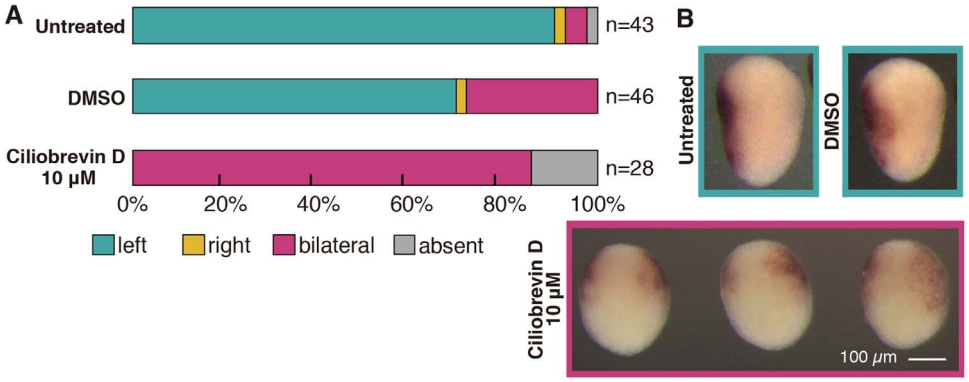

Fig. 7. *Nodal* expression in ciliobrevin D-treated embryos. (A) Proportion of embryos that showed *nodal* gene expression in the left, right, and both lateral sides. Embryos with their ventral side down were selected before neurula rotation, and then, treated with ciliobrevin D. *nodal* expression was monitored at 18 h of development. (**B**) Images of the initial tailbud embryos probed for *nodal*. *nodal* was expressed on both sides in embryos treated with ciliobrevin D, which were more spherical in shape than control tailbuds, most probably due to suppression of cell divisions.

the ascidian. But in *Drosophila*, the signals are produced locally depending on the localized molecules in the vitelline membrane. However, in the ascidian, signal molecules are present in entire vitelline membrane, and the site of contact of signal-receiving cells with vitelline membrane is spatially and mechanically controlled by the embryonic rotation. Therefore, ascidians are different with respect to mechanisms for determination of embryonic axis. On the other hand, differences in mechanisms of the L-R axis determination have been observed among ascidians species (summarized in Table 1 in Yamada et al., 2019). *Ciona robusta* embryos also show neurula rotation; however, they did not cease rotation when the left side faced the bottom. Devitellinated *Ciona* embryos express *nodal* on both sides (Shimeld and Levine, 2006; Yoshida and Saiga, 2008). When the signal molecule is identified in *Halocynthia*, it would be interesting to compare how the L-R axis is determined in *Ciona*.

In the present study, we had difficulty in separating proteins of the vitelline membrane extract by gel chromatography. We were able to select 48 proteins as potential candidates. It is possible that many protein molecules aggregated in the extract because vitelline membranes are originally composed of molecules insoluble in seawater, and therefore, they could easily aggregate. The signal molecules would be anchored to the vitelline membrane to prevent their diffusion within the perivitelline space. We carried out ion-exchange (DEAE) chromatography, and again, we detected *nodal* inducing activity in the first fraction that contained several proteins of various sizes that likely just went through the column without being trapped by the resins. We treated the extract with 6 M urea + 5 mM dithiothreitol, and performed gel chromatography in the presence of these chemicals, but we could not separate the protein molecules. Therefore, it was hard to purify vitelline membrane proteins using columns.

Nodal gene expression is auto-regulated in vertebrate embryos (Burdine and Schier, 2000; Meno et al., 1999; Schier and Shen, 2000). However, the signal molecule in the vitelline membrane of ascidians is not the nodal protein itself. Treatment of *Halocynthia* embryos with the nodal receptor inhibitor had no effect on *nodal* gene expression at the tailbud stage (Nishide et al., 2012). Six ZP proteins were included among the potential candidates of the *nodal* inducing molecules. Due to the presence of multiple EGF repeats that are specific to the ascidian vitelline membrane ZP proteins, these molecules were considered to be the most viable candidates among the *nodal* inducing molecules. However, the conventional epidermal growth factor signaling is excluded as a potential signal transducing mechanism involved in *nodal* gene activation because phosphorylated and activated MAP kinase/ERK was not detected on the left side epidermis at the neurula and initial tailbud embryos of *Halocynthia* (Nishida, 2003). Elevation in the calcium oscillation level has been observed in the left side of the node of mice and in the Kupffer's vesicle of zebrafish (Takao et al., 2013; Yuan. et al., 2015). However, elevation in calcium level was not observed in *Ciona* neurulae except for sporadic signals in the nervous system (Akahoshi et al., 2017). In *Halocynthia*, no or infrequent sporadic signals were observed in whole neurulae within the vitelline membranes and in the neurulae treated with the vitelline membrane extract (Tanaka and Nishida, unpublished observations). Therefore, it is not likely that calcium signaling might mediate *nodal* gene activation in ascidians.

To identify the *nodal* inducing signal in future, each candidate protein molecule selected in the present study would be synthesized and then the *nodal* expression assay would be carried out. As an alternative approach, cis-regulatory elements of *nodal* gene could be analyzed to identify a relevant transcription factor responsible for left side-specific expression.

Author contributions

H.N. conceived and directed this study. Y.T. and S.Y carried out the primary parts of the experiments. S.L.C contributed to initial optimization for extraction of vitelline membrane. N.H. and H.S. contributed to mass spectrometry. Y.S. carried out the experiments for revision of the manuscript. Y.T., S.Y., and H.N. wrote the manuscript.

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Declarations of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2019.01.016.

References

Aagaard, J.E., Yi, X., MacCoss, M.J., Swanson, W.J., 2006. Rapidly evolving zona pellucida domain proteins are a major component of the vitelline envelope of abalone eggs. Proc. Natl. Acad. Sci. USA 103, 17302–17307.

Akahoshi, T., Hotta, K., Oka, K., 2017. Characterization of calcium transients during early embryogenesis in ascidians *Ciona robusta* (*Ciona intestinalis* type A) and *Ciona savignyi*. Dev. Biol. 431, 205–214.

Brozovic, M., Dantec, C., Dardaillon, J., Dauga, D., Faure, E., Gineste, M., Louis, A., Naville, M., Nitta, K.R., Piette, J., Reeves, W., Scornavacca, C., Simion, P., Vincentelli, R., Bellec, M., Aicha, S., Fagotto, B., Gueroult-Bellone, M., Haeussler, M., Jacox, M., Lowe, E., Mendez, E.K., Roberge, M., Stolfi, A., Yokomori, A., Brown, R., Cambillau, T., Christiaen, C., Delsuc, L., Douzery, F., Dumollard, E., Kusakabe, R., Nakai, T., Nishida, K., Satou, H., Swalla, Y., Veeman, B., Volff, M., J-N, Lemaire, P., 2018. ANISEED 2017: Extending the integrated ascidian database to the exploration and evolutionary comparison of genome-scale datasets. Nucleic Acids Res. 46 (D1), D718–D725.

Blum, M., Feistel, K., Thumberger, T., Schweickert, A., 2014. The evolution and conservation of left-right patterning mechanisms. Development 141, 1603–1613.

Burdine, R.D., Schier, A.F., 2000. Conserved and divergent mechanisms in left-right axis formation. Genes Dev. 14, 763–776.

De Santis, R., Jamunno, G., Rosati, F., 1980. A study of the chorion and the follicle cells in relation to the sperm-egg interaction in the ascidian, *Ciona intestinalis*. Dev. Biol. 74, 490–499.

Dorfer, V., Maltsev, S., Winkler, S., Mechtler, K., Amanda, M.S., 2014. A universal identification algorithm optimized for high accuracy tandem mass spectra. J. Proteome Res. 13, 3679–3684.

Firestone, A.J., Weinger, J.S., Maldonado, M., Barlan, K., Langston, L.D., O'Donnell, M., Gelfand, V.I., Kapoor, T.M., Chen, J.K., 2012. Small-molecule inhibitors of the AAA+ ATPase motor cytoplasmic dynein. Nature 484, 125–129.

Goudet, G., Mugnier, S., Callebaut, I., Monget, P., 2008. Phylogenetic analysis and identification of pseudogenes reveal a progressive loss of zona pellucida genes during evolution of vertebrates. Biol. Reprod. 78, 796–806.

Jovine, L., Qi, H., Williams, Z., Litscher, E., Wassarman, P.M., 2002. The ZP domain is a conserved module for polymerization of extracellular proteins. Nat. Cell Biol. 4, 457–461.

Jovine, L., Darie, C.C., Litscher, E.S., Wassarman, P.M., 2005. Zona pellucida domain proteins. Annu. Rev. Biochem. 74, 83–114.

Katsumoto, S., Hatta, K., Nakagawa, M., 2013. Brief hypo-osmotic shock causes test cell death, prevents neurula rotation, and disrupts left-right asymmetry in *Ciona intestinalis*. Zool. Sci. 30, 352–359.

Kessel, R.G., Kemp, N.E., 1962. An electron microscope study on the oocyte, test cells and follicular envelope of the tunicate, *Molgula manhattensis*. J. Ultrastruct. Res. 6, 57–76.

Li, W.X., 2005. Functions and mechanisms of receptor tyrosine kinase Torso signaling: lessons from *Drosophila* embryonic terminal development. Dev. Dyn. 232, 656–672.

Martínez-Bartolomé, S., Navarro, P., Martín-Maroto, F., López-Ferrer, D., Ramos-Fernández, A., Villar, M., García-Ruiz, J.P., Vázquez, J., 2008. Properties of average score distributions of SEQUEST: the probability ratio method. Mol. Cell. Proteom. 7, 1135–1145.

Meno, C., Gritsman, K., Ohishi, S., Ohfuji, Y., Heckscher, E., Mochida, K., Shimono, A.,

Kondoh, H., Talbot, W.S., Robertson, E.J., et al., 1999. Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. Mol. Cell 4, 287–298.
Morokuma, J., Ueno, M., Kawanishi, H., Saiga, H., Nishida, H., 2002. *HrNodal*, the ascidian *nodal*-related gene, is expressed in the left side of the epidermis, and lies upstream of *HrPitx*. Dev. Genes Evol. 212, 439–446.
Namigai, E.K., Kenny, N.J., Shimeld, S.M., 2014. Right across the tree of life: the evolution of left-right asymmetry in the Bilateria. Genesis 52, 458–470.
Negishi, T., Miyazaki, N., Murata, K., Yasuo, H., Ueno, N., 2016. Physical association between a novel plasma-membrane structure and centrosome orients cell division. eLife 5, e16550.
Nishida, H., 2003. Spatio-temporal pattern of MAP kinase activation in embryos of the ascidian *Halocynthia roretzi*. Dev. Growth Differ. 45, 27–37.
Nishide, K., Mugitani, M., Kumano, G., Nishida, H., 2012. Neurula rotation determines left-right asymmetry in ascidian tadpole larvae. Development 139, 1467–1475.
Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M., Hirokawa, N., 1998. Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. Cell 95, 829–837.
Nonaka, S., Shiratori, H., Saijoh, Y., Hamada, H., 2002. Determination of left-right patterning of the mouse embryo by artificial nodal flow. Nature 418, 96–99.
Okada, Y., Takeda, S., Tanaka, Y., I-Belmonte, J.C., Hirokawa, N., 2005. Mechanism of nodal flow: a conserved symmetry breaking event in left-right axis determination. Cell 121, 633–644.
Okumura, H., Kohno, Y., Iwata, Y., Mori, H., Aoki, N., Sato, C., Kitajima, K., Nadano, D., Matsuda, T., 2004. A newly identified zona pellucida glycoprotein, ZPD, and dimeric ZP1 of chicken egg envelope are involved in sperm activation on sperm–egg interaction. Biochem. J. 384, 191–199.
Pal, K., Mukhopadhyay, S., 2015. Primary cilium and sonic hedgehog signaling during neural tube patterning: role of GPCRs and second messengers. Dev. Neurobiol. 75, 337–348.
Palmquist, K., Davidson, B., 2017. Establishment of lateral organ asymmetries in the invertebrate chordate, *Ciona intestinalis*. EvoDevo 8, 12.
Sasai, N., Briscoe, J., 2012. Primary cilia and graded Sonic Hedgehog signaling. Wiley Interdiscip. Rev. Dev. Biol. 1, 753–772.
Satoh, N., 2009. An advanced filter-feeder hypothesis for urohordate evolution. Zool. Sci. 26, 97–111.
Sawada, H., Sakai, N., Abe, Y., Tanaka, E., Takahashi, Y., Fujino, J., Kodama, E., Takizawa, S., Yokosawa, H., 2002. Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. Proc. Natl. Acad. Sci. USA 99, 1223–1228.
Sawada, H., Tanaka, E., Ban, S., Yamasaki, C., Fujino, J., Ooura, K., Abe, Y., Matsumoto, K., Yokosawa, H., 2004. Self/nonself recognition in ascidian fertilization: vitelline coat protein HrVC70 is a candidate allorecognition molecule. Proc. Natl. Acad. Sci. USA 101, 15615–15620.
Schier, A.F., Shen, M.M., 2000. nodal signalling in vertebrate development. Nature 403, 385–389.
Shimeld, S.M., Levine, M., 2006. Evidence for the regulation of left-right asymmetry in *Ciona intestinalis* by ion flux. Dev. Dyn. 135, 1534–1553.
Smith, J., Paton, I.R., Hughes, D.C., Burt, D.W., 2005. Isolation and mapping the Chicken zona pellucida genes: an insight into the evolution of orthologous genes in different species. Mol. Reprod. Dev. 70, 133–145.
Spargo, S.C., Hope, R.M., 2003. Evolution and nomenclature of the zona pellucida gene family. Biol. Reprod. 68, 358–362.
Spéder, P., Petzoldt, A., Suzanne, M., Noselli, S., 2007. Strategies to establish left/right asymmetry in vertebrates and invertebrates. Curr. Opin. Genet. Dev. 17, 351–358.
Stein, D.S., Stevens, L.M., 2014. Maternal control of the Drosophila dorsal-ventral body axis. Wiley Interdiscip. Rev. Dev. Biol. 3, 301–330.
Sugino, Y.M., Tominaga, A., Takashima, Y., 1987. Differentiation of the accessory cells and structural regionalization of the oocyte in the ascidian *Ciona savignyi* during early oogenesis. J. Exp. Zool. 242, 205–214.
Takaoka, K., Yamamoto, M., Hamada, H., 2007. Origin of body axes in the mouse embryo. Curr. Opin. Genet. Dev. 17, 344–350.
Takao, D., Nemoto, T., Abe, T., Kiyonari, H., Kajiura-Kobayashi, H., Shiratori, H., Nonaka, S., 2013. Asymmetric distribution of dynamic calcium signals in the node of mouse embryo during left-right axis formation. Dev. Biol. 376, 23–30.
Taniguchi, K., Nishida, H., 2004. Tracing cell fate in brain formation during embryogenesis of the ascidian *Halocynthia roretzi*. Dev. Growth Differ. 46, 163–180.
Thompson, H., Shaw, M.K., Dawe, H.R., Shimeld, S.M., 2012. The formation and positioning of cilia in *Ciona intestinalis* embryos in relation to the generation and evolution of chordate left-right asymmetry. Dev. Biol. 364, 214–223.
Vandenberg, L.N., Levin, M., 2013. A unified model for left-right asymmetry? Comparison and synthesis of molecular models of embryonic laterality. Dev. Biol. 379, 1–15.
Wassarman, P.M., 2008. Zona pellucida glycoproteins. J. Biol. Chem. 283, 24285–24289.
Yamada, L., Saito, T., Taniguchi, H., Sawada, H., Harada, Y., 2009. Comprehensive egg coat proteome of the ascidian *Ciona intestinalis* reveals gamete recognition molecules involved in self-sterility. J. Biol. Chem. 284, 9402–9410.
Yamada, S., Tanaka, Y., Imai, K.S., Saigou, M., Onuma, T.A., Nishida, H., 2019. Wavy movements of epidermis monocilia drive the neurula rotation that determines left–right asymmetry in ascidian embryos. Dev. Biol..
Yoshida, K., Saiga, H., 2008. Left-right asymmetric expression of *Pitx* is regulated by the asymmetric nodal signaling through an intronic enhancer in *Ciona intestinalis*. Dev. Genes Evol. 218, 353–360.
Yoshida, K., Saiga, H., 2011. Repression of Rx gene on the left side of the sensory vesicle by nodal signaling is crucial for right-sided formation of the ocellus photoreceptor in the development of *Ciona intestinalis*. Dev. Biol. 354, 144–150.
Yuan, S., Zhao, L., Brueckner, M., Sun, Z., 2015. Intraciliary calcium oscillations initiate vertebrate left-right asymmetry. Curr. Biol. 25, 556–567.