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Early development of amphioxus links evolutionary events with vertebrates

KINYA YASUI*

Department of Anthropology, National Museum of Nature and Science, Amakubo, Tsukuba, Ibaraki, Japan

ABSTRACT Comparison of early development is a powerful approach to understand how spherical embryos set up the basis for body patterning. Localization of the germ plasm likely couples with the site of gastrulation in many animals including cnidarians. A center of single or complex Wnt signaling pathway(s) is also co-localized with germ plasm and plays a role primarily in antero-posterior patterning in most animals. In addition, a Nodal signaling center appears in deuterostomes perpendicular to Wnt signaling and governs dorso-ventral patterning. Wnt and Nodal signaling pathways function as orthogonal coordinates to pattern embryos in three-dimensions. Amphioxus early embryos establish similar coordinates, but the location of Wnt signaling along the equator might modify the function of the Nodal signaling center to specify the chordate basic body pattern inverting dorso-ventral polarity, which is thought to have occurred during the evolution of deuterostomes. Surprising similarities in early developmental processes found in cnidarians and deuterostomes, and divergences of cnidarians-bilaterians, deuterostomes-protostomes, and ambulacrarians-chordates occurred one after another possibly within a geologically short period may link molecular mechanisms that gave rise to bilaterians, deuterostomes, and chordates.

KEY WORDS: *lancelet, cnidarian, signaling center, germ plasm, body patterning*

Introduction

Cephalochordate amphioxus (or lancelets) have long attracted many biologists who marvel in animal evolution, at least since the time of Russian embryologist A. O. Kowalevsky, who documented for the first time common developmental features shared by chordate embryos (Kowalevsky, 1867, 1871). When developmental biology and genetics began to be integrated in the 1980s via the introduction of molecular approaches, amphioxus was thus soon added to the list of research targets (Holland *et al.*, 1992). However, owing to the lack of an adequate background of molecular studies unlike as in fruit flies, mice, sea urchins, frogs, zebrafish, and especially ascidians, the contribution of amphioxus studies to evolutionary developmental biology has been limited unfortunately.

Among the extant animals, amphioxus displays development and anatomy most similar to those of vertebrates. Seemingly for this reason, most studies on amphioxus since the 1990s have tried to find out how amphioxus development is homologous with that of vertebrates from the viewpoint of molecules. However, it has long been known that amphioxus possesses its own specific features, for example left-right asymmetrical development. Furthermore, fossil evidence and recent phylogenetic analyses suggest a deep split of the amphioxus lineage from the other chordates, coeval with the diversification of phyla during the Cambrian Explosion (Shu *et al.*, 1999; Erwin *et al.*, 2011; Yue *et al.*, 2015). Phylogenetic analyses also suggest that many of the major divergences such as cnidarians-bilaterians, superphyla including Deuterostomia and Protostomia within bilaterians, ambulacrarians-chordates, and cephalochordates-olfactoreans (tunicates + vertebrates) occurred soon before or after the Precambrian/Cambrian boundary dated to 542 Ma (Antcliffe, 2012; Cavalier-Smith, 2017). The phylogenetic position of amphioxus thus implicates the possibility that extant amphioxus still retains unique characters that can help unravel the origin of chordates, which might have been lost in the olfactorean lineage.

Molecular developmental studies are continuously providing new approaches to help understand a large set of data obtained from a variety of animals from sponges to vertebrates. These studies, especially on cnidarians and bilaterians, emphasize common mechanisms underlying the initial organization in eggs and early embryos to coordinate the apico-blastoporal, dorso-ventral (or directive), and left-right body axes, as well as their interactions.

Abbreviations used in this paper: BMP, bone morphogenetic protein.

***Address correspondence to:** Kinya Yasui. Department of Anthropology, National Museum of Nature and Science, Amakubo, Tsukuba, Ibaraki, Japan
e-mail: furaha13@icloud.com - http://orcid.org/0000-0002-6704-883X

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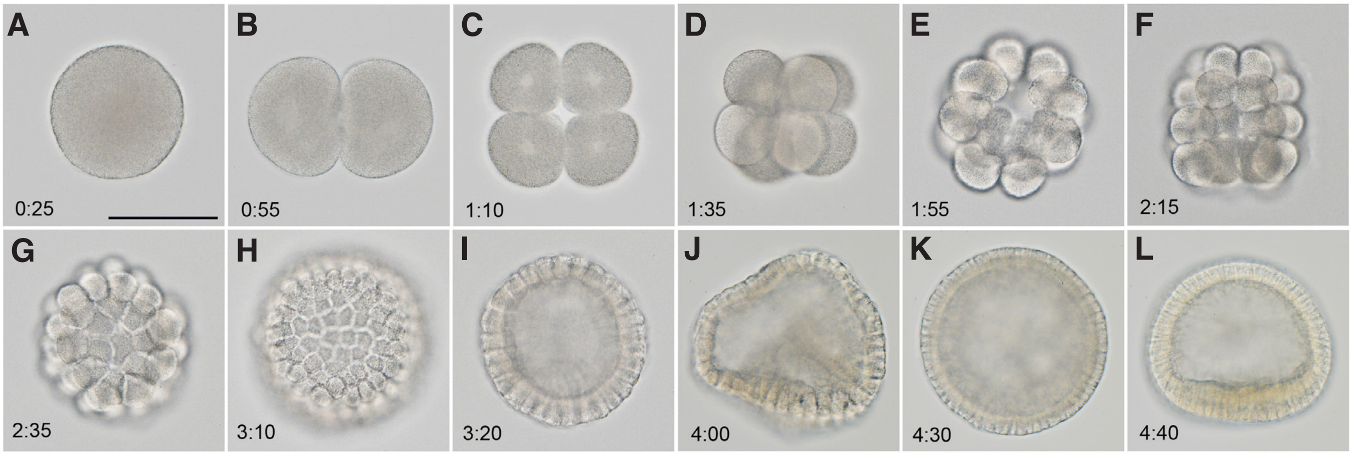

Fig. 1. Embryonic development of amphioxus *Branchiostoma japonicum* up to initial gastrula. *After developing into 128-cell blastula, cell division becomes unsynchronized* **(J)**. **(A)** *1-cell,* **(B)** *2-cell,* **(C)** *4-cell,* **(D)** *8-cell,* **(E)** *16-cell,* **(F)** *32-cell,* **(G)** *64-cell,* **(H)** *128-cell,* **(I)** *128-cell smooth surface blastula,* **(J)** *deformed blastula,* **(K)** *spherical late blastula, and* **(L)** *initial gastrula. Times post fertilization are in development at 25°C. Scale bar 100 μm.*

Viewing these common mechanisms, a common origin of ciliate larvae (Marlow *et al*., 2014), a basic pattern of bilaterian nervous system (Arendt *et al*., 2016), and the origin of chordate body pattern (Morov *et al*., 2016) have all been proposed. In this review, I integrate advances in recent understandings of early embryonic patterning in cnidarians and deuterostomes that display easily common and comparable early embryonic stages, and discuss the potential contribution of amphioxus developmental studies to the broad understanding of bilaterian evolution leading to chordates.

Amphioxus has an advantage in comparative studies of early development

As a member of the chordates, one advantage of amphioxus in comparative studies is that despite its body being reminiscent of vertebrate bodies, amphioxus develops from a spherical coeloblastula that is comparable to outgroup ambulacrarian blastulae (Giudice, 1986; Röttinger *et al*., 2015) and even remotely to some cnidarian blastulae (Lee *et al*., 2007). A fertilized egg of amphioxus starts cleaving at 40 min after sperm entry and then synchronously with 20 min intervals, producing almost equal-sized blastomeres (vegetal blastomeres are slightly larger than animal ones, in general) at 24°C in the case of *Branchiostoma japonicum* (Sino-Japanese populations formerly assigned to *B. belcheri*) (Fig. 1). Synchronized cleavage up to the 128-cell stage produces a spherical single-layered blastula. Then, likely after two rounds of unsynchronized cleavages that largely deform the blastula shape to become similar to that of an anthozoan *Nematostella* blastula (Fritzenwanker *et al*., 2007), the vegetal side of the recovered spherical blastula becomes flattened to start gastrulation (Fig. 1J-L). Although cnidarians are phylogenetically distinct from bilaterian chordates, a considerable number of cnidarian species show cleavage patterns similar to chordate radial and equal cleavage, which might suggest that radial cleavage is an ancestral character. The cleavage of amphioxus egg is commonly cited in textbooks as a typical radial and equal cleavage. Passing through this cleavage and gastrulation, the antero-posterior axis of the embryo is kept to coincide with the egg animal-vegetal axis as in cnidarians and ambulacrarians (Yu *et al*., 2003), but is never rotated unlike as ascidians and vertebrates do (Koide *et al*., 2002; Lee *et al*., 2006; Nishida, 2005).

Ambulacrarian sea urchins and hemichordate acorn worms show similar cleavage patterns to the amphioxus pattern and produce spherical blastulae, but their blastomeres are more unequal between meridian blastomeres than in amphioxus, particularly in the case of macromeres and micromeres in sea urchin embryos (Giudice, 1986; Röttinger and Lowe, 2012). In these animals, unsynchronized cleavage starts earlier than in amphioxus embryos. These differences in cleavage are apparently related to the selective inheritance of specific maternal molecules and cytoplasm into blastomeres. At the blastula stage, amphioxus and ambulacrarians attain mutually comparable spherical blastulae comprising almost equal-sized blastomeres, which enables the understanding of very basic spatial patterning of mRNAs that regulate zygotic genes for initializing body patterning under common coordinates. Their coeloblastulae also enable comparison with anthozoan development. Ascidians and vertebrates are derived in this aspect, and thus it is difficult to episodically compare their early development to that of remotely related animals.

Germ plasm and localization of maternal mRNAs

Although it is still controversial which is ancestral in germ line segregation in animals between preformation and epigenesis (Extavour and Akam, 2003; Leclère *et al*., 2012), many animals produce molecular milieu(s) called the germ plasm or "nuage" in oocytes to segregate germ cells from somatic cells. The germ plasm is usually formed near the nuclear membrane in growing oocytes and comprises mitochondria, cytoskeletons, ERs, proteins and RNA/protein complexes called RNPs (Kloc *et al*., 2014). The centrosome is reported to act as a seed for the aggregation of the germ plasm (Kloc *et al*., 2004). Maternal mRNAs and sometimes also their encoding proteins in the germ plasm represented by *vasa*, *nanos*, *piwi*, *pl10*, and *pumilio* regulate zygotic genes expression in germ line progenitors (King *et al*., 2005; Juliano *et al*., 2006; Cuykendall and Houston; 2010; Voronina *et al*., 2011). The germ plasm or a subcellular structure derived from the nuage, however, also contains mRNAs responsible for gastrulation in many animals and for endoderm and/or dorsal specification in cnidarians and deuterostomes such as those encoding proteins involved in Wnt signaling, Nodal, and T-box proteins (Zhang *et al.*, 1998; Tao *et al.*, 2005; Amiel and Houliston, 2009; Cuykendall and Houston, 2010; Gilligan, 2011). In anamniote vertebrates such as zebrafish and *Xenopus*, the nuage is formed and enlarged on the vegetal side of perinucleus as a Balbiani body and is translocated to the vegetal pole during oogenesis (Kloc and Etkin, 2005; Kosaka *et al.*, 2007).

In cnidarians, the sea anemone *Nematostella* does not show unambiguous aggregation of the germ plasm in oocytes, but mediated by an animally located germinal vesicle (nucleus in oocyte) with unknown mechanisms, transcripts from duplicated *vasa* and *nanos* genes are localized on the animal side (Extavour *et al.*, 2005) (Fig. 2). Through the gastrulation that occurs at the animal pole, *vasa* and *nanos* mRNAs are then distributed around the blastopore and in the newly forming archenteron. In the hydrozoan *Clytia*, perinuclear aggregates of the germ plasm in growing oocytes have been detected by *in situ* hybridization with germ plasm specific *piwi*, *nanos*, *vasa*, and *pl10* probes (Leclère *et al.*, 2012). These mRNAs in mature oocytes surround the female pronucleus at the animal pole like *Nematostella* oocytes and are inherited by an i-cell population around the future blastopore during cleavage. The i-cell population finally distributes into the entodermal (endodermal) inner layer, comparable to as what occurs in the case of *Nematostella* (Leclère *et al.*, 2012). As the hydrozoan i-cell population is made up of a kind of stem cell that can differentiate both to germ cells and various types of somatic cells through the adult life (Watanabe *et al.*, 2009), the behavior of the *Clitya* germ plasm is comparable to that of other preformation bilaterians. In association with the germ line mRNAs, mRNAs/proteins involved in Wnt/β-Catenin signaling (Plickert *et al.*, 2006; Amiel and Houliston, 2009) and Strabismus that initiates Wnt/PCP signaling at the initial gastrulation (Kumburegama *et al.*, 2011) are localized at the animal cortex. As a result of their localization of the maternal factors, cnidarian embryos start gastrulation at the animal pole (Lee *et al.*, 2007; Martindale and Hejnol, 2009). Furthermore, comparable to the vertebrate dorsal organizer as the Spemann's organizer in amphibians and its equivalents, the blastopore margin on the animal side of *Nematostella* gastrulae can induce a secondary embryonic axis when it is grafted to another embryo (Kraus *et al.*, 2007, 2015).

Amphioxus oocyte dynamics including the nuage and the localization of maternal factors are still poorly characterized, but some maternal mRNAs in eggs have been reported. Some germ line marker mRNAs such as *vasa*, *nanos*, *piwi1*, *pl10*, *bruno2*, and *tdrd* (tudor-domain containing) are localized near the vegetal pole, and others such as *pumilio*, *mago-nashi*, and *mex3* are near the animal poles with some ubiquitously distributed mRNAs (Wu *et al.*, 2011; Zhang *et al.*, 2013; Yue *et al.*, 2015; Dailey *et al.*, 2016). Seemingly corresponding to these bipolar distributions of the germ line markers, mRNAs related to Wnt/β-Catenin signaling are not localized to the vegetal side, from which gastrulation starts (Qian *et al.*, 2013; Wang *et al.*, 2016). Although *wnt1*, *-6*, *-9*, *-11* mRNAs are reported to be expressed maternally, their expression is too weak to detect their distribution in eggs (Qian *et al.*, 2013). These data may explain the ubiquitous nuclear accumulation of β-Catenin in cleaving embryos in amphioxus (Yasui *et al.*, 2002; Holland *et al.*, 2005). Interestingly, however, two mRNAs, *tcf* and *dsh* (dishevelled), that are involved in Wnt/β-Catenin signaling show localization on the animal side contrary to as in sea urchins and vertebrates (Lin *et al.*, 2006; Wang *et al.*, 2016).

In sea urchins, although the Balbiani body was recently reported in young oocytes, their later behavior during oogenesis is different from anamniote vertebrates and they are dispersed into the cortical region (Yakovlev, 2016) (Fig. 2). Despite the fact that sea urchin eggs do not translocate the perinuclear Balbiani body to the vegetal pole, small micromeres that are formed by the fifth cell division at the vegetal pole display features of typical germ cell precursors expressing *vasa* and *nanos* genes (Yajima nad Wessel, 2012). Furthermore, a protein involved in Wnt/β-Catenin signaling, Dsh, is localized at the vegetal pole as in *Xenopus* (Peng and Wikramanayake, 2013) and the earliest Wnt/β-Catenin signaling is activated in the vegetal hemisphere, opposite to as in cnidarian embryos (Weitzel *et al.*, 2004; Wikramanayake *et al.*, 2004).

A Tgfβ family member, Nodal is also an important molecule in the basic body patterning in many animals as will be mentioned below. Maternal *squint* (= nodal) mRNA in zebrafish (Gore *et al.*, 2005) and *nodal* mRNA in amphioxus (Morov *et al.*, 2016) are critical for dorsal specification. In sea urchin and *Xenopus* embryos, although *nodal* gene is expressed zygotically, it is regulated by other Tgfβ members, Panda (Haillot *et al.*, 2015) and Vg1 (Birsoy *et al.*, 2006), respectively, both of which are expressed maternally. In vertebrates, some mRNAs encoding maternal Tgfβ proteins are

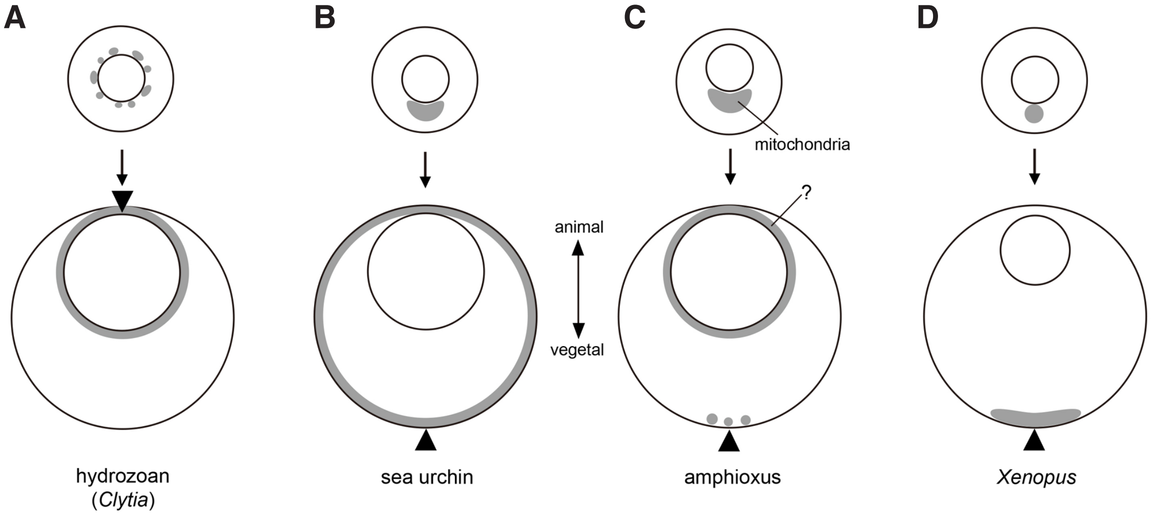

Fig. 2. Schematic drawing of germ plasm formation. *Localization of germ plasm in young and mature oocytes is indicated as gray zones. Drawing for amphioxus oocytes is tentative. Arrowheads denote the site of gastrulation.* **(A)** *Cnidarian hydrozoan,* **(B)** *sea urchin,* **(C)** *amphioxus, and* **(D)** Xenopus.

co-localized with the germ plasm at the vegetal pole, whereas amphioxus *nodal* mRNA is ubiquitously distributed in unfertilized eggs and then accumulated in the animal hemisphere after sperm entry (Onai *et al*., 2010; Morov *et al*., 2016).

As reported for cnidarians and ascidians, although maternal mRNAs of germ line markers are aggregated in nuages or the germ plasm, the majority of these mRNAs are distributed evenly in the ooplasm, and germ line progenitors can be restored when the aggregate of the mRNAs is eliminated (Takamura *et al*., 2002; Shirae-Kurabayashi *et al*., 2006; Leclère *et al*., 2012). These observations make us cautious not to underestimate restoration mechanisms during development. Also, there are mRNAs important for initial body patterning, which have been observed to behave independently of the germ plasm (Pelegri, 2003). However, it is intriguing that the distribution pattern of maternal mRNAs in amphioxus eggs is somehow intermediate between cnidarian and sea urchin/vertebrate patterns. In amphioxus, a Balbiani body-like structure is discernible as an aggregate of mitochondria in young oocytes (Figs. 2, 3), but *bucky-ball* and *oskar* genes that are involved in germ plasm formation in vertebrates or insects are absent (Marlow *et al*., 2008; Ewen-Campen *et al*., 2012). The mechanisms underlying perinuclear nuage aggregation in cnidarians and those producing the Balbiani body on the vegetal side in sea urchins and anamniote vertebrates are apparently key cues to understand the observed different strategies for the localization of maternal factors.

Patterning of Wnt signaling and anti-Wnt components in blastulae

At the blastula stage, animals that produce a single-layered spherical coeloblastula display impressive gene expression patterns in their blastulae (Fig. 4). They show apico-blastoporal axial patterning that corresponds to the vegetal-animal axis in cnidarians and to the animal-vegetal axis in bilaterians (Marlow *et al*., 2014). The inversion between cnidarians and bilaterians reflects the opposite localization of maternal factors mentioned above. The apical domain is characterized with transcription factors such as *foxq2* and *six3/6*, as well as with an Wnt receptor, *fzd5/8* (frizzled) in concentric expression, whereas the blastoporal domain is characterized with concentric expressions of wnt paralogues (Range *et al*., 2013; Cui *et al*., 2014). In the apical domain, *fzd5/8* is regarded as a mediator of Wnt that emanates from the opposite blastoporal side, and Wnt antagonists such as *dkk1*, *3* (dickkopf) and *sfrp* (secreted frizzled-like protein) are also expressed, depending on animal species (Lee *et al*., 2006; Range and Wei, 2016). Wnt signaling pathway(s) in the blastoporal domain regulates the size of the apical domain (Range *et al*., 2013), where cells extend long cilia to form an apical tuft and several types of sensory/neurosecretory cells differentiate to constitute an apical organ at larval stages (Marlow *et al*., 2014). Many protostome animals develop from rather specialized eggs. However, eutrochozoans that produce trochophore larvae also display a comparable gene expression pattern in the apical domain on the animal side, although Wnt signaling on the vegetal side is not well documented (Marlow *et al*., 2014).

In amphioxus blastulae, of the cardinal transcriptional factors in the apical domain, *foxq2* and *six3*, only *foxq2* is expressed broadly on the animal side towards the equatorial zone (Fig. 5). Initial *foxq2* expression in almost the whole embryonic body and receding towards the animal hemisphere suggests an interaction with Wnt8 signaling. On the other hand, *six3/6* expression is completely different from typical apical expression and occupies the dorso-vegetal region and then gastrulating archenteron (Fig. 5). These expression patterns may be related to the apical expression of the Wnt antagonist *dkk3* (Yu *et al*., 2007) and ubiquitous expression of the Wnt receptor *fzd5/8* (Qian *et al*., 2013). On the blastoporal side, typical concentric expression of wnt paralogues does not appear, but instead the earliest expression detectable by *in situ* hybridization is circular expression of *wnt8* along the equator at the late blastula stage (Yasui *et al*., 2001; Morov *et al*., 2016). Interestingly in amphioxus blastulae, Wnt antagonists *dkk1/2/4*, *sfzp3/4*, and *sfzp2*-like are expressed on the blastoporal side (Yu *et al*., 2007). These gene expression profiles in amphioxus blastulae are atypical compared with those showing counter-gradients between Wnt signal and anti-Wnt components in other animals. Consistently, amphioxus embryos show no specific nuclear β-Catenin gradient but instead ubiquitous nuclear accumulation in blastulae (Yasui *et al*., 2002; Holland *et al*., 2005) and do not develop an apical tuft on the apical side.

In animals that develop to spherical coeloblastula and then gastrulate in the manner of invagination or its variants, the Wnt expression domain(s) at a pole defines the future blastopore margin. Beside Strabismus in *Nematostella* (Kumburegama *et al*., 2011), however, it is still unknown how and which Wnt signaling functions in gastrulation in each animal. In amphioxus embryos, only *wnt8* expression has been confirmed in blastulae (Yasui *et al*., 2001; Yu *et al*., 2007; Morov *et al*., 2016). Its circular expression along the equator is soon followed by *brachyury1* expression in the same domain, and the flattening of the vegetal plate expands to the equatorial region (Fig. 1L), suggesting that Wnt8 signaling plays a major role in amphioxus gastrulation. Many wnt paralogues are expressed maternally, but in *wnt8* this is not the case (Qian *et al*., 2013).

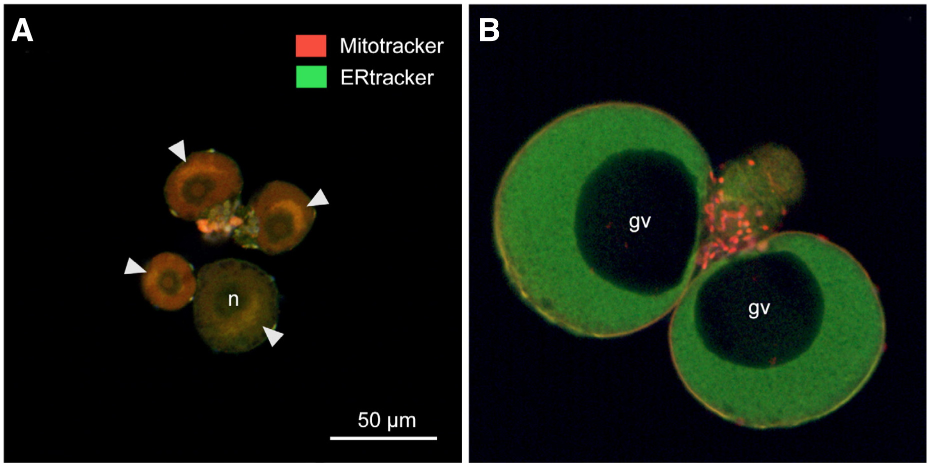

Fig. 3. Mitochondrial distribution in amphioxus oocytes. (A) *Mitochondrial aggregates are found on the opposite side of attaching site to germinal epithelium (vegetal side) in young oocytes (arrowheads).* **(B)** *There are no signals of Mitotracker in growing oocytes. Abbreviations: gv, germinal vesicle; n, nucleus. Scale bar, 50 μm.*

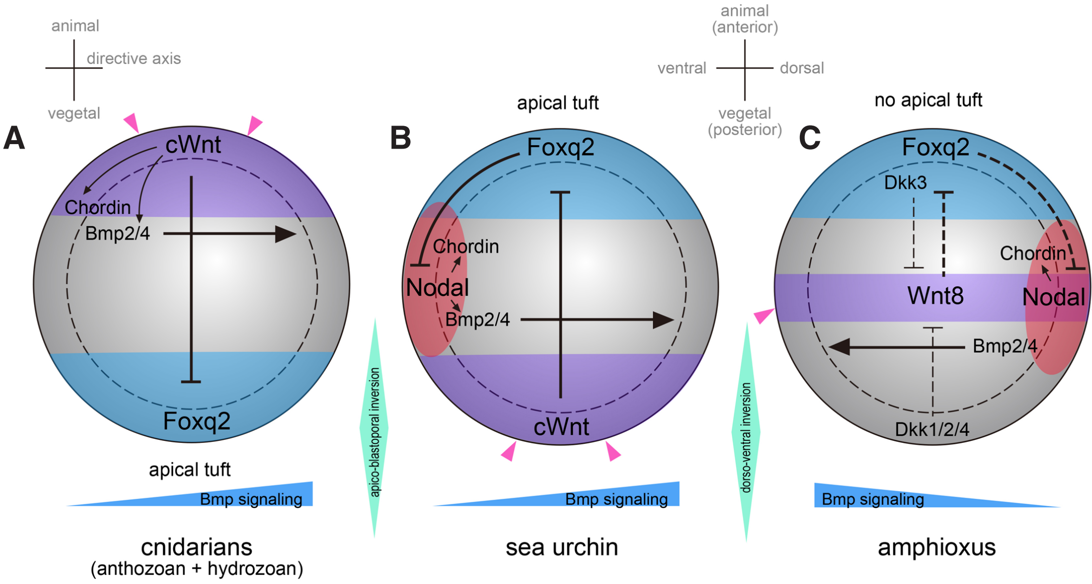

Fig. 4. Comparison of orthogonal Wnt and Nodal (Bmp) signalings. (A) *Cnidarian,* **(B)** *sea urchin, and* **(C)** *amphioxus blastulae. Representative genes (or their products) and their interactions are indicated. Arrowheads denote the site of gastrulation. Apico-blastoporal inversion between cnidarians and sea urchins, and dorso-ventral inversion between sea urchins and amphioxus are indicated.*

Because Wnt signaling on the blastoporal side likely functions as the initiator not only of gastrulation, but also of the apical specification to pattern embryonic apico-blastoporal axis, it is important to understand how the amphioxus system is derived from the typical pattern found in sea anemone and sea urchin embryos (Lee *et al.*, 2007; Röttinger, 2012; Range *et al.*, 2013; Watanabe *et al.*, 2014).

Chordin and Bmp in dorso-ventral specification

The apico-blastoporal specification by Wnt signaling leads to antero-posterior body patterning, but this signaling itself does not give rise to dorso-ventral patterning. Bmp (Dpp; Decapentaplegic) and Chordin (Sog; Short gastrulation) play central roles in the dorso-ventral patterning in bilaterians (Sasai *et al.*, 1995; Biehs *et al.*, 1996). In the comparison between *Xenopus* and *Drosophila*, it was found out that *chordin* (*sog*) was expressed consistently side where the nerve cord develops and *bmp4* (*dpp*) was expressed on the opposite side. These two genes thus have become a pair of good markers for dorso-ventral polarity (Lowe *et al.*, 2006). In sea urchin embryos, however, both *bmp2/4* and *chordin* are activated by Nodal signaling and co-expressed on the ventral (oral) side (Lapraz *et al.*, 2009). In this situation, Bmp2/4 function is blocked by Chordin co-existing on the ventral side, but is accomplished on the dorsal (aboral) side by a Chordin-Bmp2/4 complex releasing Bmp2/4. Thus Bmp2/4 signaling on the dorsal side and Chordin blocking of this signaling on the ventral side correspond to protostome polarity.

Although sea anemone embryos do not differentiate dorso-ventral polarity, they display a secondary axis called the directive axis perpendicular to the primary animal-vegetal (blastoporal-apical) axis.

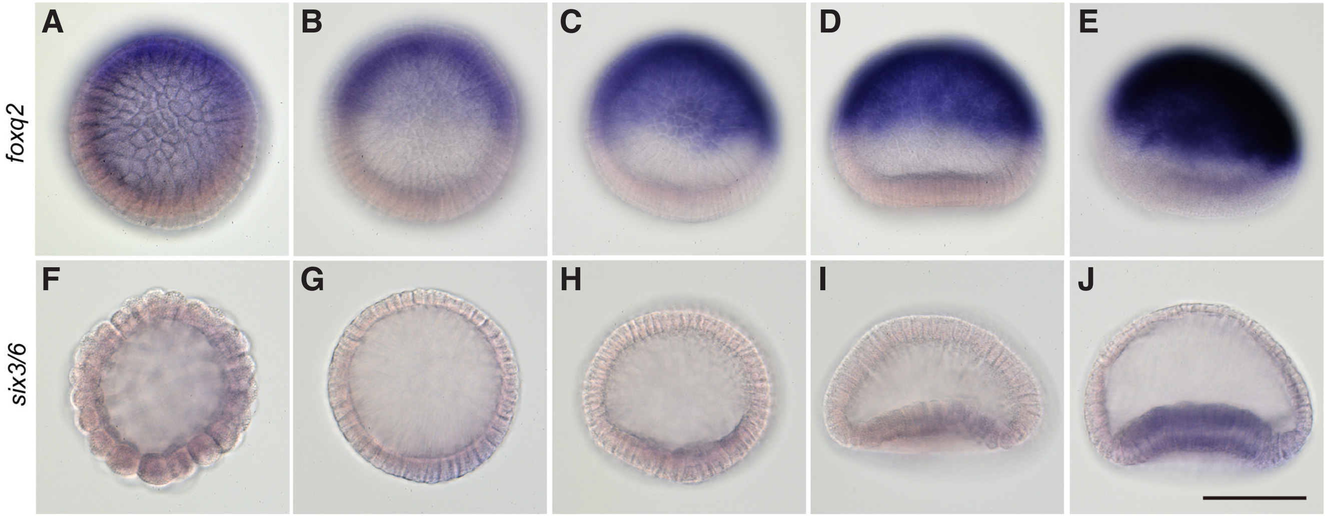

Fig. 5. Expression pattern of *foxq2* and *six3/6* at blastula and gastrula stages. (A-E) *Whole-mount* in situ *hybridization probed with* foxq2. **(A)** *Early blastula,* **(B)** *late blastula,* **(C)** *onset of gastrulation,* **(D)** *gastrula with flattened vegetal plate, and* **(E)** *invaginating vegetal plate.* **(F-J)** *Whole-mount* in situ *hybridization probed with* six3/6. **(F)** *128-cell stage, and* **(G-J)** *correspond to* **(B-E)** *in stage. Scale bar, 100 μm.*

Interestingly, sea anemone embryos first express both *bmp2/4* and *chordin* genes around the blastopore margin and then translocate these expressions to one side of the future directive axis, where *chordin* is expressed in the outer layer (ectoderm) and *bmp2/4* is in the inner layer (entoderm) (Rentzsch *et al*., 2006; Saina *et al*., 2009). The gradient of Bmp2/4 signaling, however, is highest on the opposite side as in sea urchin embryos, and GLWA positive neurons in the circular oral nervous system appear specifically on the weaker side (= *chordin* expression side) of Bmp2/4 signaling (Watanabe *et al*., 2014). The asymmetrical expression of *bmp2/4* and *chordin* on the same side and the counter-gradient of Bmp2/4 signaling in sea urchin and sea anemone embryos are comparable to each other, and may be a retained ancestral feature (Lapraz *et al*., 2009). Furthermore, the asymmetrical development of GLWA positive neurons in the oral nervous system on the *chordin* side is reminiscent of the neuronal patterning in bilaterian trunk nervous systems (Watanabe *et al*., 2014), or more specifically of the central nervous system developing on the dorsal *chordin* side in chordates as Bmp2/4 signaling at the mid-ventral region in amphioxus embryos induces primary sensory neurons in the ectoderm (Lu *et al*., 2012).

Amphioxus dorso-ventral patterning is the same as that of vertebrates, and has been deduced from their expression patterns (Panopoulou *et al*., 1998; Morov *et al*., 2016), Chordin from the notochord most likely suppresses Bmp2/4 signaling in the dorsal ectoderm and functions to differentiate it into the neural plate as seen in vertebrates. However, both *bmp2/4* and *chordin* are expressed in the archenteron on dorsal side and Bmp2/4 signaling is activated on the ventral side (Morov *et al*., 2016). This pattern is the same as in sea urchin embryos, but the direction is opposite along the dorso-ventral axis.

In sea anemone embryos, the activation of both *bmp2/4* and *chordin* are dependent on Wnt/β-Catenin signaling (Watanabe *et al*., 2014), and in sea urchin embryos both genes are regulated by Nodal signaling (Lapraz *et al*., 2009) that is indirectly regulated by Wnt/β-Catenin signaling (Range *et al*., 2013). In hydra budding, Nodal signaling, which is utilized in the laterality of budding, is reported to be a downstream target of Wnt/β-Catenin signaling (Watanabe *et al*., 2014). Amphioxus *chordin* is regulated as a downstream target gene by Nodal signaling as seen in sea urchin embryos (Yu *et al*., 2007; Morov *et al*., 2016), but the upstream regulator(s) of zygotic *bmp2/4* in amphioxus is unknown, although blocking of Nodal signaling affects the *bmp2/4* expression pattern, suggesting the involvement of Nodal signaling (Morov *et al*., 2016). These lines of circumstantial evidence suggest that both *bmp2/4* and *chordin* expression on the same side and the counter-gradient of Bmp2/4 signaling are ancestral features that have been retained in cnidarians and deuterostome sea urchins and amphioxus (Fig. 4).

Nodal signaling at the initial embryonic patterning

Nodal signaling was originally recognized as a regulator for mesoendodermal specification, as well as for antero-posterior and left-right axes specification before and during gastrulation in vertebrates (Schier and Shen, 2000). Of these functions, the role in left-right asymmetrical development has been highlighted (Saijoh *et al*., 2000; Duboc *et al*., 2005), and a similar Nodal signaling function was found even in a cnidarian hydra as a unit involving Pitx was reminiscent of its bilaterian counterpart, suggesting a deep origin of this signaling unit in animals (Watanabe *et al*., 2014).

In sea urchin embryos, another important role of Nodal signaling has been elucidated. A center of Nodal signaling is established in spherical blastulae perpendicular to the blastoporal-apical (vegetal-animal) Wnt/β-Catenin signaling regime (Duboc *et al*., 2005; Yaguchi *et al*., 2008; Molina *et al*., 2013; Range *et al*., 2013). Accordingly, in sea urchin blastulae, orthogonal signaling systems, Wnt/β-Catenin signaling along the antero-posterior axis, and Nodal signaling along the dorso-ventral axis regulate embryonic patterning. As the Nodal signaling center is established by zygotic Nodal-Lefty interaction (Duboc *et al*., 2005), it remains unknown how maternal factors regulate the formation of the center, although an asymmetrical redox gradient affected by mitochondria distribution in fertilized eggs (Coffman *et al*., 2001, 2004, 2009, 2014) or a maternal Tgfβ member Panda (*Paracentrotus* anti-nodal dorsal activity) (Haillot *et al*., 2015) have both been proposed as anti-initiators of *nodal* expression. This asymmetrical Nodal signaling activates *bmp2/4* and *chordin*, as well as *not* and *goosecoid*, and in turn Goosecoid activates *brachyury* and *foxa*, producing the dorso-ventral patterning (Molina *et al*., 2013). The Nodal signaling domain itself becomes the oral (ventral) ectoderm in sea urchin embryos (Duboc *et al*., 2005). The ventral *nodal* expression in sea urchin embryos is also indirectly regulated by the perpendicular Wnt/β-Catenin signaling as Wnt/β-Catenin indirectly downregulates *foxq2* on the animal side and Foxq2 suppresses Nodal autoregulation (Yaguchi *et al*., 2008; Range *et al*., 2013).

Amphioxus *lefty* has been known as one of the earliest genes expressed zygotically (Onai *et al*., 2010). Its expression pattern, reminiscent of sea urchin *lefty*, suggests that the amphioxus dorso-ventral patterning that involves Nodal signaling is similar to sea urchin mechanisms. Actually, *nodal* expression domain in amphioxus blastulae becomes matched with *lefty* domain across the equator and likely represents orthogonal signaling systems with some vegetal-animal signaling comparable to those in sea urchin embryos (Yaguchi *et al*., 2008; Morov *et al*., 2016). The asymmetrical Nodal signaling domain in the amphioxus spherical blastula regulates *chordin*, *goosecoid*, *not-like*, and *brachyury1* during gastrulation and then is located on one side of the margin of the blastopore, showing the same topology as the vertebrate dorsal organizer like the Spemann's organizer in amphibians (Morov *et al*., 2016).

Dorso-ventral inversion in bilaterians was proposed by Jeoffroy Saint-Hilaire in 1822. Recently, this idea has been supported as this inversion occurred during deuterostome evolution because a deuterostome hemichordate retains the same dorso-ventral polarity as that of protostomes (Lowe *et al*., 2006). The amphioxus Nodal expression domain is comparable to that in sea urchin blastulae, which further clarifies how and when the dorso-ventral inversion occurred in the bilaterian lineage. It is highly plausible that the dorso-ventral inversion occurred in the last common ancestor of chordates by converting the ancestral gene regulatory network for mouth specification to one for dorsal specification (Morov *et al*., 2016).

Nodal signaling in sea urchin embryos later displays a right-handed asymmetrical expression in prism larvae and induces *pitx2* in the mesoderm precursor cell population at the tip of archenteron on the right side (Duboc and Lepage, 2008). In *Branchiostoma japonicum* embryos, *nodal* expression at the mid-dorsal blastopore margin likely induces *pitx2* expression at the same region, which is shared with vertebrate embryos (Yasui *et al*., 2000).

The mid-dorsal expression of *nodal* ceases but asymmetrical expression in the future anterior somitic region appears in early neurulae, with the left side stronger than the right side (Yu *et al*., 2002 for *B. floridae* and personal observation for *B. japonicum*). This asymmetrical expression soon activates *pitx2* in the left first somite primordium (Yasui *et al*., 2000). Although in embryos of extant sea urchins and amphioxus asymmetrical *pitx2* expression occurs on the opposite side, it is originally the same right side when considering the dorso-ventral inversion. Furthermore, in amphioxus the asymmetrical expression of the Nodal-Pitx gene regulatory network (GRN) in the left first somite induces a laterally located larval mouth under a similar mechanism as that for coelomic pore (hydropore) formation in ambulacrarians (Kaji *et al*., 2016). This diversion is necessary for amphioxus (and broadly for chordates) as amphioxus utilized the ancestral gene regulatory network for oral specification to specify new dorsal structures (Morov *et al*., 2016). Remarkably, in vertebrates and probably in olfactoreans, the Nodal-Pitx GRN derived from the mid-dorsal blastopore margin continuously functions at the anterior-most of neurulae and forms a median mouth by inducing the anterior pan-placode (Schlosser, 2005; Soukup *et al*., 2013). These observations suggest that the deuterostome Nodal-Pitx GRN shares a common origin with its hydra counterpart (Watanabe *et al*., 2014). The parallel Nodal-*pitx2* regulation between sea urchin and amphioxus embryos is important in understanding how amphioxus larvae acquired left-right asymmetrical development.

Amphioxus development not only looks forward, it also looks back

As penta-radial echinoderm and bilateral amphioxus adults are largely different from each other, developmental studies on these two animals had, until recently, not been efficiently interactive. However, recent studies on the ventral Nodal signaling center in sea urchin embryos suggest a deep common origin with the vertebrate dorsal organizer (Molina *et al*., 2013; Lapraz *et al*., 2015). Supporting this, amphioxus embryos likely retain a developmental process to show how the ventral organizer can become the dorsal organizer (Morov *et al*., 2016). The linkage of the early development between chordates and ambulacrarians raises an important possibility; that the bilateral body of chordates does not need to have evolved from an animal that had acquired an adult bilateral body like enteropneust-type hemichordates.

Currently, it is commonly believed that the echinoderm ancestor was a bilateral animal in the morphological sense with a penta-radial body pattern in adults that was secondarily derived. Most extant echinoderms that perform metamorphosis are interpreted as manifesting a recapitulation of their evolutionary history of ancestral bilateral larvae and derived penta-radial adults. However, the earliest known echinoderm fossils, helicoplacoids, are already radially symmetrical animals (Dornbos and Bottjer, 2000), and morphologically bilateral echinoderm fossils appear later than these fossils (Zamora *et al*., 2012; Smith *et al*., 2013). Comparative studies on early development involving a wide range of animals but displaying a similar developmental strategy have shown surprisingly similar molecular strategies in their developmental processes. Cnidarian anthozoan and hydrozoan embryos show Wnt/β-Catenin signaling gradients along the apico-blastoporal axis, and *foxq2* and *six3/6* are expressed on the apical (vegetal) side (Chevalier *et al*., 2006; Sinigaglia *et al*., 2013; Marlow *et al*., 2014; Watanabe *et al*., 2014). High Wnt/β-Catenin or Wnt/PCP signaling on the animal side induces gastrulation on this side, and the blastopore margin can induce a secondary embryonic axis like the vertebrate Spemann's organizer when grafted (Kraus *et al*., 2007, 2015). These molecular mechanisms are mostly applicable to sea urchin development, although the location where the mechanisms work is inverted in terms of the animal-vegetal axis, and include the observation that vegetal micromeres can induce secondary gastrulation when transplanted to the animal pole (Ransick and Davidson, 1993). The asymmetrical expression of Bmp2/4 and Chordin on the same side are also found both in cnidarians and deuterosotmes. In sea urchin blastulae, however, the Nodal expression domain appears on the future ventral side and establishes orthogonal coordinates with Wnt/β-Catenin signals from the vegetal side (Yaguchi *et al*., 2008; Wei *et al*., 2012; Li *et al*., 2014). These molecular mechanisms in sea urchin embryos are in turn found in amphioxus embryos, although Wnt/β-Catenin signaling along the animal-vegetal axis has apparently been modified, and this modification has likely given rise to the chordate body plan in this animal (Morov *et al*., 2016). Both differences found between cnidarians and sea urchins, and between sea urchins and amphioxus are likely related to the variation in localization of maternal factors mediated by nuages or a Balbiani body during oogenesis.

Amphioxus development thus may provide insights not only into the origin of vertebrates, but also into the origin of bilaterians. Although speculations on "urbilaterians" sometimes imagine a bilateral animal geared with full set of modern bilaterian features (De Robertis, 2008; Arendt *et al*., 2016), reminiscent of Owen's ideal archetype of the vertebrate skeleton (Owen, 1866), genome-wide studies are now providing similarities rather than differences between cnidarians and bilaterians (Putnam *et al*., 2007; Dubuc *et al*., 2012; but see Boero *et al*., 2007; Schwaiger *et al*., 2014)), raising the possibility that bilaterian ancestors retained a radially symmetrical appearance. This is also supported by the fact that many bilaterians such as lophophorates, annelids and their close relatives, priapulids, and echinoderms that display radially symmetrical appearances in each superphylum clade branch off basally in the phylogenetic tree (Dunn *et al*., 2008). One remarkable difference between cnidarian and bilaterian clades is found instead in development as mentioned above; opposite localization of maternal factors in eggs along the animal-vegetal axis and the resultant inverted embryonic development. Gastrulation from the animal pole has also been reported for ctenophoran embryos (Freeman, 1977; Martindale and Hejnol, 2009) and both cnidarians and ctenophorans develop radially symmetrical bodies. The inversion of the site of gastrulation in terms of the animal vegetal axis is well known, as is the dorso-ventral inversion. However, molecular and subcellular mechanisms underlying the apico-blastoporal inversion and how they are related to segregate the bilaterian clade from radially symmetrical animal clades have not yet been elucidated. Given that early animal diversifications occurred rapidly around the Precambrian/Cambrian boundary (Rokas *et al*., 2005; Antcliffe, 2012; Cavalier-Smith, 2017), comparative study involving cnidarians, ambulacrarians, amphioxus, and protostome animals that may retain less-derived early developmental patterns, such as chaetognaths (Takada *et al*., 2002; Carré *et al*., 2002), phoronids (Pennerstorfer and Scholtz, 2012), and priapulids (Wannberg *et al*., 2008) may provide data for better understanding the bilaterian, deuterostome, and chordate origins. Cues for understanding these origins might couple with each other in oogenesis and early developmental processes.

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