

Chapter 10

Regeneration in Hemichordates and Echinoderms

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Abstract Hemichordates are closely related to echinoderms, which are remarkable for their powers of regeneration. Among hemichordates, some enteropneust worms show dramatic regeneration, though this process has not been as well studied as in echinoderms. Unlike echinoderms, which are pentamerous in adult form, hemichordates exhibit bilateral symmetry throughout the life cycle. Adult body regeneration in hemichordates may therefore show similar molecular patterning to chordate regeneration. In this chapter, we review the original literature about regeneration in hemichordates. We present our results from *Glossobalanus berkeleyi* and *Ptychodera flava*, the latter of which reliably regenerates anterior structures in the laboratory. When *P. flava* is bisected, the wound at the anterior end of the posterior half heals, followed by outgrowth of a blastema that becomes the new proboscis. After the proboscis develops, the mouth opens, the collar folds up, and gill slits are formed by tissue remodeling in the posterior worm fragment. Renewed interest in hemichordate regeneration, combined with the fact that it can be elicited in the lab, sets the stage for using molecular markers for stem cells and differentiated tissue in order to characterize the cellular and molecular events occurring during hemichordate regeneration. Hemichordate data presented here suggests that the ability to regenerate using stem cells may be common in ambulacrarian deuterostomes, the sister group to chordates. Understanding the molecular basis of regeneration in hemichordates may lead the way to methods for stimulating regeneration in vertebrates, including in humans for therapeutic purposes.

Keywords Hemichordates · Deuterostomes · Regeneration · Stem cells

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10.1 Introduction

Regeneration is a phenomenon widespread among metazoans. Most animal groups, with nematodes and cephalochordates as possible exceptions, include some species that regenerate certain tissues (Sánchez Alvarado 2000). Morgan classified regeneration into two different types: regeneration that requires cell proliferation (epimorphosis), and regeneration that does not require cell proliferation (morphallaxis) (Morgan 1898; Morgan 1901; Sánchez Alvarado 2000). Epimorphosis is frequently seen in the form of a blastema, a mass of proliferating undifferentiated cells that precedes regeneration. Morphallaxis, on the other hand, involves remodeling of existing tissues without extensive cell proliferation. Epimorphic blastema regeneration is the characteristic mode of regeneration found in vertebrates. Limbs and tails of anuran tadpoles (Dent 1962; Shimizu-Nishikawa et al. 2003; Mochii et al. 2007), urodele amphibians (Iten and Bryant 1976; Brockes 1997; Echeverri and Tanaka 2005) and fins of zebrafish (Stoick-Cooper et al. 2007; Yokoyama et al. 2007) form blastemas when replacing missing structures. In tunicates, it appears that regeneration occurs most frequently through epimorphosis. In colonial tunicates, whole body regeneration is accomplished via epimorphosis without a blastema, a process similar to asexual vascular budding (Goldin 1948; Rinkevich et al. 2007; Ballarin and Manni 2009; Sköld et al. 2009). Neural regeneration in the solitary adult tunicate, *Ciona*, probably involves non-blastemal epimorphosis and morphallaxis as well (Schultze 1900; Bollner et al. 1993; Bollner et al. 1995; Bollner et al. 1997).

Because regeneration in a given animal may employ both epimorphosis and morphallaxis, the terms distalization and intercalation have been proposed by Agata et al. (2007) to describe regeneration processes. Frequently, the first step during regeneration is wound healing. At this point, whether or not a blastema is formed, there must be a new patterning axis established in order for the new tissues to be replaced correctly. This process is called distalization, and occurs whether regeneration is accomplished via epimorphosis or morphallaxis. Next, tissues that were lost must be replaced, and this process is called intercalation. Intercalation also can involve epimorphosis or morphallaxis.

A third distinction that can be made between regeneration modes is whether or not regeneration results in simply repairing a damaged individual or results in two complete individuals – resulting in asexual reproduction. Regeneration is considered bi-directional when bisection of an animal will result in two fully functional animals, and unidirectional if only one half regenerates. Within deuterostomes, bi-directional regeneration is characteristic of some echinoderms (Emson and Wilkie 1980; Mladenov and Burke 1984; Vickery et al. 2001b; Vickery et al. 2002; Eaves and Palmer 2003; Knott et al. 2003; McGovern 2003; Rubilar et al. 2005; Candia Carnevali 2006) and possibly of hemichordates (Gilchrist 1923; Packard 1968; Petersen and Ditadi 1971) but not of solitary ascidians or vertebrates (chordates).

Here, we begin by reviewing regeneration mechanisms and asexual reproduction in echinoderms, since they are closely related to hemichordates, and the processes of regeneration in them are much better understood. Next, we will review original literature concerning asexual reproduction and regeneration in hemichordates,

present new data from studies on regeneration in enteropneust worms that is ongoing in our laboratory, and suggest where we expect future studies to be most fruitful.

10.2 Regeneration and Asexual Reproduction in Echinoderms

Within ambulacraria (echinoderms and hemichordates) (Swalla and Smith 2007), echinoderms have been documented to have extensive regeneration (Candia Carnevali 2006). There are five extant classes of echinoderms: crinoids (sea lilies), asteroids (sea stars), ophiuroids (brittle stars), holothuroids (sea cucumbers) and echinoids (sea urchins and sand dollars). Some species in each echinoderm class have been reported to regenerate (Candia Carnevali et al. 2009; D'Ancona Lunetta 2009). Crinoids are the most basal class of echinoderms and their ability to regenerate new arms has been well documented (Candia Carnevali et al. 1997; Candia Carnevali et al. 1998; Thorndyke et al. 2001a; Thorndyke et al. 2001b; Patruno et al. 2003), even in the fossil record (Oji 2001), suggesting that regeneration is an ancestral trait of echinoderms.

Both bi- and unidirectional regeneration modes are found in echinoderms. Examples of unidirectional regeneration are found in sea star arms and radial central nerve cords (Cuenot 1948; Thorndyke et al. 2001a) and sea cucumber gut and muscle regeneration after spontaneous evisceration (Dolmatov and Ginanova 2001; Garcia-Ararras and Greenberg 2001). Of all classes of echinoderms, adult sea urchins have the most limited regenerative capacity, yet they are still able to regenerate several of their body parts, including spines and pedicellariae (Heatfield and Travis 1975a,b; Drager et al. 1989; Dubois and Ameye 2001).

Some adult asteroids, ophiuroids, and holothuroids are able to regenerate bi-directionally when arms are cut or autonomized (Emson and Wilkie 1980; Mladenov and Burke 1984; Mazzone and Byrne 2001; Mazzone et al. 2003). This regeneration mechanism furthermore allows some species to reproduce asexually. Spontaneous autonomous fission in adults is the primary means of reproduction in some species (McGovern 2003; Rubilar et al. 2005), which probably leads to clonal populations. Echinoderm larvae of nearly every class (echinoids, asteroid, ophiuroids, and holothuroids) commonly clone themselves both in culture and nature through asexual budding (Bosch 1988; Bosch et al. 1989; Jaekle 1994; Balser 1998; Vickery and McClintock 2000; Eaves and Palmer 2003; Knott et al. 2003; Sköld et al. 2009). Larval cloning in echinoids and ophiuroids appears to occur by a recapitulation of usual developmental processes based on visual observation of the process (Eaves and Palmer 2003; Knott et al. 2003). It will be especially informative to examine how closely echinoderm adult fission and larval cloning mimic normal development on a cellular and molecular basis.

In addition to the natural process of larval cloning in planktotrophic larvae, in some cases, surgically bisected echinoderm larvae can regenerate bi-directionally (Vickery et al. 2001b, 2002). Bi-directional regeneration in bisected larva is seen in sea urchins (echinoids) and sea stars (asteroids). Sand dollars (echinoids) were observed to regenerate completely the posterior half, but the anterior half

was reported to only partially regenerate over two weeks (Vickery et al. 1999). Mesenchymal cells were seen migrating into the cut site, and were similar in appearance to mesenchymal cells forming the blastema in adult echinoderm arm regeneration (Bonasoro et al. 1998; Thorndyke et al. 1999; Candia Carnevali and Bonasoro 2001). Larval halves lacking coeloms and hence, lacking coelomocytes, were still able to regenerate. This indicates that coelomocytes are not required for this process, at least in echinoderm larvae (Vickery et al. 2002). Subtractive hybridization was done to isolate genes involved in the process of larval regeneration after bisection and revealed nine differentially expressed genes (Vickery et al. 2001a). At the time of publication in 2001, eight of these genes had no known homology with any gene in GenBank, indicating the potential power of this technique in marine invertebrates for discovering new genes important in regeneration. Whether regeneration occurs in injured holothuroid, crinoid, and ophiuroid larvae remains to be seen. Holothuroid (Hörstadius 1925, 1928, 1973; Dolmatov 1991) and non-feeding crinoid larvae (Runnström 1925) that have been bisected have had limited success in achieving full regeneration, but they have not been studied as intensively as other echinoderm classes for their regenerative capacity. Ophiuroid larvae, though known to clone from the cast off larval arms (Balsler 1998), have not yet been shown to regenerate following surgical bisection. It is also unknown whether or not crinoid larvae or hemichordate tornaria larvae (which are morphologically similar to echinoderm larvae) are able to regenerate or clone.

10.3 Developmental Genes Implicated in Echinoderm Regeneration

Molecular understanding of regeneration in echinoderms has only recently been explored. So far, only a few genes have been implicated in being differentially regulated during regeneration in echinoderms, including *Hox1*, and *BMP2/4* and *univin*, from the TGF- β superfamily of signaling molecules (Table 10.1). *Hox1* is expressed at low levels in normal sea star radial nerves, and is upregulated during regeneration of the nerve (Thorndyke et al. 2001a,b). *Hox* gene expression has also been seen in other regeneration systems. For instance, *Hox* genes are expressed during planarian (Bayascas et al. 1997; Bayascas et al. 1998; Saló et al. 2001) and urodele amphibian limb regeneration (Simon and Tabin 1993; Stocum 1996; Brockes 1997; Torok et al. 1998; Carlson et al. 2001; Nicolas et al. 2003). It is thought that *Hox* expression is important for repatterning the body or limb axis during regeneration, as during embryonic development. Experiments with urodele amphibians show that there are *Hox* genes expressed during regeneration that are regeneration specific (Brown and Brockes 1991; Stocum 1996; Torok et al. 1998; Carlson et al. 2001; Christen et al. 2003).

Bone morphogenetic proteins (BMPs) are part of the TGF- β (transforming growth factor- β) superfamily of signaling molecules (Hogan 1996; Newfeld et al. 1999). BMPs play important roles in animal embryonic development as well as in wound healing (O’Kane and Ferguson 1997) and regeneration in the tail and limb

Table 10.1 Developmental genes expressed during echinoderm arm regeneration

Taxon	Gene	Developmental expression	Nonregenerating adult expression	Regeneration expression pattern	References
<i>Asterias rubens</i> (sea star)	<i>Hox1</i>	?	Lower levels (rt-PCR) in arm radial nerve	Upregulated (rt-PCR) expression arm radial nerve	Thorndyke et al. 2001a
<i>Amphiura filiformis</i> (brittle star)	<i>univin</i>	?	Uniform expression in mesenchyme of arm radial water canal	Separate proximal and distal expression regions in radial water canal mesenchyme at 3 weeks after amputation	Thorndyke et al. 2001b; Bannister et al. 2005
<i>Antedon Bifida</i> (crinoid)	<i>BMP2/4</i>	?	Detectable by rt-PCR but not by <i>in situ</i> in normal adult arm	Early in blastema, then in coelomic canal epithelium, later in most proximal newly formed tissue	Patruno et al. 2003

of frog tadpoles (Beck et al. 2006). *BMP2* and *BMP4* are normally expressed during frog tail and limb development, and if either are antagonized after tail and limb amputation, these structures fail to regenerate (Beck et al. 2006). Echinoderms have two TGF- β superfamily members that have been implicated in echinoderm arm regeneration: *BMP2/4* (Patruno et al. 2003) and a TGF- β gene related to *Xenopus Vg1* (Range et al. 2007), *univin* (Bannister et al. 2005), although the functional role of these genes in normal post embryonic echinoderm development is unknown. In a crinoid, *BMP2/4* is expressed early in arm regeneration in the blastema, later in the coelomic canal epithelium, and in advanced stages, expression is found only proximally where new tissue is being differentiated (Thorndyke et al. 2001a,b,c; Patruno et al. 2003). A TGF- β similar to sea urchin *univin* is expressed during several stages of arm regeneration in brittle stars (ophiuroids), and expression is seen in coelomocytes in both normal and regenerating adults (Bannister et al. 2005).

Both *BMP2/4* and *univin* are known to be important in early embryonic development in echinoids. *Univin* promotes embryonic skeletal growth in sea urchin embryos and larvae (Zito et al. 2003). In early development *univin* is upstream of *nodal* signaling and is important in the dorsoventral (oral-aboral) axis formation of the sea urchin embryo (Range et al. 2007). *BMP2/4* is also implicated in patterning the dorsoventral axis of sea urchins (Duboc et al. 2004). While it is not yet known if *BMP2/4* or a gene similar to *univin* is implicated in hemichordate regeneration, some evidence exists for similar embryonic function for *BMP2/4* in hemichordates and echinoderms. Expression of *BMP2/4* in morphologically similar echinoderm holothuroid and hemichordate ptychoderid larvae was

seen specifically in the hydropore region (Harada et al. 2002), suggesting that they are homologous structures. *BMP2/4* expression has not been reported in indirect developing hemichordate *Ptychodera flava* embryos (Harada et al. 2002) but is important for body patterning in the direct developing hemichordate, *Saccoglossus kowalevskii*. *BMP2/4* expression in *S. kowalevskii* begins during gastrulation, where it is expressed on one side of the embryo. Expression continues throughout development in the same place, but it narrows to a stripe on the ectoderm at the dorsal midline by the juvenile stage (Lowe et al. 2006). Knock down of *BMP2/4* expression in *S. kowalevskii* results in a ventralized embryo since the mouth forms circumferentially and gill slits that normally form on the dorsal side are lacking, whereas overexpression of *BMP2/4* results in a dorsalized embryo lacking a mouth (Lowe et al. 2006). Therefore, *BMP2/4* plays an important role in dorsal-ventral axis formation in sea urchin and direct developing hemichordate embryos.

Hemichordates are less well characterized than echinoderms in their regenerative abilities, but some species of enteropneust worms have remarkable regeneration (Hadfield 1975). However, this subject has received little research attention in the intervening decades. Our lab is currently studying regeneration in several different enteropneust hemichordate species.

10.4 Sexual and Asexual Reproduction in Hemichordates

Regeneration ability often correlates with reproductive mode. Animals that are able to reproduce asexually are often also able to overcome injury via regeneration. Therefore, asexual reproduction and regeneration ability have been considered related phenomena (Sánchez Alvarado 2000; Bely and Wray 2001; van Bekkum 2004; Saló 2006). For instance, in groups such as tunicates where some forms reproduce only sexually, and others both asexually and sexually, those that are able to asexually reproduce are highly regenerative compared to obligate sexual reproducers (Kawamura et al. 2008; Tiozzo et al. 2008).

Hemichordates follow one of two distinct life histories, the solitary enteropneust worms (Fig. 10.1A,B), and the colonial pterobranchs (Fig. 10.1C,D). Solitary enteropneusts comprise the indirect developing families Ptychoderidae (Fig. 10.1A), Spengelidae (not shown), and a direct developing family Harrimaniidae (Fig. 10.1B) (Cameron et al. 2000). Despite the fact that all enteropneust hemichordates are obligate sexual reproducers, and besides developmental mode differences (indirect vs. direct), they are distinguished by differences in morphology. In the adult enteropneusts, the anterior proboscis of ptychoderids is much shorter and wider than the long thin proboscis of harrimaniids (Fig. 10.1A,B) (Kowalevsky 1866; Spengel 1893; Hyman 1959). Also in ptychoderids, the gill bar skeleton has adjacent gill bars connected by small synapticalae, their gonads are contained within prominent ridges or wings in the branchial region of the trunk, and in the posterior trunk hepatic sacculles or outpockets of the gut are found (Fig. 10.1A). However, no synapticalae, genital ridges, or hepatic sacculles are present in harrimaniids (Hyman 1959; Aronowicz and Lowe 2006).

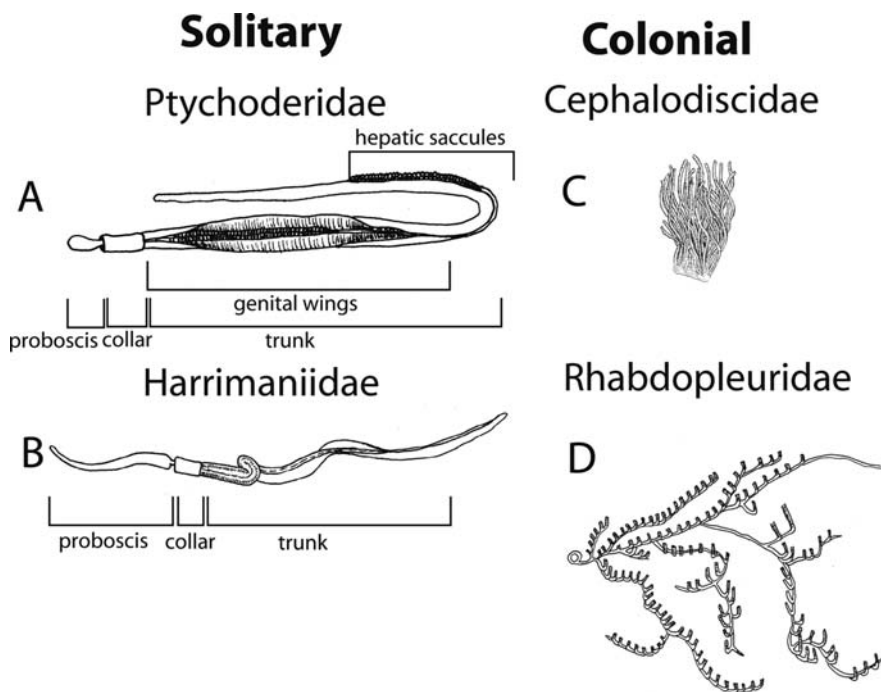


Fig. 10.1 Comparison of the body plans between two solitary enteropneust worm families, Ptychoderidae (**A**) and Harrimaniidae (**B**), and two colonial pterobranch families, Cephalodiscidae (**C**) and Rhabdopleuridae (**D**). (**A**) modified from (Hyman 1959), (**B**) modified from (Marion 1886), (**C**) modified from (Andersson 1907), and (**D**) modified from (Schepotieff 1907a). Ptychoderid worms (**A**) have a shorter, stouter proboscis compared to harrimaniid worms (**B**), and possess hepatic sacs and genital ridges or wings (**A**) that are lacking in harrimaniids (**B**). Colonial hemichordates form colonies by budding asexually. *Cephalodiscus* species (**C**) can be either sessile or pelagic, but all form small colonies. In contrast, *Rhabdopleura* species (**D**) grow within a characteristic cartilaginous coenecium, and individuals will not be viable if buds are separated from the colony

In contrast, the colonial pterobranchs are extremely morphologically divergent from solitary enteropneusts and include the direct developing families Cephalodiscidae (Fig. 10.1C) and Rhabdopleuridae (Fig. 10.1D) (Cameron et al. 2000). Pterobranch hemichordates reproduce both asexually and sexually (Fig. 10.2A), but regeneration ability has not yet been documented. Cephalodiscid pterobranch zooids are usually seen with 1–14 buds growing from the most posterior end of the stalk, the number of buds depending on the species (Figs. 10.1C and 10.2A). Adult asexually reproduced cephalodiscid zooids secrete their own coenecium (external collagenous tube) and thereby remain attached to their parent zooid although the living tissues of individual parent and offspring may or may not remain physically linked (Masterman 1900; Schepotieff 1908; Lester 1985; Schiaparelli et al. 2004) (Fig. 10.2A). By contrast, budding in rhabdopleurid pterobranchs occurs along the stolons that connect individuals to one another, so that

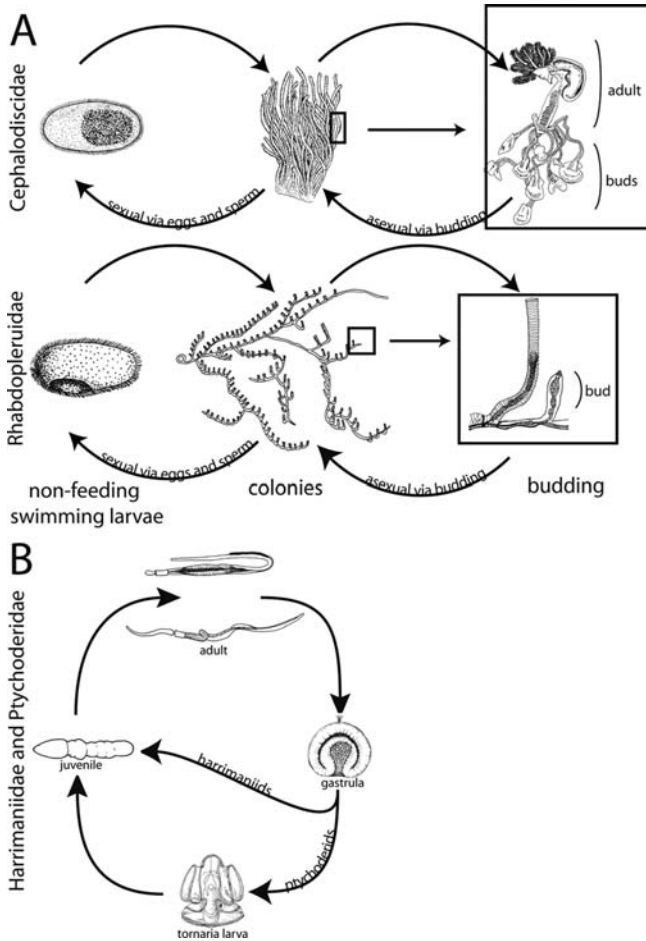


Fig. 10.2 Life cycles of colonial and solitary hemichordates. A. Colonial hemichordate life cycle. *Cephalodiscus* larva modified from (Schepotieff 1909), *Cephalodiscus* colony modified from (Andersson 1907), *Cephalodiscus* individual modified from (John 1931); *Rhabdopleura* larva modified from (Lester 1988); *Rhabdopleura* colony modified from (Schepotieff 1907b), budding *Rhabdopleura* modified from (Schepotieff 1907b). B. Solitary hemichordate life cycle (adult hemichordates modified from (Hyman 1959)). Both cephalodiscids and rhabdopleurids can reproduce asexually via budding (individual shown on the right magnified in box) and sexually via eggs and sperm (left side) (A). When reproducing sexually, both cephalodiscids and rhabdopleurids develop into swimming, non-feeding larvae and then directly develop into settled zooids. The larva of *Rhabdopleura* is characterized by a ventral groove on the anterior end, and the *Cephalodiscus* larva has a prominent yolk mass localized to the posterior end. Asexual budding in cephalodiscids occurs with new individuals budding off of the parent stalk (A, top right), while in rhabdopleurids, budding occurs from the tip or lateral edges of the growing stolon that connects individual zooids in a colony (A, bottom right). Solitary hemichordates reproduce sexually (B). Harrimaniids go through direct development, whereas ptychoderids develop indirectly first into a feeding planktonic tornaria larva (bottom) before settling and developing into a juvenile

the individuals are distinct (Figs. 10.1D and 10.2A). Both terminal and lateral buds develop along a given stretch of stolon (Schepotieff 1907b; Stebbing 1970; Dilly 1975) (Fig. 10.2A). This type of clonal reproduction and attachment of adult zooids is comparable to social ascidians which form asexual buds from stolons that connect individual zooids (Zeng et al. 2006; Tiozzo et al. 2008). The differences in budding between groups of pterobranchs results in clumped grouping of zooids in cephalodiscids (Fig. 10.1C) compared with more dispersed individuals in colonies of rhabdopleurids (Fig. 10.1D).

Solitary hemichordates, on the other hand, are obligate sexual reproducers (Fig. 10.2B). It is possible, however, that fragile enteropneusts that are broken by wave action or predation are, in some species, able to recover via regeneration of resulting pieces, which may lead to two or more new individuals from a single severed worm. Although both harrimaniids and ptychoderid solitary enteropneusts are obligate sexual reproducers, they are not equally able to regenerate.

10.5 Regeneration in Solitary Enteropneust Worms

We focus here on regeneration in solitary enteropneust hemichordates, since very little is documented for colonial hemichordate regeneration. First, we describe the normal developmental mode in hemichordates, and then their regeneration potential. The direct developing harrimaniids progress from embryogenesis directly into a juvenile worm (Fig. 10.2B) (Bateson 1884, 1885; Hyman 1959; Cameron et al. 2000), although they do spend a short period of time as a non-feeding lecithotropic larva. Ptychoderids are indirect developers, and following embryogenesis, pass through a planktonic feeding stage (tornaria) prior to settling and developing adult structures (Fig. 10.2B) (Metschnikoff 1869; Hadfield 1975; Urata and Yamaguchi 2004).

Regeneration has been reported just once in a direct developing harrimaniid (Tweedell 1961). In *Saccoglossus kowalevskii*, regeneration is fairly limited in lab conditions as they are only able to regenerate the most anterior structures after amputation, that is, within the proboscis and collar (Fig. 10.1B). In contrast, several ptychoderid species (*Ptychodera flava*, *Glossobalanus minutus*, *Balanoglossus australiensis*, and *Glossobalanus crozieri*) have been reported to have remarkable regeneration (Willey 1899; Dawydoff 1909, 1948; Rao 1955; Packard 1968; Petersen and Ditadi 1971). Most of the published observations have been from posterior trunk pieces containing only gonad, gut, and hepatic sacculations that are able to regenerate all new anterior structures, including the proboscis and collar, which contain the heart, kidney, collar nerve cord, stomochord, and proboscis skeleton, and the branchial region containing the pharyngeal slits and skeleton (Willey 1899; Dawydoff 1909, 1948; Rao 1955; Packard 1968; Petersen and Ditadi 1971).

All of the detailed reports of ptychoderid regeneration have several features in common. Anterior regeneration from an amputated trunk appears to incorporate both epimorphosis and morphallaxis (Dawydoff 1909; Rao 1955). We have repeated

many of the observations of Dawydoff (1909) and Rao (1955) in studies of regeneration in *Ptychodera flava* over eight days in running seawater tables at Kewalo Marine Laboratory in Honolulu, HI. The first step, wound closure, is accomplished by the endoderm growing together with ectoderm, and we see this occurring in all amputated worms within two days (Figs. 10.3A,B,D,E and 10.4D) (Rao 1955). Based on histological data, Rao (1955) observed mesenchyme cells or coelomic cells from the coelomic fluid of trunk region migrate into the wound site. Epimorphosis is characteristic of the beginning of the regenerative process: within two days the wound has closed, then at four days, cells have moved into the space between the endoderm and ectoderm (Figs. 10.3F and 10.4E). A small blastemal structure was seen at day four in six of seven animals bisected. This blastema precedes the formation of the proboscis and appears to be the distalization step in hemichordate regeneration. Next, the proboscis begins to grow out from the proliferated tissue, which at day six, was present in six out of eight amputated animals along with a mouth opening (Fig. 10.3G–I). Rao (1955) concluded that the mouth only breaks through once the ectoderm differentiation is complete. We have observed endoderm on lateral sides of the proboscis evaginated by day six (Fig. 10.4G), although no collar rudiments were visible externally. Then, by day eight, collar buds were visible externally in five out of seven amputated worms. During this time, around day six to eight, we have also observed the stomochord forming via endoderm evagination as in normal development (Figs. 10.4I and 10.5B) (Rao 1955). These collar halves later fuse first on the ventral side (Fig. 10.3H), and secondarily on the dorsal side (Fig. 10.3I,J). The dorsal fusion of the two collar halves creates a new dorsal collar nerve tube. Around this time, mesenchyme cells with ectoderm and endoderm form a rudiment that eventually gives rise to the proboscis and heart/kidney complex (Figs. 10.3G–I and 10.4A,I). We have noticed that the proboscis and collar tissue are much lighter in color in regenerated *Ptychodera* than in non-regenerated adults (Fig. 10.3G–J), indicating that this is newly proliferated tissue that is not yet pigmented. Further evidence for the role of cell proliferation in this process is the presence of PCNA positive nuclei in the proboscis ectoderm, mesenchyme (Fig. 10.5A,C,E), and evaginating endoderm (Fig. 10.5A,B,D) in a day six regenerating animal. It remains to be determined whether or not the mesenchyme are an undifferentiated population of stem cells or whether they dedifferentiate into stem cells once wounding has occurred. Evidence for tissue remodeling to make way for new gill slits to form in the regenerating trunk comes from the paucity of cells in the endoderm that are posterior to evaginating collar endoderm in a day six regenerate (Fig. 10.4F). We propose that these missing cells may have undergone apoptosis and stem cells will later proliferate and remodel the endoderm to form gill endoderm.

Dawydoff (1909, 1948), who examined regeneration in *Glossobalanus minutus*, noted that while any region of an amputated trunk would regenerate anterior structures, those amputated more anteriorly regenerate more readily. Also, we have seen that the anterior two body portions of *P. flava*, the proboscis and collar, regenerate more rapidly (within two weeks), than structures of the third body portion, the

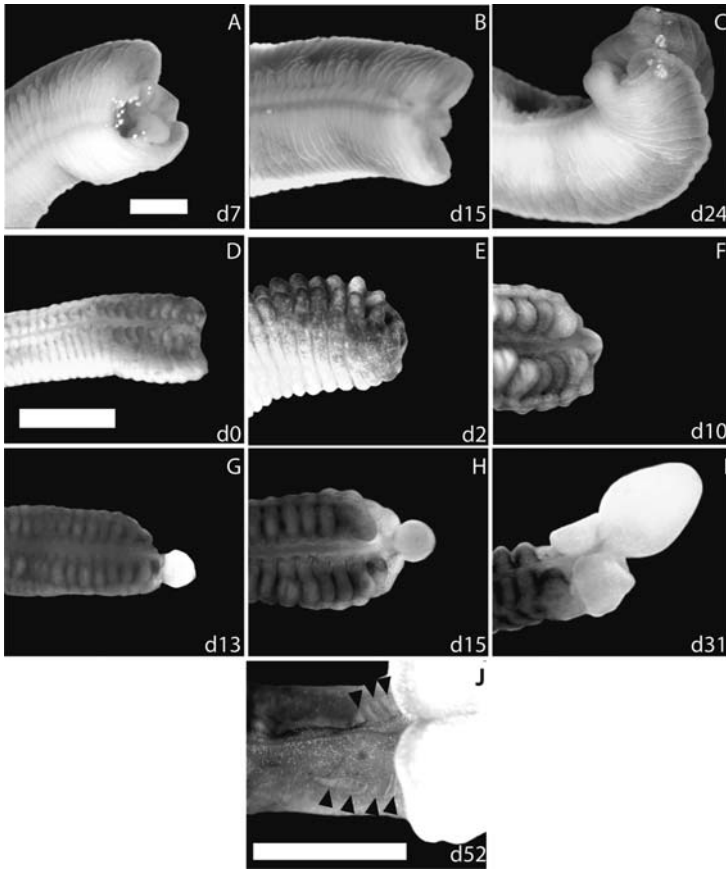


Fig. 10.3 Regeneration in the Ptychoderidae. Anterior regeneration is compared in *Glossobalanus berkeleyi*. (A–C) and *Ptychodera flava* (D–J). Regeneration in a single *Glossobalanus berkeleyi* individual over 24 days that begins, but does not progress (A–C). When *Glossobalanus berkeleyi* is bisected (A, day 7) the posterior half is able to heal the wound in the cut end at the anterior (B). Here, wound healing is complete at 15 days (B). Regeneration appears to be stalled at this step, since subsequently no regeneration blastema forms, and no regrowth occurs (B,C). In this case, the closed end reopened and became necrotic within 24 days (C). Stages of regeneration in a selection of *P. flava* individuals (D–J). The timing of regeneration is slightly variable in *Ptychodera flava*, but it always follows the same sequence. When *P. flava* is bisected (D; day 0), the open end of the posterior half heals within 2 days (E), and a regeneration blastema forms within 5–10 days (F). Development of anterior structures is accomplished rapidly thereafter with a proboscis/mouth forming first in 9–15 days (G), closely followed by the emergence of the two halves of the collar within 15 days (H). By 31 days the collar is completely joined (I). Once the proboscis and collar are formed, gill slits are added in an anterior to posterior fashion by remodeling existing trunk tissue (J). Arrowheads indicate developing gill slits in a day 52 *P. flava* regenerate. Scale bar equals 2 mm in A, D, and J

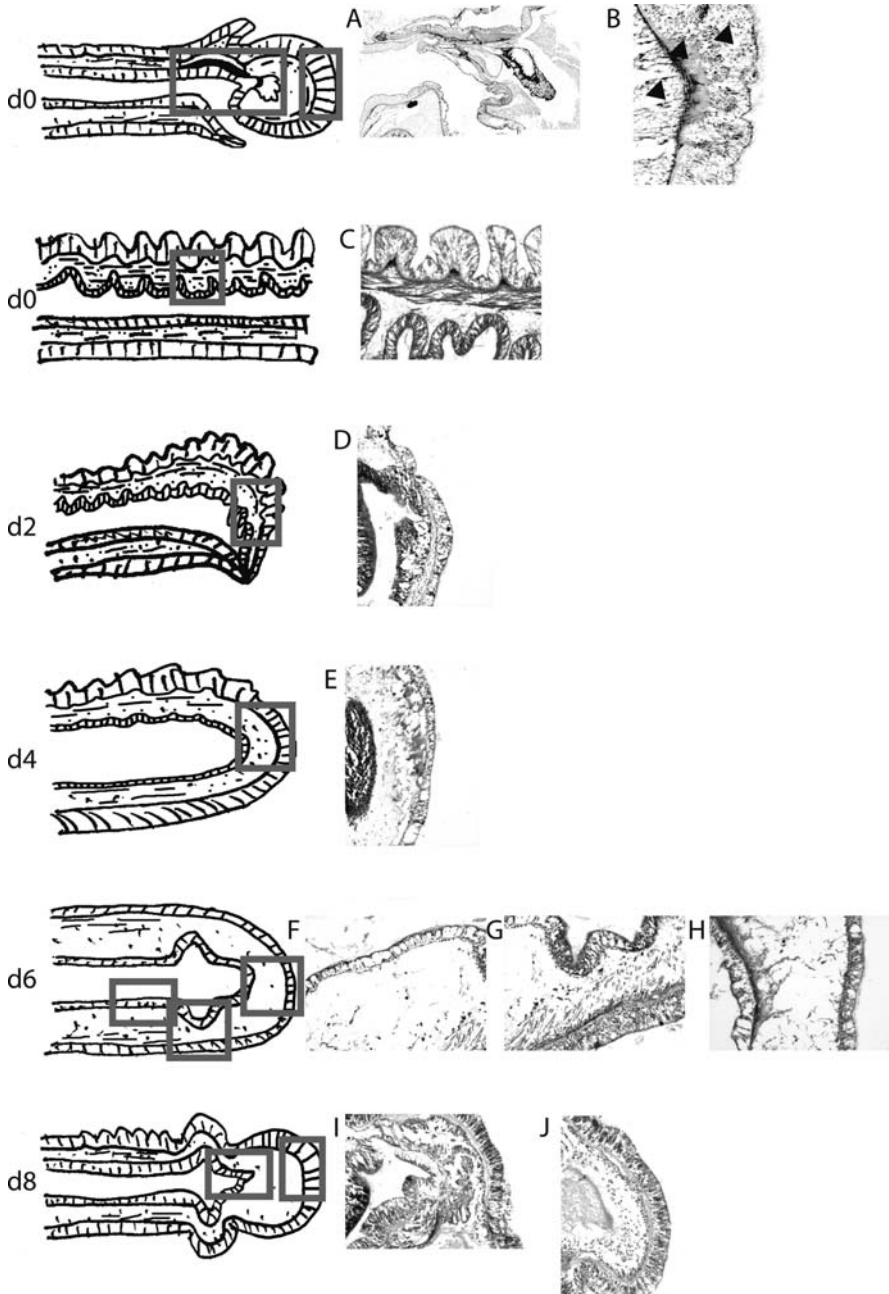


Fig. 10.4

trunk. In one instance, we observed a worm that had been regenerating for nearly two months with nine to ten gill slits in the trunk (Fig. 10.3 J). While ptychoderids in general may have greater powers of regeneration than harrimaniids, it is possible that not all ptychoderid species regenerate. We have, on several occasions, cut individuals of a Pacific Northwest ptychoderid hemichordate, *Glossobalanus berkeleyi*, and have not yet seen complete regeneration (Table 10.2; Fig. 10.3A–C). In *Ptychodera flava*, regeneration proceeded very reliably in the lab (Table 10.3), and in each case, regeneration began by forming the most anterior structures first, with more posterior structures following. The proboscis formed first, followed by the collar, then gill slits began to develop in an anterior to posterior manner, similar to how they form in normal development (Fig. 10.3 J) (Aronowicz and Lowe 2006; Rychel et al. 2006; Rychel and Swalla 2007). In conclusion, anterior regeneration in *P. flava* is a complex process likely involving both cell death of old tissues followed by cell proliferation and tissue remodeling. The source of the stem cells for the new tissues generated in the proboscis and collar is still unknown, but our lab is actively pursuing their identity. We are beginning with a cellular and molecular characterization of hemichordate regeneration and expect this to be an excellent model system once the *Ptychodera flava* genome is sequenced.

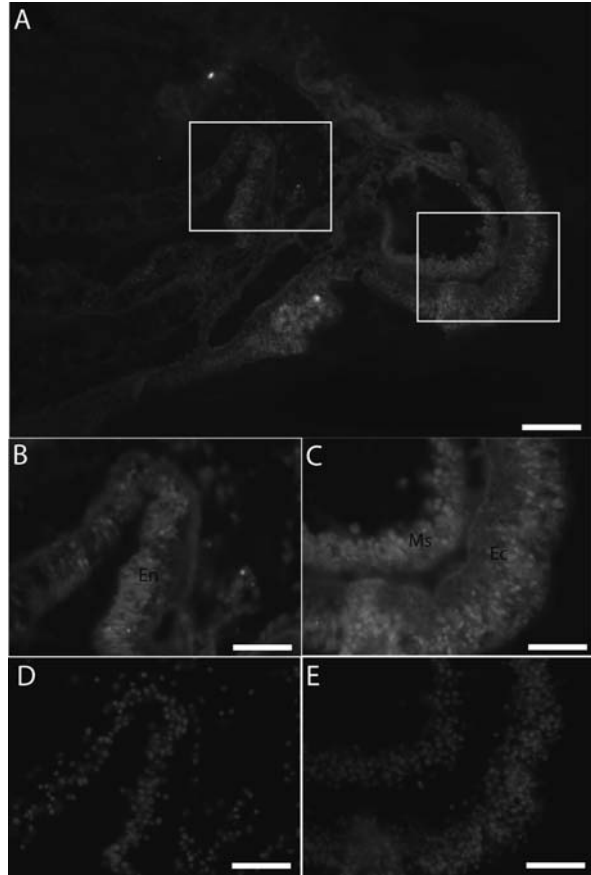
10.6 Future Experiments and Prospectus

Some hemichordate species show extensive regeneration of the anterior structures of the worm and represent a new model system to study stem cells and regeneration. In *Ptychodera flava*, the entire proboscis, mouth, and collar regenerate from the posterior half within two weeks after bisection of the worm, and gill slits are



Fig. 10.4 Trichrome stained longitudinal sections in regenerating *Ptychodera flava*. Boxes on the line drawing on the left indicate the area shown in the magnified sections on the right. The anterior of a normal adult worm (d0), the proboscis, contains the heart/kidney/stomochord (A). In the proboscis tip differentiated muscle, nervous layer, and ectoderm are seen, and are indicated by arrowheads (B). In the posterior hepatic region of a normal worm (d0), differentiated ectoderm, muscle and gut endoderm are present along with mesenchymal cells between ectoderm and endoderm (C). At day two of regeneration (d2), ectoderm and endoderm have grown together, and the ectoderm is very thin relative to normal proboscis ectoderm. Mesenchymal cells are sparse, but they all have long filopodia (D). At day four of regeneration (d4), the ectoderm has thickened and mesenchymal cells with very abundant extensions proliferate in the space between the ectoderm and endoderm (E). At day six (d6) in sections lateral to where the proboscis has formed, spaces between cells are visible in more posterior endoderm (F). More anteriorly, evaginating collar endoderm is present (G). Mesenchymal cells with long extensions are still present in the space between endoderm and ectoderm at the anterior end of the section (H). At day eight (d8), evaginating stomochord endoderm is present surrounded by mesenchyme cells that will form the heart/kidney rudiment (I), and the mesenchyme cells in the proboscis are smaller, with fewer extensions than mesenchymal cells seen in earlier stages (J). At eight days, the regenerated proboscis ectoderm is thicker than six days (J), but not as thick as a normal adult (B)

Fig. 10.5 Six day regenerating *Ptychodera flava* cell proliferation. In a longitudinal section stained with an anti-PCNA antibody, cells with nuclei in S phase (PCNA and DAPI positive) are seen concentrated at the anterior of the animal (A). Boxes in (A) indicate where higher power magnifications are seen in (B–E). Cell nuclei that are positive for PCNA (B,C) and DAPI (D,E). PCNA labels cells in S phase, suggesting that cell division is high in the evaginating endoderm (B,D), as well as proboscis ectoderm and mesenchyme (C,E). En: endoderm, Ms: mesenchyme, Ec: ectoderm. Scale bar in (A) is 100 μ m, scale bar in (B–E) is 50 μ m



slowly added over a longer time scale. In our observations, the anterior structures are regenerated more quickly than more posterior ones, and this would be selectively advantageous to allow eating quickly. We have on several occasions bisected the harrimaniid, *Saccoglossus bromophenolus*, and have not observed any regeneration. This combined with evidence from *Saccoglossus kowalevskii* (Tweedell 1961) suggest that the direct developing harrimaniids show much less regeneration than the ptychoderid worms that have planktonic tornaria larvae. Since these families are also separated phylogenetically (Cameron et al. 2000), it is difficult to know whether regeneration differences are due to phylogenetic history, or if reduced regeneration potential is a consequence of direct development. We know that echinoderms vary in regenerative ability, since among the classes of echinoderms, echinoids have the most limited regenerative ability as adults. It would be interesting to see if echinoderms varied in their regenerative abilities in a phylogenetic way within classes and/or if the differences seen are also linked to direct development. It is unknown

Table 10.2 *Glossobalanus berkeleyi* amputation trials

Trial	Number amputated	Percent of survival 7 days	Percent of survival 14 days	Percent of survival 21 days	Longest number of days followed
1	4	100	100	100	52
2	3	100	100	67	27
3	10	50	50	20	25

Table 10.3 *Ptychodera flava* regeneration concurrent trials

Trial	Number amputated	Trial length in days	Percent of survival at end of trial	Percent of survivors regenerating	Typical regeneration stage at end of trial	Percent of animals at typical regeneration stage
1	10	2	80	100	wound closure	100
2	10	4	40	100	blastema	86
3	10	6	80	100	small proboscis + mouth	75
4	10	8	70	100	larger proboscis + 2 collar buds	71

whether hemichordate tornaria larvae are capable of cloning, a common ability in brittle star, sea star, sea urchin, and sea cucumber larvae. If they can, then it suggests this is an ancestral feature of dipleurula ambulacrarian larvae; while if not, then this would be a phenomenon that would be restricted to echinoderm larvae.

In light of the current excitement about stem cells and regeneration for medical purposes, the study of the cellular behaviors and molecular mechanisms of hemichordate stem cells and regeneration is relevant since they have a body plan with many features in common with vertebrates (Aronowicz and Lowe 2006; Rychel and Swalla 2007; Swalla 2007). The hemichordate body plan develops in an anterior to posterior fashion, similar to vertebrates, and expression of the *Hox* genes begins right at the first gill slit (Lowe et al. 2003; Aronowicz and Lowe 2006; Swalla 2006) just as *Hox* gene expression begins dorsal to the first gill slit or arch in the mid-brain of vertebrates. These results show that an A-P axis based on *Hox* expression is conserved between hemichordates and vertebrates. The coelomic cells that are necessary for regeneration in echinoderms and hemichordates are likely made up of mesenchymal or stem cells, that are capable of differentiating into a variety of cell types. The in-depth study of these cells will be fascinating. We hope to understand how they are mobilized, multiply and then subsequently differentiate after injury in hemichordates.

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