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Regeneration in the Hemichordate *Ptychodera flava*

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When the body of *P. flava* is severed, the animal has the ability to regenerate its missing anterior or posterior as appropriate. We have focused on anterior regeneration when the head and branchial regions are severed from the body of the worm. After transection, the body wall contracts and heals closed in 2 to 3 days. By the third day a small blastema is evident at the point of closure. The blastema grows rapidly and begins the process of differentiating into a head with a proboscis and collar. At 5 days the blastema has increased greatly in size and differentiated into a central bulb, the forming proboscis, and two lateral crescents, the forming collar. Between 5 and 7 days a mouth opens ventral to the differentiating blastema. Over the next few days the lateral crescents extend to encircle the proboscis and mouth, making a fully formed collar. By 10 to 12 days a new head, sized to fit the worm's body, has grown attached to the severed site. At about this time the animal regains apparently normal burrowing behavior. After the head is formed, a second blastema-like area appears between the new head and the old body and a new branchial region is inserted by regeneration from this blastema over the next 2 to 3 weeks. The regenerating tissues are unpigmented and whitish such that in-situ hybridization can be used to study the expression of genes during the formation of new tissues.

Key words: animal model, brain evolution, SEM, gill, in-situ hybridization, regeneration, acorn worm

INTRODUCTION

Hemichordates are among animals such as hydra, planaria, and starfish that have amazing regenerative capabilities and can form a complete animal from random portions cut from the body. Hemichordate regeneration is primarily epimorphic, with the regenerated portion fit to the original body parts to form a complete whole. Among animals with a dorsal hollow neural structure, hemichordates are the only group that exhibit such a flexible regenerative capability (Cori, 1902; Dawydoff, 1907; Kuwano, 1902; Packard, 1968; Rao, 1955; Spengel, 1893; Tweedell, 1961; Willey, 1898), although some colonial tunicates can carry out whole-body regeneration by forming a complete new zooid from the vascular system left when the zooids of the colony are excised (Rinkevich et al., 2007). Reports dating from the end of the 19th century indicate that *P. flava* regenerates a head when the anterior region is severed from a portion of the body (Spengel, 1893; Willey, 1898). Although reports vary, descriptions in general indicate that when the body of a hemichordate is severed, a head and other anterior missing parts are regenerated on an anterior facing wound and tail parts on a posterior facing wound, with the new structures filling out the complete body in a coordinated fashion. We conducted an extensive survey of both anterior and posterior regeneration when the body of *P. flava* is severed at various locations along the anterior-posterior axis, and conclude this species is able to regenerate the missing anterior and posterior parts through an epimorphic process wherever the body is severed.

In recent years, as an understanding of stem cells in higher organisms has coalesced into a robust scientific field, excitement about regeneration and regenerative medicine has grown considerably. It seems reasonable that an understanding of the molecular gene circuitry that is activated and deployed in animals that can regenerate may provide medical science with clues about how to unlock latent regenerative pathways in humans. Extensive studies in our lab on molecular expression during development of *P. flava* (Tagawa et al., 2001) and in other labs on other hemichordates (Lowe et al., 2003) have shown that the cohort of genes involved in neurogenesis in hemichordates (Harada et al., 2000; Harada et al., 2002; Tagawa et al., 2000; Tagawa et al., 1998; Taguchi et al., 2000; Taguchi et al., 2002) is the same as that specifying neural development in chordates and humans. Although the diffuse nervous system in hemichordates is in many ways much more ancestral than the more evolved chordate nervous system, its most centralized component is a dorsal hollow nerve track formed by neurulation just as in chordates (Morgan, 1893), and the organization of domains of expression of genes specifying neural structures is very similar to that of chordates (Lowe et al., 2003; Tagawa et al., 2000). Thus a molecular analysis of head regeneration in *P. flava* is a good focus for pursuing the potential for invertebrate regeneration to provide keys to

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latent regenerative capabilities in the human neural gene programs.

Already studies in the regeneration of lower animals, especially planarians, are producing extensive and fascinating insights from molecular and genetic approaches for understanding the gene circuitry involved in regeneration (Birnbaum and Sanchez Alvarado, 2008; Sanchez Alvarado, 2006). In-situ hybridization is being used to show localization of the expression of developmental genes in the regenerate (Agata et al., 2003; Rentzsch et al., 2007); RNAi has proven valuable for establishing how specific genes are involved in regeneration (Gurley et al., 2008; Petersen and Reddien, 2008); expression profiling of regenerating tissue is offering insights into the ontology of the gene sets mobilized in the regenerative process (Rinkevich et al., 2007); and the elucidation of the molecular features defining stem cells (Jaenisch and Young, 2008; Morrison and Spradling, 2008) that are central to regeneration are all revitalizing and revolutionizing regeneration research. As we learn to apply powerful modern molecular techniques to regeneration in animals, the study of regeneration in hemichordates will provide a unique perspective in the search for clues about latent regenerative potential.

MATERIAL AND METHODS

Ptychodera flava were collected from areas of fine to coarse clean sand on the extensive flat shallow reef at Paiko, Oahu, Hawaii, where tidal depths range from 0 to 0.7 meters. Worms were exposed by disturbing the sand to a depth of about 4 to 10 cm with a wave of the hand in the water over the sand. Worms ranged from 2 to 20 cm in depth. About 2% of the worms collected exhibited signs of recent anterior regeneration irrespective of the season. The animals were maintained in the laboratory in 60-liter seawater aquaria filled with reconstituted sea water (Instant Ocean) at 26°C and equipped with 3-cm-deep coarse (1- to 3-mm grains) coral gravel bottom filters driven by aeration. The aquaria were kept clean, and 50% of the water was changed out every two weeks. Intact animals or anterior pieces with heads that had posterior parts removed by transection were kept in closed plastic boxes with tops and bottoms of heavy 1-mm mesh screen covered with a layer of fine clean sand (2 to 5 mm). Recently transected posterior bodies were kept in clean glass bowls in the aquaria until about 12 days of regeneration, at which point the animals regained burrowing behavior and could crawl out of the bowls. They were then moved to the closed plastic boxes with mesh tops and bottoms and the layer of fine sand. In all experiments, freshly collected animals were severed at various body levels with an orthogonal cut by using sharp surgical scissors.

Samples for scanning electron microscopy (SEM) were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer with 0.35 M sucrose, washed in 0.1 M cacodylate buffer and 0.4 M sucrose, post fixed in 1% OsO_4 0.1 M cacodylate buffer, critical point dried, mounted on aluminum stubs, coated with gold/palladium, and observed with a Hitachi S-800 Field Emission SEM with digital image capture in the Pacific Biosciences Research Center Biological Electron Microscopy Facility, University of Hawaii at Manoa.

Fixation of samples and in-situ hybridization were carried out as previously described for hemichordate embryos and larvae (Lowe et al., 2004; Tagawa et al., 1998) using a *PfSoxB1* probe (Taguchi et al., 2002).

RESULTS

In dozens of collections ranging from 50 to 300 *P. flava* individuals obtained over several years from the reef at Paiko in Honolulu, about 1 to 10 of the animals would have heads of white tissue that contrasted with the more pigmented orange, reddish, greenish or brown posterior portion of the animal's body. A few of these animals had only small or rudimentary heads that rapidly developed into full-sized heads when the animals were brought into the lab. These light colored heads appeared to represent anterior regeneration of naturally wounded animals. A series of preliminary experiments was conducted in the laboratory to define the parameters of this regeneration. Tests were conducted to determine the aquarium temperature optimal for the good health of the animals and for regeneration. At 19°C the worms appeared healthy, but little or no regeneration was observed. As the temperature was raised, the animals remained healthy and regeneration became more robust in both rate and extent. The optimal well being of the animals appeared to be achieved between 26°C and 30°C. An aquarium temperature of 26°C was chosen for all experiments.

Animals maintained in lab aquaria were transected at various levels and regeneration was observed. As shown in Fig. 1, robust and morphologically normal anterior (Fig. 1 A) and posterior (Fig. 1B) regeneration was observed in nearly 100% of animals severed at all levels. A general perception from these experiments is that regeneration is epimorphic, with new tissue added seamlessly onto the old tissue to provide the missing parts, without any regression or reorganization of the old tissue. Regenerates after about 12 weeks regained normal pigmentation and could not be distinguished from animals that had not regenerated. Fig. 2 shows a striking example of the integration of tissue, where a worm transected at mid-collar to remove the proboscis and anterior collar regenerated new tissue, which was well integrated with the old tissue, to replace the lost parts. Minor exceptions to full and integrated regeneration were observed. Anterior regeneration failed in about 20% of pieces shorter than about 3 cm in length from the tail region, and a small number of large worms severed at more anterior sites produced two adjacent heads at the cut site. Animals with severed branchial baskets often degraded the severed portion of the branchial basket during anterior regeneration, although maintenance of the severed branchial basket and its integration into the epimorphic regenerate were also often observed.

For standardized anterior regeneration experiments, we used small to medium worms, 5 to 12 cm in length, transected 1 to 3 mm behind the posterior terminus of the gill basket, as marked in Fig. 3A. Upon transection, the posterior of the animal ceases to show directed movement or burrowing behavior. For about 48 hours, the wound remains expanded, as shown in Fig. 3B, exposing the open gut cavity surrounded by layers of the body wall. By 48 hours the cut edges of the body wall are noticeably swollen, creating a smooth, rounded edge of tissue. This rounded, swollen edge of body wall can be seen in the semicircle of body wall still extending from the partially closed wound shown in Fig. 3C. By 72 hours the wound has closed, as shown in Fig. 3D, and an indistinct whitish spot, the forming blastema, not visible in Fig. 3D, appears in the closure area, generally in a slightly dorsal position on the cut end. When these freshly accumulating blastema cells are examined by SEM they

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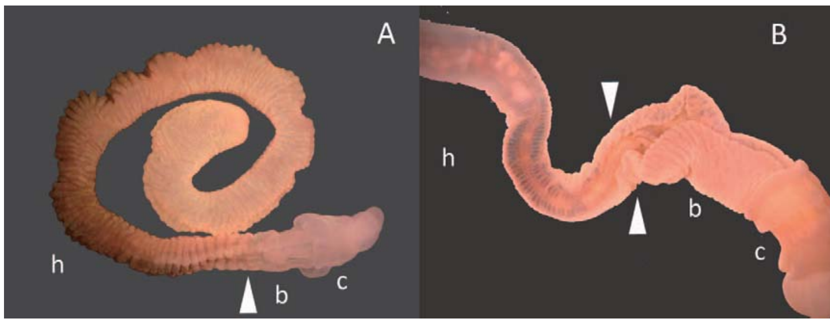

Fig. 1. Anterior regeneration at 25 days **(A)** and posterior regeneration at 22 days **(B)** after transection just posterior to the branchial basket. Arrowheads mark site of original transection in each photograph. In (A), the regenerated whitish tissue consists of the newly formed proboscis and collar as well as the differentiating branchial region with the nascent gill basket with gill slits. In (B), the new posterior part of the worm that has formed behind the branchial basket, as marked by the arrowheads, has developed pigmentation and a differentiating hepatic region. c, collar; b, branchial region; h, hepatic region.

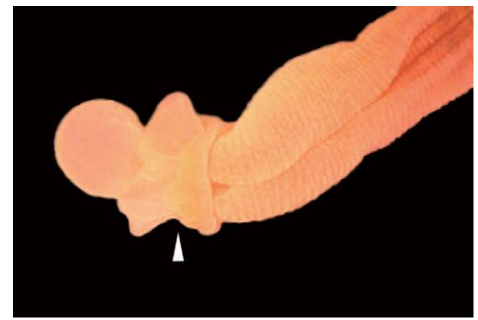

Fig. 2. Worm transected mid-collar at 11 days post-operation. Note that the new tissue integrates with the old tissue and only replaces the portion of the collar and the proboscis that was removed. Arrowhead marks site of transection.

appear to have exposed surfaces with three distinct surface morphologies and with, at most, only a single short cilium (Fig. 4A). The three cell types, in order of abundance, are cells with a blebbing or bubbly-appearing surface, cells covered with microvilli, and cells with a smooth surface. These exposed cells can be compared with the epithelial cells from the original surface nearby, which have a forest of cilia (Fig. 4B), as will the surface cells of the regenerate at later stages, and as do the epithelial cells of *P. flava* wherever we have examined them (not shown).

The accumulation of cells increases rapidly to a well-developed blastema by 4 days (Fig. 3E). By 5 days, as shown in Fig. 3F, the rapidly growing blastema has transformed into a flattened bulge of almost heart shaped central tissue (see also Fig. 5 of a fixed specimen at 5 days), the nascent proboscis, and two lateral ridges that will develop into the collar. About this time, the new pharyngeal opening of the gut is evident as a nascent mouth ventral to the blastema. During the next few days, the proboscis assumes its definitive shape, and the lateral ridges of the blastema expand ventrally to surround the mouth and dorsally to complete the collar. The appearance at 7 days is shown in the photograph in Fig. 3G and the SEM image in Fig. 5. The new head, attached directly to the tissue at the original cut surface, continues to grow rapidly and attains a size appropriate for the size of the worm by about 12 days, as seen in Fig. 3H. The animal, without any evidence of a regenerating new branchial region, begins to exhibit normal burrowing behavior and intake of sand grains.

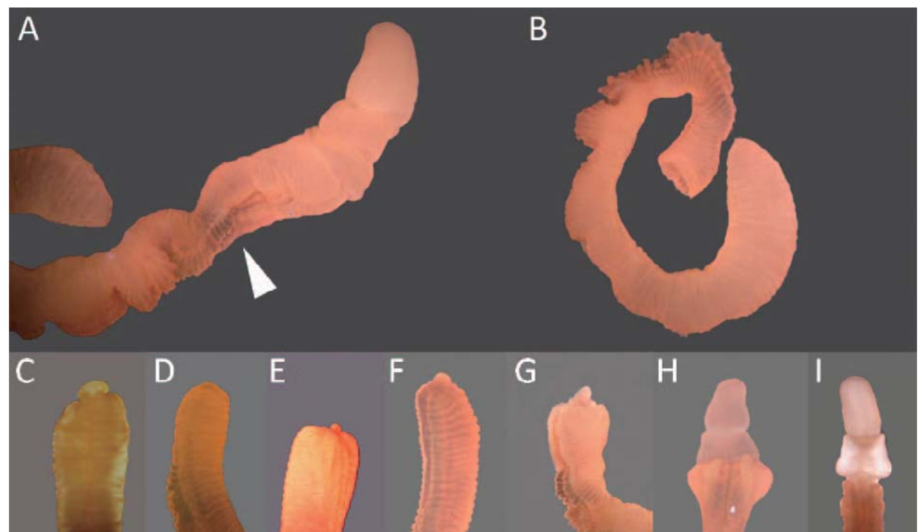

Fig. 3. Progression of anterior regeneration after transection of *P. flava* just posterior to the gill basket. **(A)** Intact worm marked at site of transection by arrowhead at posterior end of branchial basket. **(B)** Posterior body of severed worm just after transection. **(C–I)** Progression of regeneration from 2 days to 17 days. **(C)** Two days after transection, showing a bulge on one side of the swollen edge of the severed body wall as the wound is pulling closed. **(D)** Three-day transected anterior end, which has healed closed and exhibits a tiny blastema not apparent in this photo. **(E)** Four-day regenerate with closed wound and a growing blastema. **(F)** Five-day regenerate with a slightly flattened middle that is the nascent proboscis and small ridges on each side which will become the collar. **(G)** Seven-day regenerate with nascent proboscis and prominent collar ridges on each side. **(H)** Full-sized head grown to fit body diameter at 12 days. **(I)** Seventeen-day regenerate showing the early stages of formation of the branchial region as an insert of tissue between the regenerated head and the old body.

The first sign of regeneration of the missing pharyngeal region appears soon after the completion of the head. First there seems to be an insert of white tissue between the apparently completely regenerated head and the old body. This insertional blastema grows rapidly, as shown at 17 days in Fig. 3I, into a new pharyngeal region and branchial basket over the next few weeks, as illustrated at 25 days in Fig. 1A.

To determine whether gene expression in the regenerating tissue could be examined by in-situ hybridization, we

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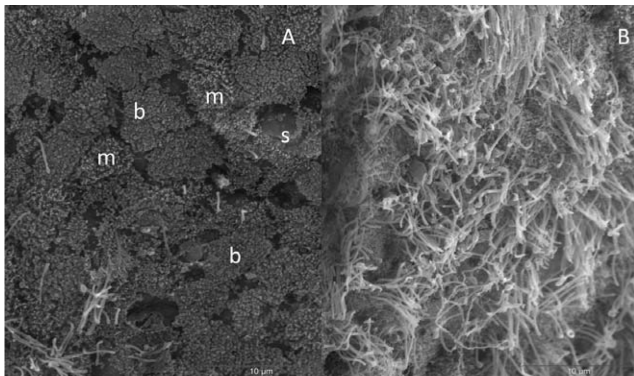

Fig. 4. Scanning electron microscope images of regenerating blastema cell surfaces and adjacent epithelial surface. **(A)** Exposed blastema cells at 3 days have a few single cilia; the blastema appears to have cells with at least three surface textures: b, blebbing cell; m, microvillar cell; s, smooth cell. **(B)** Cells of the original surface are covered with cilia, as are the surfaces we have observed on all older *P. flava* epithelia.

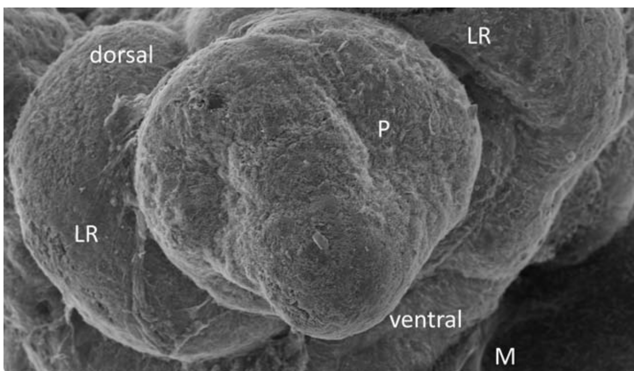

Fig. 5. Seven-day blastema with nascent proboscis (P) with lateral ridges (LR) on each side that will grow, extend around the mouth (M), and fuse to form the collar.

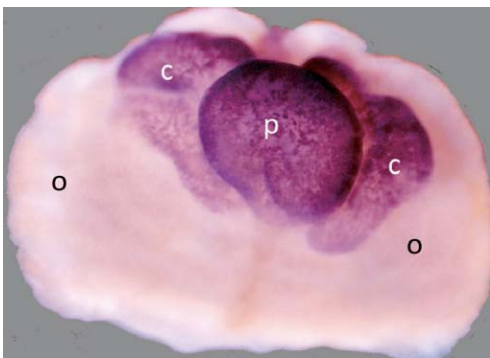

Fig. 6. In-situ hybridization, with a *PfSoxB1* probe, of 5-day regenerating head. The cells in the blastema tissue, which is organized as a central nascent proboscis and at this stage is flattened and somewhat heart shaped, and the two lateral ridges that will form the collar, show signals for SoxB1 expression. No signals were evident in the cells of the old tissue upon which the blastema formed. p, nascent proboscis; c, nascent collar ridges; o, original tissue.

performed this procedure following protocols developed for *P. flava* embryos and larva using a probe for the *PfSoxB1* gene (Taguchi et al., 2002). It appears, as shown in Fig. 6, that cells throughout a 3-day blastema exhibit a *SoxB1* signal when incubated with an antisense *PfSoxB1* probe. The blastema tissue shows a strong signal for the expression of *SoxB1*, while there is no evidence of a signal in the original tissue upon which the blastema formed. There is also no signal from a sense-strand control probe in tissue cut from the site immediately after transection or in a 3-day blastema (data not shown). Standard in-situ hybridization procedures can be adapted to study the course of gene expression during regeneration in *P. flava.*

DISCUSSION

These observations support and extend the many prior observations that extensive regeneration occurs in hemichordates (Cori, 1902; Dawydoff, 1907; Kuwano, 1902; Rao, 1955; Rychel and Swalla, 2008; Spengel, 1893; Willey, 1898). We observed universal regeneration whereever we transected the worm. Prior reports have indicated certain limits to regeneration after transection. These in some cases could represent species differences, but our experience, not discussed in detail, indicates that the health of the animals, and clean aquaria and holding conditions for the wounded animals, are critical for success in observing universal regeneration at all levels.

Our observations that an average of about 2% of animals at our collection site exhibit signs of anterior regeneration, and that visible signs of regeneration lasted for about 3 months in the regenerated animals in our aquaria, suggest that about 10% of *P. flava* individuals in this location lose their heads every year.

The rapidity and completeness of regeneration in *P. flava* is quite striking. The process of anterior regeneration can be divided into three phases. During the first three days after the wounding that removes the anterior parts, the anterior tissue swells and is mobilized, and the wound closes and heals shut. When the wound closes after about three days is the first suggestion of a group of cells assembled into a blastema to begin the process of reforming the lost parts of the body.

The second phase involves a very rapid increase in the cell mass of the blastema, without any evidence of remodeling of original tissue, and the epimorphic molding and differentiation of the blastema cells into the new head over the next 10 days. We could not determine whether the cells for the new head are derived from cell division or from mobilization of cells migrating out of the original body parts. Staining with Proliferating Cell Nuclear Antigen antibody has suggested there is rapid cell division at this time (Rychel and Swalla, 2008). If the cells are derived from the old body, we have seen no evidence that suggests this involves the release of cells or remodeling from the old tissues in the original body. The new head appears to just be added to the stump of the original body, which maintains its original appearance, as far as visual observation can tell.

The third phase of regeneration is the formation of a new branchial region. This begins at about 12 days after the new head is made and the animal begins to exhibit burrowing behavior. At that time, an insert of blastema-like tissue

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appears between the new head and the old body. This insert expands rapidly and differentiates into a branchial region with a new gill basket. The full restoration of the missing body parts is achieved about 5 weeks after original transection. It is another 5 weeks before the regenerated tissue regains enough pigmentation such that visual evidence of regeneration is lost.

The source and nature of the cells in the hemichordate blastema, a central issue in the more studied regeneration systems over the years, will only be resolved with further studies. The high expression of the *SoxB1* gene in the *P. flava* blastema cells supports the idea that blastema cells are multipotent stem-like cells, since *SoxB1* is now recognized as a gene important for producing iPS cells (Takahashi, et al., 2007). In hydra and planarians, the stem cells can be recognized cytologically and are known to be scattered throughout the tissue and to move to the site of regeneration to establish the blastema (Birnbaum and Sanchez Alvarado, 2008), which then grows by proliferation. However, the regeneration blastema in amphibians appears to be formed by the partial dedifferentiation of cells in the tissue into stem cell-like states, and these cells then enter the blastema and form the regenerated tissue (Kragl et al., 2009).

The demonstration that gene expression during hemichordate regeneration can be approached by in-situ hybridization is encouraging. The finding that the *SoxB1* gene is expressed in the blastema cells, but not in the tissue upon which the blastema forms, tends to confirm the general idea that the blastema is an accumulation of stem cells derived by mobilization of such cells from their niches in the adult tissue or are cells induced to differentiate into stem cells by the wounding process. This result confirms the potential of in-situ hybridization and other powerful molecular approaches coupled with the genomic information that will soon be available from sequencing of the *P. flava* genome (Tagawa et al., in preparation) to contribute to significant progress in the study of regeneration in this system.

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