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Siphon Regeneration Capacity is Compromised During Aging in the Ascidian *Ciona intestinalis*

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Abstract

The ascidian *Ciona intestinalis* has a short life span and powerful regeneration capacities. The regeneration of the oral siphon (OS) involves wound healing, blastema formation, cell proliferation, and replacement of eight oral pigment organs (OPO), the latter via differentiation and migration of stem/precursor cells from localized siphon niches in the siphon. The restoration of OPO pattern during OS regeneration occurs with a high degree of accuracy through three successive cycles of amputation. It is shown here that oral siphons of the largest and oldest members of a wild *Ciona* population do not completely regenerate their siphons after amputation. The loss of regeneration capacity was accompanied by reduced cell proliferation. In contrast to arrested OS outgrowth, the stem/precursor cells responsible for OPO replacement “over-differentiate” after OS amputation in the oldest animals, the typical number of OPO is increased from eight to twelve-sixteen, and malformed OPO are produced. Also in contrast to younger animals, the oldest animals of the population show arrested OPO development after two consecutive cycles of amputation and regeneration. We conclude that there is a size and age threshold in *Ciona* after which the regenerative capacity of the OS is compromised due to effects of aging on cell proliferation.

Keywords

Ascidian; *Ciona intestinalis*; siphon regeneration; oral pigment organs; cell proliferation

1.1 Introduction

Regeneration is considered to be one of the major unsolved problems of biology (Morgan, 1901; Goss, 1969; Sánchez Alvarado, 2000). In recent years, however, previously intractable questions in regenerative biology have begun to be answered by taking advantage of invertebrate models with powerful regeneration capacities (Reddien et al., 2005; Cebriá et al., 2007; Dupont and Thorndyke, 2006; 2007; Peterson and Reddien, 2008; 2009). As the secrets of regeneration are beginning to be revealed, a related problem comes into sharp focus: the loss or fading of regenerative potential during aging.

While some vertebrates have substantial regenerative capacities early in life, the capacity to accurately replace lost parts often recedes and subsequently disappears with maturity. Frogs

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are able to replace missing appendages at the tadpole stage, but this capacity is lost after metamorphosis (Givan et al., 2002). Juvenile opossums show retarded posterior development, and consequently the more slowly developing hind limbs, in contrast to the more developmentally advanced forelimbs, are capable of regeneration (Mizell, 1968). Young children are sometimes able to replace the tips of severed fingers, but this ability recedes as they mature (Illingworth and Barker, 1980). Viewing regeneration as part of the broader field of tissue wounding and repair, it is commonly observed that the ability to close and repair wounds decreases with age (Reed et al., 2003). These and other studies suggest that the ability to replace lost parts after injury is compromised during aging.

Because most vertebrates have limited regeneration abilities, complex tissues and organs, and relatively long life spans, alternative animal models with short life cycles and more powerful regeneration capacities will be useful to resolve the relationships between regeneration and aging. Here we employ the tunicate (ascidian) *Ciona intestinalis* as a model to study the effects of aging on tissue repair and regeneration. *Ciona* has powerful regeneration capacities, and recent phylogenetic analysis has placed the tunicates as the closest living relatives of vertebrates (Bourlat et al., 2006; Delsuc et al., 2006). Thus, understanding the relationship between regeneration and aging in *Ciona* may provide insights into this phenomenon in vertebrates.

Ascidians are chiefly known for their determinative style of embryonic development in which isolated blastomeres lack the regulative capacity to form complete tadpole larva (Jeffery, 2001). However, this inability to replace lost parts is reversed after metamorphosis when juveniles begin to show regenerative capacities (Carnevali and Burighel, 2010). Among the organs capable of regeneration are the neural complex (including the brain, or cerebral ganglion, of the central nervous system) (Hirschler, 1914; Bollner, et al., 1992; Dahlberg et al., 2009) and the oral and atrial siphons (Fox, 1924; Von Haffner, 1933; Sutton, 1953; Whittaker, 1975; Auger et al., 2010). The siphons contain pigmented organs on their distal margins, which have been proposed to function as sensory receptors (Dilly and Wolken, 1973). There are usually eight oral pigment organs (OPO) located in notches between muscular lobes in the oral siphon (OS). After OS amputation, the pattern of eight equidistantly spaced OPO is replaced with complete accuracy: first, new pigment cells differentiate from local stem/precursor cells located in niches located within the “stump” of the amputated siphon, then the differentiated pigment cells migrate distally and accumulate in lines along the wound surface, next the lines of pigment cells condense into pigmented spots, and eventually a complete OPO consisting of a pigmented cup surrounding a pit of epidermal cells with a basal neural component is reformed (Auger et al., 2010). *Ciona* siphon and OPO regeneration resemble vertebrate limb regeneration in that cell proliferation in a blastema, stem cell recruitment from local sources, and intercalation, proximal-distal polarization, and precise restoration of patterned elements are all part of the process.

Ciona has a life span of about a year in the wild, where several successive generations are normally present (Berrill, 1947; Millar, 1952; Dybern, 1965; Peterson et al., 1995). The tadpole larvae metamorphoses a few days after fertilization, juvenile development takes about ten days, and adults grow isometrically throughout the rest of their life (Berrill, 1947; Millar, 1953). The correlation between body length and age led to the discovery of an inverse relationship between aging and brain, OS, and OPO regeneration (Dahlberg et al., 2009; Auger et al., 2010), showing that the capacity to replace lost parts fades during the adult life in ascidians, just as it does in vertebrates. However, the question of whether regenerative capacity is eventually lost during aging has not been previously addressed. Here it is shown that OS regeneration and OPO replacement is compromised in the oldest animals of a natural *Ciona intestinalis* population.

2.1 Materials and Methods

2.1.1. Biological Materials

Ciona intestinalis was collected in the wild by diving at a site near Brest, France and maintained in aquaria with running natural seawater at Station Biologique, Roscoff, France. Clusters of animals attached together to a support (shells, stones, etc) and containing each of the size classes described in the Results were collected from the same site three separate times during the months of July and August and were used in the experiments within three days after collection. Collections at a single site where all animals are subjected to the same environmental conditions abrogated the possibility that size differences were result of different different microhabitats rather than different ages.

2.1.2. Body Length Determination

Body length was measured in animals that were anesthetized by treatment for 15 min at 18°C with 2 mg/ml tricaine methanesulphonate (MS222; Sigma-Aldrich, St. Louis, MO) dissolved in seawater. The flaccid animals were allowed to flatten in a container containing MS222/seawater and their length from the base of the body to the distal margin of the OS was measured under a dissection microscope.

2.1.3. Siphon Amputation and Regeneration

Amputation of the OS was carried out on anesthetized animals (Auger et al., 2010). For each animal, the length of the OS, from its base to its distal margin, was recorded. The siphon was then severed in a plane perpendicular to its long axis at a point midway between its base and opening, thus leaving an amputated “stump” consisting of the proximal half of the original siphon.

After siphon amputation, the animals were removed from MS22-containing seawater and allowed to recover for 30 min in a tank containing seawater without anesthesia. Recovered animals were recognized by elicitation of muscular contractions and “squirting” after probing. Animals that did not recover in 30 min were discarded and excluded from further analysis. The regenerating animals were maintained in a large aquarium containing natural running seawater (16°C) for up to one to two months.

Regenerating animals were re-anesthetized and assayed for the extent of OPO replacement and/or siphon regeneration periodically. The extent of OPO regeneration was determined by direct microscopic observations and/or photography of living or fixed OS flat mounts (Auger et al., 2010). The percent siphon regeneration was calculated for each animal by dividing the length of the regenerated siphon by half the length of the original siphon.

2.1.4. Cell Proliferation

Cell proliferation was determined by staining OS flat mount preparations with phosphohistone-H3 antibody. Regenerating animals were fixed in 4% paraformaldehyde, washed in PBS-0.1% Tween-20, stained with a 1:50 dilution of phosphohistone-H3 antibody (Upstate Biotechnology, Lake Placid, NY) for 1.5 h, and then with a 1:400 dilution of rhodamine-labeled goat anti-rabbit IgG for 1.5 h. Flat mount preparations of the stained specimens were prepared and photographed. Labeled cells were counted in 400 μm^2 areas located immediately above and below the amputation plane.

2.1.5. Explant Culture

Explants of OS mid-pieces were prepared as described in Figure 5A and cultured at 18°C in sterile 12-well culture plastic plates containing 10% GIBCO Neurobasal culture medium

(Invitrogen, Carlsbad, CA), 10 µg/ml kanamycin (Sigma-Aldrich), and 50 µg/ml gentamycin (Sigma-Aldrich) in Millipore-filtered seawater. The culture medium was replaced daily for 14 days. To assess the extent of red/orange pigment cell differentiation, the explants were removed from culture medium, flat mounts were prepared, and cells were counted in 100 µm² regions of the oral siphon region band (ORB).

3.1. Results

3.1.1. Size and Age Classes

The life span of *Ciona intestinalis* is about a year under natural conditions and includes several co-existing generations (Berrill, 1947; Millar, 1952; 1953; Dybern, 1965; Peterson et al., 1995). Moreover, continuous growth during this period is proportional to age, allowing animals of different ages from the same microhabitat to be distinguished by size (Fig. 1A). A total of 136 animals collected as clusters in the wild from the same site were separated into four size classes: 5–8 cm (Class 1), 8–10 cm (Class 2), 12–15 cm (Class 3), and 16–20 cm (Class 4). Although the precise age of animals obtained in the wild is unknown, body length was used as a proxy for age (Millar, 1953), accordingly Class 4 animals were designated as the oldest animals in the group, and Class 1 animals as the youngest animals in the group.

3.1.2. Age Related Changes in Siphon Regeneration

The OS was amputated at a position about half the distance between the connection to the body and the distal margin, and the extent of regeneration was determined after one month (Fig. 2A–C). As shown in Figure 2C, most Class 1–3 animals completed OS regeneration by one month post-amputation (PA), although some of the smaller Class 1 animals actually exceeded 100% OS regeneration during this period because they grew more rapidly than larger animals. In contrast, Class 4 animals regenerated only about 10–20% of the original siphon length during the one-month assay period (Fig. 2C). Even after 2 months, these Class 4 animals had not progressed much farther in OS regeneration than during the previous month (Fig. 1C). The difference in regeneration of siphon lengths seen in Class 4 versus Class 1–3 animals was significant ($p = 0.000$; One way Anova), but there was no significant difference between the extent of OS regeneration in Class 4 animals after one or two months PA. The results show that there is a size threshold in which *Ciona* are unable to completely regenerate an amputated OS.

3.1.3. Cell Proliferation is Decreased During OS Regeneration in Old Animals

To determine whether the arrest of OS regeneration in old animals involves a change in cell proliferation, regenerating animals were fixed at 3, 6, and 15 days PA, stained with phosphohistone-H3 antibody, a marker of dividing cells (Hans and Dimitrov, 2001), and the number of stained cells was determined in regions of the OS above and below the amputation plane. Because minimal differences were observed in OS regeneration between Class 1, 2, and 3 animals (Fig. 2C), only Class 3 and 4 animals were compared in these and subsequent experiments. As shown in Figures 2D, I and 3, phosphohistone-H3 stained cells were significantly decreased in number in the region immediately above the amputation plane at 3 ($p = 0.001$), 6 ($p = 0.002$), and 15 ($p = 0.01$) d PA in Class 4 relative to Class 3 animals. The differences in cell proliferation were not reflected in the region immediately below the amputation plane, suggesting the largest animals were still in a state of active growth. Significant increases ($p = 0.001$ at 3 and 6 d PA, and 0.02 at 15 d PA) were also seen in the number of dividing cells located above the amputation plane compared to those located below it in Class 3 but not Class 4 animals (Fig. 3), confirming the existence of a regeneration blastema in the former. The results suggest that a large decrease in cell proliferation is responsible for the reduction in regenerative capacity of old animals.

3.1.4. Age Related Changes in OPO Regeneration

Animals of each class had an OS consisting of red/orange OPO located in clefts within a yellow pigment band (Fig. 1B, C). OPO are replaced during the first 7–10 days of OS regeneration (Auger et al., 2010). To investigate the possibility of age related changes in OPO regeneration, the OS of Class 3 and 4 animals were amputated as described above (Fig. 2A, B) and OPO replacement was assessed periodically for one month PA. In Class 3 animals (Fig. 2E–H), OPO replacement occurred as described previously (Auger et al. 2010): red/orange pigment cells differentiated throughout the OS (Fig. 2E), subsequently some of these cells migrated distally and formed pigment lines along the siphon margin (Fig. 2F), then each pigment line coalesced into a red/orange cup-shaped organ (Fig. 2G) and developed into a complete OPO consisting of a pit of epidermal cells surrounded by a cup of red/orange pigment cells and adjacent bands of yellow pigment (Fig. 2H). In contrast, OPO regeneration in Class 4 animals differed from the normal pattern in several ways (Fig. 2J–M). First, the number of red/orange cells in the amputated OS was more than in Class 3 animals (Fig. 2J, E; Table 1). Second, large aggregations of red/orange pigment cells were observed at the siphon margin later during replacement, and the spacing between these centers was not equidistant (Fig. 2K), as was observed in young animals. Third, the aggregation centers formed multiple cups of red/orange pigment cells, usually appearing as adjacent pairs or triplets (Fig. 2J). Fourth, the multiple pigment cups eventually produced patches of large malformed OPO composed of a mixture of red/orange and yellow cells (Figs. 2L, M). Lastly, instead of the typical number of 8 OPO regenerated in Class 3 animals, in most Class 4 animals the number of replaced OPO ranged from 12 to 16 (Table 2).

A key aspect of OS regeneration is that the number and spacing of OPO is reproduced with complete fidelity during at least three cycles of amputation and regrowth (Auger et al., 2010). The regeneration of larger numbers of OPO in Class 4 animals after one OS amputation cycle (Fig. 2I–K) suggested that the fidelity of regeneration could be changed in old animals. To investigate this possibility, Class 3 and 4 animals were subjected to two consecutive amputation and regeneration cycles, and the number of replaced OPO was determined in individual animals after the end of each cycle. Although eight OPO are typically found spaced around the OS opening in natural *Ciona* populations, a low proportion of animals can have seven or nine OPO (Millar, 1953; Auger et al., 2010). Animals exhibiting the latter phenotypes in the population were identified and included in this experiment to bolster variation in OPO number. In Class 3 animals, the number of OPO was accurately replaced during two amputation and regeneration cycles: each animal with 7 original OPO regenerated 7 OPO, each animal with 8 OPO regenerated 8 OPO, and each animal with 9 OPO regenerated 9 OPO (Table 2). Class 4 animals behaved differently. First, as described above, Class 4 animals typically generated up to 12–16 OPO after a single amputation and regeneration cycle (Table 2; also see Fig. 2K–M). Second, following the second amputation and regeneration cycle, some OPO were not entirely reformed in Class 4 animals (Fig. 4). In these cases, OPO replacement arrested at the stage when red/orange pigment cells formed lines along the siphon opening. The lines subsequently merged with yellow pigment cells (Fig. 4A, C); and later the regenerated OS showed a band of superimposed red/orange and yellow pigment cells in place of an OPO (Fig. 4B, D).

In summary, the results show that changes in OPO number, structure, and accuracy of replacement occur after OS amputation in old animals.

3.1.5 Local Source of Pigment Cells for OPO Regeneration

After OS amputation, red/orange pigment cells appear to “over-differentiate” in the siphon stump, forming excess OPO in Class 4 relative to Class 3 animals (Fig. 2E, J; Table 2).

There are two possibilities to explain this phenomenon: (1) either stem/precursor cells responsible for red/orange pigment cell differentiation are more abundant in the siphon niches of Class 4 animals, or (2) the local cells are joined by additional stem/precursor cells migrating into the siphon niches from the body. Siphon explant experiments were conducted to distinguish between these possibilities. Explants were prepared in two steps (Fig. 5A): (1) the distal 50% of the OS was amputated, and the resulting distal siphon fragment was discarded, and (2) another amputation was carried out in a plane perpendicular to the first amputation plane at a level midway between it and the siphon base. The siphon fragments derived from the second amputation (explants) were placed in culture and observed over the next 14 days. Class 3 explants developed OPO on their distal margins (Fig. 4B), as described previously (Auger et al., 2010), but explants from Class 4 animals did not develop distinct OPO during the culture period. However, explants from Class 4 animals differentiated a larger number of red/orange pigment cells than their counterparts from Class 3 animals (Table 1; Fig. 5C, D), similar to the situation observed *in vivo*. The results suggest that excess red/orange pigment cells appear in old animals as a result of local differentiation in the OS rather than because of re-enforcement by migratory stem/precursor cells from the body and that the stem/precursor cell niches of old animals are overpopulated with OPO progenitors.

4.1. Discussion

This investigation has revealed changes and defects in OS and OPO regeneration after siphon amputation in the largest and oldest individuals of a natural *Ciona* population. The primary change is in the ability to re-grow the OS to full length, which is greatly diminished in the largest and oldest animals in the population. Within a two-month period after siphon amputation, these animals were unable to re-grow the OS to the extent seen in younger animals during a single month. The alternative explanation for our results, saturation of growth rate at old age, is not supported by experiments showing that the extent of cell proliferation was not significantly different in the amputated siphon stump young and old animals.

Several changes were also detected in the process and pattern of OPO regeneration between young and old animals (summarized in Fig. 6). First, old animals differentiate an excessive number of red/orange pigment cells in their siphons, which subsequently migrate to the distal opening of the regenerating OS, resulting in the formation of multiple and structurally malformed OPO. Second, because of these supernumerary OPO the normal pattern of OPO spacing is defective along the siphon margin of old animals. Lastly, after two consecutive cycles of OS amputation and regeneration, old animals do not reform complete OPO, but instead produce bands of pigment cells with not distinguishable OPO. This contrasts to young animals in the same population that restore the normal structure, number, and pattern of OPO with remarkable fidelity, even after several successive cycles of OS amputation and regeneration.

As a result of this study, we now understand that *Ciona* proceeds through three different periods during its life cycle with respect to regenerative capacity. During the first period, the highly determinative mode of development lacks a program for regeneration of embryonic or larval cells and tissues (Jeffery, 2001). During the second period, which is initiated by metamorphosis, regenerative abilities are activated in juveniles and adults, and some tissues and organs can be completely reformed following their removal or injury (Carnevali and Burighel, 2010). During the third period, which begins near the end of the normal life span, regenerative capacity, at least in the case of the OS, is significantly modified or lost in the oldest members of natural populations. Therefore, just as has been described in vertebrates,

the capacity to replace injured tissues and organs by regeneration gradually fades and disappears during aging in *Ciona*.

The activation of cell proliferation in a blastema is an important aspect of new siphon outgrowth during regeneration in young animals (Auger et al., 2010). In contrast, phosphohistone-H3 antibody staining showed that very little cell proliferation occurs in the amputated siphon of old animals. Therefore, the primary cause of arrested OS regeneration in old animals is likely to be a dramatic decline in cell proliferation. In the place of dividing cells, the amputated siphon of old animals is largely populated by an excess of non-proliferating red/orange pigment cells. The differentiated pigment cells, which are the precursors of the pigmented cups of the OPO, are produced by stem/precursor cells located in specific niches within the ORB, eight longitudinal siphon stripes in register with OPO and extending from its distal tip to its base (Auger et al., 2010). A similar inference concerning the importance of reduced cell proliferation in the modification of tissue repair processes was reached by comparing short-lived and long-lived species of marine bivalves (Strahl et al., 2010; Philipp and Abele, 2010). Thus, reduced cell division could be a general phenomenon responsible for decay in regenerative capacity during aging.

A local source of stem/precursor cells for OPO replacement in old animals is strongly supported by the explant studies reported in this investigation. Old animals have a surplus of stem/precursor cells in their siphon niches that can be activated to differentiate into pigment cells and ultimately produce multiple OPO. However, after a second cycle of amputation and regeneration in many cases old animals are unable to re-form complete OPO. The fact that bands of pigment cells replace fully formed OPO suggests that aging also affects the ability of the siphon epidermis to participate in OPO replacement. Alternatively, it is possible the quality of red/orange pigment cells is compromised after the second amputation in old animals, such that they are unable to induce OPO formation. Further studies on the development of OPO components during the second cycle of amputation and regeneration in old animals will be necessary to resolve this issue.

The excessive differentiation of red/orange pigment cells and formation of multiple OPO in the absence of siphon regrowth in old animals suggests that siphon regeneration consists of at least two distinct processes that are uncoupled during aging. The first process, siphon regrowth, which is dependent on cell proliferation, is arrested in old animals. The second process, replacement of OPO, is independent of cell proliferation (Auger et al., 2010) and actually enhanced in old animals, at least after one cycle of amputation and regeneration. The reason for “over-differentiation” of pigment cells and OPO could be related to the uncoupling described above; undifferentiated precursor/stem cells could continue to accumulate in the siphon niches despite a gradual slowing of siphon growth late in the life cycle. Thus, when the OS is amputated in the oldest members of the population and fails to re-grow to full length, the accumulated red/orange pigment cells are present at levels above those required to re-produce a normal complement of OPO in the truncated siphon.

The only other case in which “over-differentiation” and changes in OPO pattern has been seen in *Ciona* is when amputation is carried out at the base of the OS, or below its connection to the body (Auger et al., 2010), rather than within the siphon itself, as was done in all of the experiments reported here. This situation was also explained by a surplus of undifferentiated stem/precursor cells located in the body of the animals, which were recruited to participate in siphon regeneration from a long distance source. Presumably these cells multiply somewhere else in the body prior to their recruitment into the siphon niches, perhaps in the endostyle, which is located immediately below the OS and known to be a source of stem cells in *Botryllus schlosseri*, another ascidian species (Voskoboinik et al.,

2008). It follows that overpopulation of the siphon stem cell niche in old animals could be the consequence of an aging endostyle.

The results reported here contribute to a surprisingly small literature on the relationship between aging and regeneration. Two regenerating organs affected by age have now been identified in *Ciona*, the OS and the brain. The OS has been shown to decrease its regeneration rate during the life cycle and ultimately to cease overall regeneration as animals reach old age, while showing a high level of inaccuracies in replacing OPO. In contrast, the brain is currently known only to decrease in rate of regeneration during the life cycle, and in future studies it will be interesting to determine whether the capacity to accurately replace this organ is also compromised in old animals.

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Highlights

The oldest animals in wild *Ciona intestinalis* do not regenerate the oral siphon
Lack of regeneration is accompanied by dramatically decreased cell proliferation
Stem/precursor cells for siphon pigment organs over-differentiate in old animals
Overdifferentiation is due to excessive stem/precursor cell accumulation

Figure 1.

Ciona intestinalis. A. Examples of the four classes of animals studied in this investigation. 1: Class 1. 2: Class 2. 3: Class 3. 4: Class 4. B. Oral (OS) and atrial (AS) siphons showing OPO arranged equidistantly in clefts embedded within yellow pigment bands along the openings of each siphon. Scale bar: 5 mm. C. A single orange OPO surrounded by a band of yellow pigment cells in the OS. Scale bar: 100 μ m.

Figure 2.

Age related changes in OS and OPO regeneration. A. A diagram showing the plane of OS amputation. B. An animal with an amputated OS (compare to Fig. 1B, the same animal before OS amputation). Scale bar: 5 mm. C. Histograms showing the percent OS regeneration (See Materials and Methods) measured one or two months PA. The black bar (far left) shows the extent of OS regeneration in Class 1 animals (N = 20), the dark grey bar (middle left) shows the extent in Class 2 animals (N = 31), the light grey bar (middle right) shows the extent in Class 3 animals (N = 40), and the white bar (far right) in Class 4 animals (N = 45) at one month PA. The orange bar shows the extent of regeneration in Class 4 animals (N = 24) at two months PA. Error bars: standard error of the means. D–M. Age related changes in cell proliferation and OPO regeneration in Class 3 (D–H) and 4 (I, M) animals. D, I. Cell proliferation determined by staining with phosphohistone H3 antibody at 6 days PA (also see Table 1). Arrows indicate stained cells. The progress of OPO regeneration determined at 2–4 (E, J), 5–8 (F, K), and 9–14 days PA (G, L), and at one month PA (H, M). Scale bar in D is 50 μ m; magnification is the same in D, E, I, J. Scale bar in F is 200 μ m; magnification is the same in F–H and K–M.

Figure 3.

The role of cell proliferation in OS regeneration in Class 3 and 4 animals determined by phosphohistone H3 antibody staining. The number of phosphohistone H3 stained cells are significantly higher in the region above the siphon amputation plane compared to the region below it in Class 3 animals (gray bars) but not in Class 4 (red bars) animals. A: amputation plane. dpa: days post-amputation. Asterisks represent significant differences. N is shown below each bar.

Figure 4.

Age related changes in the fidelity of OPO pattern after two cycles of OS regeneration. Class 4 animal subjected to two cycles of OS amputation and photographed 15 days (A, C) or 30 days (B, D) PA after the second amputation showing permanent development of lines (arrows), rather than OPO, along parts of the margin of the regenerating OS. A, B. Scale bar is 2 mm. C, D. Scale bar is 100 μ m. See Table 1 for OPO fidelity in Class 3 animals after one and two cycles of OS amputation and Class 4 animals after one cycle of OS amputation. Other abbreviations are the same as in Figure 1.

Figure 5. Local sources of pigment cell differentiation and OPO regeneration in OS explant cultures. A. Explant method. Step 1: Distal portion of OS removed and discarded. Step 2. Distal portion of siphon stump removed and placed into explant culture. B. Explant from Class 3 animal showing OPO development. C, D. Explant from Class 4 animal showing excessive numbers of red/orange pigment cells. ORB: OPO regeneration band. LMB. Longitudinal muscle band. Scale bar in B is 100 μ m; B and C are the same magnification. Scale bar in D is 20 μ m.

Figure 6.

Summary diagram of differences in OPO replacement in young (Classes 1–3, left) and old (Class 4, right) animals after one cycle of OS amputation and regeneration. The left and right panels represent events along the margin of OS opening during five consecutive stages of OPO replacement (1–5, left). The thick black horizontal lines represent the OS margin. The downward protrusions of this line in stages 4 and 5 (black to green lines) represent the formation of the epidermal pit component of the OPO with more pits formed in old compared to young animals. The upward protrusion of this line in stage 4 and 5 represent the outgrowth of siphon lobes, which occurs only in young animals. ORB represents the niche in which stem/precursor cells differentiate in red/orange pigment cells during OPO replacement. LMB represents the region of longitudinal muscle bands, and the thin vertical blue lines are muscle fibers. Red spheres: red/orange pigment cells. Yellow spheres: yellow pigment cells. White spheres: other cells. The excess numbers of red/orange pigment cells form supernumerary pigment cups and OPO whose pattern of equidistant spacing is changed in old compared to young animals.

Table 1

Red/orange pigment cell differentiation during OS regeneration

Class	Intact OS ^a				Explant OS ^b			
	N	Mean	+/-SEM ^c	Significance ^d	N	Mean	+/-SEM	Significance
3	5	10.00	+/-2.51	p = 0.015	4	22.7	+/-4.09	p = 0.022
4	7	31.86	+/-5.10		5	57.00	+/-10.79	

^a3 days PA.

^b10 dpa.

^cSEM: standard error of the mean. Two tailed t test.

Table 2

Fidelity of OPO replacement during repeated cycles of OS regeneration

Class	N	Cycle	Beginning OPO Number			Regenerated OPO Number										
			7	8	9	>9	7	8	9	..12	13	14	15	16		
3	15	1	2	12	1	0	2	12	1	0	0	0	0	0		
		2	2	12	1	0	2	9*	1	0	0	0	0	0		
4	15	1	1	10	1	0	0	1	1	4	1	1	4	3		
		2	0	1	1	13	Most regenerated bands (See Figure 4) *									

* animal(s) died during a cycle