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沖縄科学技術大学院大学

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Regeneration in the Enteropneust Hemichordate, *Ptychodera flava*, and Its Evolutionary Implications

Asuka Arimoto[1] and Kuni Tagawa[2]

1: Marine Genomics Unit, Okinawa Institute of Science and Technology Graduate University, 1919-1 Tancha, Onna, Okinawa, 904-0495, JAPAN

2: Marine Biological Laboratory, Graduate School of Science, Hiroshima University, 2445 Mukaishima, Onomichi, Hiroshima, 722-0073, JAPAN

Corresponding authors:

Asuka Arimoto: asuka.arimoto@oist.jp, TEL: +81-98-966-8653, FAX: +81-98-966-8622

Kuni Tagawa: e-mail: kuni@hiroshima-u.ac.jp, TEL: +81-848-44-6055, FAX: +81-848-44-5914

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Abstract:
Hemichordates are marine invertebrates that are closely related to chordates, but while their body plans are comparable to those of chordates, they possess a remarkable capacity for regeneration, even as adults. A small fragment is sufficient to form a complete individual. Unlike echinoderms, their larvae transform directly into adults; therefore, hemichordate systems offer clear morphological and molecular parallels between regeneration and development. Morphological events in regeneration are generally similar to organogenesis in juveniles. Nonetheless, comparative analysis of gene expression in these two morphological phenomena suggests that hemichordate regeneration is regulated by regeneration-specific mechanisms, as well as by developmental mechanisms. Dependency upon resident pluripotent/multipotent stem cells is a significant difference in metazoan regeneration, and such stem cells are essential for regeneration in many lineages. Based on the present gene expression study, regeneration in acorn worms is more closely related to that in vertebrates, because it employs endogenous stem cell-independent transdifferentiation.

(150/250 words)
Keywords: evolution, hemichordate, pluripotency, regeneration, transdifferentiation

Introduction:

Hemichordates, a phylum within the Deuterostomia, are marine invertebrates. Echinoderms, including sea cucumbers and sea stars, are commonly used as model systems for studying invertebrate deuterostome regeneration. While echinoderms lose their bilateral body plans as adults, hemichordates maintain bilateral body plans throughout their life cycles. This trait is reasonable for comparing regenerative capabilities based on body plan and/or cell-type complexity among bilaterian lineages. Some acorn worms with high regenerative capacity can regenerate missing parts within two weeks. Recently, genomes of acorn worm taxa exhibiting direct and indirect development were decoded and massive cDNA libraries were constructed. In addition, molecular analytical techniques (e.g. *in situ* hybridization and knock-down analysis) are being developed to establish platforms for understanding molecular mechanisms of hemichordate regeneration. In this review, we summarize classic reports and modern molecular studies on hemichordate regeneration and compare mechanisms underlying regeneration among bilaterians.

Overview of hemichordates and regeneration

Hemichordates are marine invertebrate deuterostomes, a sister group to echinoderms. They exhibit bilateral body plans through their life cycles. The phylum Hemichordata consists of two major groups, enteropneust acorn worms and pterobranchs, which have very different morphologies. Acorn worms attain adult sizes ranging from several millimeters to two meters and live on the seafloor, including the intertidal zone and deep seas (Worsaae *et al*. 2012; Cannon *et al*. 2013). Pterobranchs are tiny animals and their known habitats are very limited. The number of classified pterobranch species is limited due to the difficulty of finding them (Tassia *et al*. 2016). Bulk gene analyses based on whole genome sequences suggest that extant deuterostomes comprise three major groups: chordates, hemichordates, and echinoderms. Morphological characters of the common ancestor of deuterostomes are hypothesized to resemble those of extant acorn worms (Simakov *et al*. 2015; Cannon *et al*. 2016; Rouse *et al*. 2016).

Two different developmental patterns, i.e. direct and indirect development, are observed in acorn worm embryogenesis (Fig. 1A). Planktonic larvae of indirect developers, known as tornaria, have typical dipleurula-type morphology (Garstang 1928). Tornaria larvae do not form any adult rudiments during metamorphosis because larval structures transform into adult forms (Agassiz 1873). Direct developers become juveniles with complete sets of adult structures after undergoing embryogenesis that resembles larval metamorphosis of indirect developers (Burdon-Jones 1952).

Adult enteropneusts are vermiform animals. Most species do not have prominent structures (e.g. appendix); however, during the reproductive season, gonads of species belonging to the family Ptychoderidae swell and become extended lobes called genital wings. The bodies of adult worms are divided into three regions: an anterior proboscis (protosome), a middle collar (mesosome), and a trunk (metasome) (Fig. 1B). The mouth is located on the ventral side of the anterior region of the collar. The digestive tract passes straight through the trunk and the anus is located at the posterior end of the body.

Hemichordates display several features that may be comparable to specific chordate attributes, such as gill slits on the dorsal side of the anterior trunk. In addition, a stomochord in the proboscis, a notochord, and a dorsal nerve cord forming a hollow tube in the mesosome, imply a close relationship between hemichordates and chordates. Although structural homologies of these organs are still controversial (Satoh *et al*. 2014; Tagawa 2016), formation of gill slits and pharyngeal arches in deuterostomes is regulated by an orthologous gene cluster (Ogasawara *et al*. 1999; Gillis *et al*. 2012; Simakov *et al*. 2015).

Bodies of acorn worms are covered with characteristic aromatic mucus, containing phenol bromide and/or indole bromide (Ashworth & Cormier 1967; Higa & Scheuer 1977). The body is easily broken off by friction or shearing, but missing parts are readily regenerated. Regenerative capacity varies, depending on the species, and some hemichordates can regenerate complete individuals from fragments. Moreover, there are species that reproduce asexually through regeneration (Gilchrist 1923; Packard 1968; Miyamoto & Saito 2010). Even fragments that lack the central nervous system or pharyngeal gill slits can regenerate intact animals. In these regards, hemichordates differ greatly from chordates, in which regeneration is strictly limited.

Morphological characters of hemichordate regeneration

The first described hemichordate was *Ptychodera flava* (Eschscholtz 1825), a species that we have been studying; however, it was originally thought to be a sea cucumber. Later Kowalevsky (1866) identified gill slits in acorn worms, and Metschinikoff described hemichordate developmental stages (1869, 1870). Bateson (1885) proposed that the Hemichordata be classified as a subphylum of the Chordata, based on morphological and developmental characters, but later the subphylum Hemichordata was elevated to the status of a phylum, where it remains.

The most distinctive ability exhibited by acorn worms is the reconstruction of intact individuals from fragments that result from cutting at an arbitrary position along the anterior-posterior (AP) axis. Unlike planarians, there are no reports that hemichordates can regenerate from small cell masses, but this ability is frequently used to regenerate themselves after injury and reproduce new individuals. Intertidal species may depend on frequent regeneration. For example, in a population at Oahu, Hawaii, about 2% of collected specimens were regenerating or had recovered from injury within the preceding several months (Humphreys *et al*. 2010). Furthermore, even species known to employ sexual reproduction show biased sex ratios in several habitats (Rao

1954; Ritter & Davis 1904). These observations suggest that some populations may maintain their populations by asexual reproduction through fragmentation and regeneration. Another mode of asexual reproduction has also been reported, in which fragments called regenerands are produced by autotomy and each regenerand regrows into a new individual (Gilchrist 1923; Packard 1968; Miyamoto & Saito 2010). In wild specimens, regeneration sites can often be identified based on different pigmentation of newly formed tissue. The frequency of such observations suggests that most regeneration in nature is due to injury.

The degree of regenerative capability also varies widely among acorn worms. For example, *Glossobalanus minutus* can only regenerate a lost anterior part, and reproduction does not occur if the posterior part is lost (Dawydoff, 1909). In a regeneration study of *Saccoglossus kowalevskii*, only the posterior part could regenerate a missing proboscis and collar when it was cut at the collar region, while only the anterior could regenerate when it was cut in the center of the trunk (Tweedel, 1961). This means that in *S. kowalevskii*, the direction of regeneration depends upon the location of the injury.

In contrast, our model organism, *Ptychodera flava*, can regenerate in both directions, irrespective of amputation site, when we used worms immediately after collection. In this process, original structures are restored sequentially starting with the most distal end of the missing part (Fig. 2). This type of regeneration fits the model called “distalization and intercalation” (Agata *et al*. 2007). The success of regeneration can exceed 90% or, but extremely rarely, individuals with multiple proboscises and/or collars are seen (Fig. 3A). The frequency of errant regeneration in nature is estimated at <0.03% (Nishikawa, 1985), but can be increased by surgically perturbing the cut surface (unpublished data). This suggests that some factors governing axis polarity and/or patterning at the site of the injury affect regeneration (Fig. 3B).

Molecular mechanisms of acorn worm regeneration and their association with development

Molecular biological techniques, such as *in situ* hybridization, have been applied to various non-model organisms in recent years and comparative studies have yielded numerous important insights. *Ptychodera flava* is the first hemichordate to be studied not only in relation to development, using molecular biology techniques, but also with respect to regeneration, because of its remarkable capabilities. Several species of both direct and indirect developers, including *P. flava*, have been utilized for comparative developmental analyses. Studies of axial patterning in embryogenesis of a direct developer, *S. kowalevskii*, found ectodermal AP patterning that is conserved among hemichordates and vertebrates (Lowe *et al*. 2003; Aronowicz & Lowe 2006; Pani *et al*. 2012; Darras *et al*. 2018). Larval and adult body plans of an indirect developer, *Schizocardium californicum,* have been compared by analyzing over 20 transcription factors involved in ectodermal AP patterning (Gonzalez et al. 2017). The results suggest that the Hox and Wnt gene families are important in patterning the AP axis during hemichordate development, as in most bilaterians. Expression patterns of these genes, particularly *hox* genes, during trunk formation of juveniles, are clearly conserved between direct and indirect developers (Fig. 3B).

Whether these axial pattering genes show similar expression patterns during regeneration is still unclear; however, the comparison of *hedgehog* gene expression patterns between regeneration and embryogenesis in *P. flava* suggests that there may be regeneration-specific mechanisms for specifying positional information (Arimoto & Tagawa 2015). *Hedgehog* is known as a marker that is expressed at the anterior end of larvae during bilaterian metamorphosis, and it is also expressed in the pharyngeal region from late metamorphosis into the young juvenile stage (Pani *et al*. 2012; Miyamoto & Wada 2013). It is reported that the anterior expression of *hedgehog* disappears in juvenile *Balanoglossus simodensis*, which is closely related to *P. flava* (Miyamoto & Wada 2013). Therefore, transient anterior expression of *hedgehog* might be expected in regeneration, if molecular mechanisms that form adult anterior structures during metamorphosis are simply reused. However, in expression analysis using *in situ* hybridization, *hedgehog* expression was confirmed only in the pharynx and was not detected at the anterior tip during regeneration (Arimoto & Tagawa 2015). The absence of *hedgehog* expression at the anterior tip of the blastema supports the idea that the regenerative rudiment and anterior structures in metamorphic larvae have different natures. These results also imply that the blastema is not formed by simply repeating the metamorphic process. Assuming that, *P. flava* has at least two types of mechanisms, i.e. adapting metamorphosis or regeneration, to form adult anterior structures.

A major unresolved question in acorn worm regeneration concerns how cells in the regenerating region are supplied. A previous study using *P. flava* suggested that mesenchymal cells contribute to some of the newly formed tissues (Rychel & Swalla 2008). These cells migrated to the injured site soon after amputation and proliferating cells became restricted to the anterior regenerating end. This process was followed by apoptosis and then rudiments of missing structures started to form. We visualized cell division during regeneration of *P. flava* by labeling with EdU (an analog of thymidine) and found that cell division is highly activated in the dorsal region of the regeneration site (unpublished data). These results imply that the location of the blastema is determined by the dorsal nerve cord; thus, hemichordate regeneration may be nerve-dependent, as in several other groups of animals. Further analysis is required to determine whether migrating cells are themselves pluripotent or whether dividing cells near the wound site are derived from migratory cells.

We conducted an analysis of comprehensive gene expression in *P. flava* regeneration. We compared ESTs from six early developmental stages and four adult tissues and found that gene expression profiles of regenerating tissues tend to be down-regulated compared to other EST libraries (Tagawa *et al*. 2014; unpublished data). Luttell et al. (2016) also reported gene expression profiles using RNA-seq in early regenerative stages, showing that potential homologs of vertebrate somatic cell reprogramming factors, such as *pou*, *klf*, and *sox*, are expressed in a stage-specific manner during regeneration. These potential homologs of hemichordate and vertebrate reprogramming factors are classified into closely related gene families; nevertheless, they do not show direct orthology. However, the flexibility of the combination of vertebrate reprogramming factors (Brouwer et al. 2016) suggests that these homologs possibly regulate somatic cell reprogramming in hemichordates. The next-generation sequencing data is consistent with our results of gene expression patterns from *in situ* hybridization (unpublished data). Together, these results indicate that re-acquiring a pluripotent state may be a key to regeneration in hemichordates.

Comparison of regulatory mechanisms of regeneration among bilaterians

The blastema is formed during animal regeneration and can easily be distinguished morphologically and histologically from differentiated cells. In anterior regeneration of *P. flava*, the blastema is formed on the anterior surface of the wound-repair surface and missing structures are reformed sequentially from the distal end of the proboscis, collar, or trunk, starting from the blastema (Willey 1898; Fig. 2).

Furthermore, in the field, many individuals are seen undergoing regeneration and sexually mature individuals without gonads are rarely encountered (Humphreys et al. 2010). From these observations, it is assumed that gonads can be regenerated even if reproductive organs are completely lost, that is to say, all cell types including totipotent germ cells can be recovered by regeneration.

Cells involved in acorn worm regeneration seem to be pluripotent; however, the source of these pluripotent cells is unknown. There are two possible origins (Fig. 4A). One is that such stem cells are maintained in the adult body, as in planarians, and that they give rise to the entire range of cell types and organs. The other is that differentiated cells achieve pluripotency or multipotency through de-differentiation, as in vertebrate regeneration. Our current findings, including gene expression studies in *P. flava* described above, support the latter (Fig. 4B).

Body axis patterning in acorn worms is determined in early embryogenesis in both direct and indirect developers, and body axes do not change before or after metamorphosis, unlike in echinoderms. In a previous study (Arimoto & Tagawa 2015), we proposed that anterior regeneration might be driven by regeneration-specific mechanisms, different from those employed in embryogenesis. Are similar mechanisms used for reconstruction of body axes and organ position in regeneration?

We have shown that a secondary AP axis can be induced anomalously by surgery, as mentioned earlier. On the other hand, modification of dorsal-ventral (DV) axis patterning in regeneration has not been reported. DV axis determination in acorn worm embryogenesis is regulated by highly conserved mechanisms in bilaterians, e.g. antagonism of BMP and Chordin which are known as a dorsalizing and a ventralizing factor in non-chordates, respectively, as well as AP axis determination (Lowe *et al.* 2006; Röttinger & Martindale 2011). However, since surgery does not induce DV axis remodeling during regeneration, positional information in the DV axis may not be reconstructed during regeneration, but may reflect information existing in the trunk. It is reported that muscles are essential to reconstruction of body axis information in planarian regeneration (Witchley *et al.* 2013); similar mechanisms may also exist in acorn worms.

Hedgehog genes, described above, also function throughout the body during protostome regeneration. In planarians, the most well-understood regeneration model system, *hedgehog* is involved in determination of the AP axis in opposition to *wnt* signals (Rink *et al*. 2009; Yazawa *et al*. 2009). In polychaete annelids, *hedgehog* functions as a segment polarity gene when regenerating lost somites (Niwa *et al*. 2013). The expression pattern of *hedgehog* observed in *P. flava* regeneration is different from the wnt/hedgehog antagonism model in regeneration in the aforementioned protostomes (Arimoto & Tagawa 2015). Therefore, molecular mechanisms for body axis determination and/or position information in acorn worm regeneration differ from those of protostomes.

In deuterostome regeneration, adults with true bilateral body plans are extremely vulnerable to bisection transverse to the AP axis. Separation of the gill and anus regions is invariably fatal, even in non-vertebrate chordates (Somorjai *et al*. 2012; Fig. 4A). Since such tendencies also exist in some species of acorn worms, comparison of molecular responses among hemichordates with different regenerative abilities might reveal the evolutionary history of susceptibility to AP axis amputation.

New technologies accelerating studies of hemichordate regeneration

To understand molecular mechanisms of regeneration in hemichordates, it is necessary to develop optimized experimental systems for functional analysis for the model animals being investigated. In acorn worm embryogenesis, it is possible to inhibit signaling pathways and then to induce abnormal morphogenesis using inhibitors commonly used for other marine invertebrates (Darras *et al*. 2011). Although there are no reports of treating hemichordates with such inhibitors during regeneration, in principle, this could be done to see whether they have similar effects to those observed during embryonic development. Skillful microinjection using short interfering RNAs or recombinant proteins has been employed to study early developmental embryos of *S. kowalevskii* (Lowe *et al*. 2006). This system provided gain- or loss-of-function analysis with high target specificity. Such targeted functional assay systems for regenerating individuals have not been established yet, but gene transfer into regenerating tissues by electroporation may be a promising technique for regeneration analysis.

Identifying the source and the capacity for differentiation of regenerating cells are the two most important points necessary to understand mechanisms of regeneration in hemichordates. As a first step, it is necessary to analyze cell lineages of regenerative tissues and then to grasp the restriction of differentiation potential of those cells involved in regeneration. Currently use of the CRISPR/Cas system to create knock-out/knock-in animals, might be one of the most powerful methods (Sasaki *et al*. 2014; Lin & Su 2016). In addition, single-cell analysis using next-generation sequencing, e.g. pseudo-time course analysis, would help to identify the sources of cells in regenerated structures (Guo *et al*. 2017). It is also desirable to obtain more comprehensive RNA-seq data, to increase sampling frequency, and to cover the major morphogenetic events during regeneration. Comparisons of comprehensive RNA-seq data among various animals with high temporal resolution could illuminate mechanisms underlying limitations of regeneration in each animal.

Conclusions:

The specificity of hemichordate regenerative capability enables hemichordates to reconstruct intact individuals from fragments, even when the AP axis is completely cut at any given position. Generally, such damage is lethal to bilateral adult deuterostomes, such as chordates. The high regenerative ability of *P. flava* is thought to be driven not only by the same mechanisms as in embryogenesis, but also by regeneration-specific mechanisms that directly specify the structure of adult organs.

Whether hemichordate regeneration depends on resident pluripotent stem cells as in planarian regeneration, is open to challenge. Based on our current gene expression study of reprogramming factors using *in situ* hybridization and RNA-seq analyses, however, acorn worm regeneration is more closely related to that of vertebrates, occurring by transdifferentiation. It is interesting to consider the evolutionary change in regenerative cell source from resident pluripotent stem cells found in many metazoan lineages to de-differentiated stem cells, characteristic of chordate lineages.

Comparative analysis among species and/or higher taxa is expected to yield insights into evolutionary changes in regenerative capability, as well as genetic factors that limit regenerative flexibility among deuterostomes. Such knowledge may help to enhance regenerative capability even in species with limited regenerative ability, such as humans.

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Figure legends:

Figure 1. (A) A lifecycle of hemichordates. Gray and green arrows indicate developmental and regenerative processes, respectively. Indirect developers, such as *Ptychodera flava*, make tornaria larvae and the larvae transform into vermiform juveniles. Direct developers do not have larval stages; thus, the juveniles are directly developed from the gastrulae. Fragments of adult worms can make complete individuals through regeneration. (B) A dorsolateral view of a mature female *Ptychodera flava*, an enteropneust hemichordate. The body surface is fully covered with colorless mucus. The yellowish mucus is secreted when the animal is exposed to physical stimuli. This mucus has a characteristic odor. The trunk, posterior to the collar is elastic and gonads of mature worms are elongated in the middle of the hepatic region where two lines of hepatic saccules are aligned. At the dorsal midline between the anterior gonads, two lines of pharyngeal gill slits form the branchial region. The branchial region represents the dorsal side of the pharyngeal region so that gill slits are also called pharyngeal gills. Whole-body regeneration is elicited by a cut at an arbitrary position along the anterior-posterior axis. Through this process, more than one individual is reproduced. An arrowhead indicates the anterior tip of the proboscis. The inset shows a lateral view of anatomical scheme of anterior structures. a, anus; c, collar; dn, dorsal nerve cord; go, gonads (genital wings); gs, gill slits (pharyngeal gills); h, hepatic saccules; m, mouth; pr, proboscis; s, stomochord.

Figure 2. Anterior regeneration of *Ptychodera flava*, from Humphreys et al. (2010). (A) Intact worm before amputation between the branchial and hepatic regions. (B) Posterior trunk just after amputation. (C) At two days post-amputation (dpa), the area around the wound is swelling and the hole is almost closed; however, the digestive tract is still open to the outside. (D) The wound repair process finishes at 3 dpa. A tiny shield blastema appears on the tip of the anterior end. (E) The blastema becomes visible to the naked eye at 4 dpa. This visible part becomes the proboscis. (F) At 5 dpa, two ridges of the collar rudiment are formed on both sides of the most prominent mass of the blastema. (G) Lateral ridges of the blastema completely cover the collar region at 7 dpa. The mouth opens at the ventral region of the regenerating proboscis. (H) Regeneration of functional proboscis and collar is complete at 12 dpa. The worm starts digging in the

sand with the recovered proboscis. (I) At 17 dpa, regeneration continues to form the missing branchial region. This process intercalates new tissues between the regenerated anterior structures and the original posterior trunk. (J) Anatomical schemes of anterior regeneration. In this figure, left is anterior and top is dorsal. Arrowheads indicate the position of blastema. A shaded part at 7 dpa corresponds to the regenerating collar. (K) Regenerative capability of each part of the body based on a previous report (Nishikawa 1977). Blue bars correspond to regions that remain in initial fragments. “Yes” and “No” indicate observed regeneration succeeded or not in each direction, respectively. “Autotomy” means that cutting in the point can induce regenerative autotomy. “Partially” means that regenerated individuals showed abnormal morphologies of the structure which was recovered in the direction.

Figure 3. Expression patterns of body-axis patterning genes and axial patterning error during anterior regeneration. (A) A regenerating individual with a secondary AP axis anomaly induced by surgery. Black and white arrowheads indicate primary and secondary regenerating structures, respectively. The speed of regeneration of secondary structure is slower than that of primary structure. (B) Expression patterns of body-axis patterning genes conserved between direct and indirect developers during the juvenile stage. Various members of the HOX family, including NKL, PRD, SINE, and TALE, are associated with development of the proboscis and collar. Positional information of the trunk is mainly regulated by *hox* genes of the HOXL subclass. The dorsoventral axis is determined by antagonism of BMP-Chordin as in protostome bilaterians. Expression patterns are based on previous reports (Lowe *et al*. 2003; Aronowicz & Lowe 2006; Lowe *et al*. 2006; Röttinger & Martindale 2011; Gonzalez *et al*. 2017).

Figure 4. Regenerative capabilities and mechanisms in metazoan lineages. (A) Comparison of regenerative capabilities among solitary metazoans based on Lai and Aboobaker (2018). Whole-body regeneration is a common characteristic of metazoans; however, residential pluripotent/multipotent stem cells are required for the process in many phyla. In hemichordate regeneration, it seems that differentiation potential of somatic cells is recovered, as in chordate regeneration. In chordates, organ or tissue regeneration depends on stem cells, and large scale structural regeneration is driven by de-differentiation of somatic cells. Parentheses indicate that dependencies are supported

by indirect evidence. Question marks mean that dependency types have not been identified. NA, not applicable. (B) A possible model of hemichordate regeneration based on our present knowledge. Wound repair completes before regeneration starts in a strict sense, i.e. before blastema formation. Pluripotency-associated genes are expressed in parallel with the repair process. De-differentiated cells form a blastema in the first step of regeneration. Missing tissue is reconstructed by differentiation of the blastema with cooperation of unknown regeneration-specific and conserved developmental mechanisms.