Regeneration, Stem Cells, and Aging in the Tunicate *Ciona*: Insights From the Oral Siphon

William R. Jeffery

Eugene Bell Center for Regenerative Biology and Tissue Engineering, Marine Biological Laboratory, Woods Hole, MA, USA.
Department of Biology, University of Maryland, College Park, MD USA.
Tel: 301-405-5202
Fax: 301-314-9358
Jeffery@umd.edu

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Abstract

Regeneration studies in the tunicate *Ciona intestinalis* have recently been focused on the potential of adult stem cells to replace injured tissues and organs during the adult life cycle using the oral siphon (OS) as a model. The OS has oral siphon pigment organs (OPO) along its rim and an underlying network of muscle fibers in its tube. Different regeneration processes are triggered by OS amputation at the tip, along the tube, or at the base. One process involves the replacement of OPO without new cell division by direct differentiation of locally deployed stem cells or stem cells that migrate from the branchial sac. Another process involves blastema formation by the migration of progenitor cells produced from branchial sac stem cells. The capacity for complete and accurate OS regeneration declines continuously during the adult life cycle. Finally, after an age threshold is reached, OS regeneration ceases in old animals. The loss of regeneration capacity in old animals involves the depletion of stem cells in the branchial sac, the inability of brachial sac progenitor cells to migrate to the sites of regeneration, and defective OPO replacement. The significance of the OS model for studying regeneration, stem cells, and aging will be enhanced by the application of molecular methods.

Key Words: *Ciona intestinalis*, oral siphon, regeneration, adult stem cells, progenitor cells, branchial sac, blastema, aging

1. **Introduction**

The ability to replace injured tissues and organs is a key feature of many different animals (Brockes and Kumar, 2008). However, regenerative potential has been repeatedly lost during the evolution of some animal groups (Bely, 2010; Poss, 2010). For example, although certain vertebrates, such as teleost fishes and urodele amphibians, are able to completely replace severed appendages, anuran amphibians, birds and mammals do not have this capacity, at least as mature adults. The loss of regenerative capacity has a developmental basis. Anuran tadpoles can replace severed limbs prior to metamorphosis, but not as adult frogs (Given et al., 2002). Young opossums can replace their slowly developing hind limbs but not their rapidly developing forelimbs (Mizell, 1968). Some human children also have the capacity to replace severed digits but this ability disappears later during childhood (Illingsworth and Barker, 1980). Even species with powerful regeneration and tissue repair capacities as young adults show subsequent reduction in this ability during aging (Reed et al., 2003; Poss, 2010; Seifert and Voss, 2013; Sousounis et al., 2014). Important clues about how and why the ability to regenerate has been lost during evolution might be obtained by studying the decay of regenerative potential during aging, a process termed “regenerative aging”.

To study regenerative aging we need to understand the general principles of regeneration in an animal that has extensive tissue and organ replacement capacities. Questions to be answered include: 1) what steps are necessary for regeneration, 2) what cells and molecules are involved in replacing tissues and organs, and 3) how are regenerative processes regulated? Then, we need to understand how aging affects regeneration. Ultimately, it would be desirable to obtain enough

information to reverse regenerative aging. This knowledge then might also be used to rescue regeneration in animals that have evolved restricted regenerative capacity, such as mammals.

Achieving these goals requires a model animal with favorable attributes for studying regenerative aging. These attributes include powerful regenerative capacities during youth and more restricted capacities during aging. Other favorable attributes would be: the ability to study aging in the laboratory over a reasonably short time period, a structurally simple organism capable of rapid and accurate regeneration, and -since a long term goal is the reversal of lost regenerative processes in vertebrates- the model animal should be closely related to vertebrates.

One of the most suitable organisms for this type of study is the ascidian tunicate *Ciona intestinalis* (Kourakis and Smith, 2015). First, it can be grown in laboratory culture from eggs to adults (Cirino et al., 2002; Joly et al., 2002), offering the opportunity of testing regenerative potential in individual animals at any stage of the life cycle. Second, *Ciona* has a relatively short life cycle of about 1.5 years (Berrill, 1947, Millar, 1952; Dybern et al, 1965; Peterson et al., 1995; Kourakis and Smith, 2015). Third, it has a fairly simple body plan and contains fewer different types of tissues and organs than vertebrates (Millar, 1953). Fourth, it has a long history of use as a model in developmental biology (Satoh, 1994; 2013), and a large molecular toolkit is available for studying this organism (Stolfi and Christiaen, 2012). Fifth, *Ciona*, like other tunicates (Berrill, 1951; Rinkevich et al., 1995; Tiozzo et al., 2008), has powerful regenerative capacities and displays regenerative aging (Jeffery, 2015a). Lastly and most importantly, *Ciona* belongs to a chordate group (tunicates) that has been inferred as the closest living relative of the vertebrates (Bourlat et al., 2006; Desuc et al., 2006).

There is a long history of regeneration research in adult *Ciona* (Jeffery, 2015b) but few studies have considered regenerative phenomena during the life cycle or in the context of stem

cell function and aging. Here, we review recent studies on regeneration, stem cells, and aging in *Ciona* using the oral siphon as a model.

2. **Life Cycle, Adult Organization, and Growt**h

Ciona is a solitary ascidian with a life cycle consisting of larval and adult stages (Fig. 1A). The motile non-feeding larva, which consists of a trunk (or head) and a tail, is formed from an egg within about a day after fertilization by rapid stereotypical cleavages and post-gastrulation cell movements (Satoh, 1994). Like other ascidians, embryonic development in *Ciona* is strictly determinate: ablation or removal of blastomeres results in missing parts of the embryo or larva (Jeffery, 2001). Likewise, excision of larval parts, such as the tail (Takamura et al., 2002), does not result their replacement. Therefore, regenerative potential is not apparent during the embryonic and larval stages of the *Ciona* life cycle.

After a free swimming period, the larva settles head first on a substrate and undergoes metamorphosis, a process in which the larval tail is retracted into the trunk, and the latter then undergoes re-organization and differentiation of new body parts to form a sessile filter feeding adult (Fig. 1A, B). The newly formed distal body tissues and organs first become capable of regeneration after metamorphosis (Jeffery, 2015b). The mechanisms responsible for the conversion of a non-regenerating larva into a regenerating adult are unknown in *Ciona* and other tunicates.

Adult *Ciona* have an elongate vase-like body consisting of proximal (toward the attached base) viscera, a large pharynx containing a branchial sac, and two distal siphons (Fig. 1B). The tunic, a protective layer containing cellulose-like material that is a defining feature of the tunicates, covers the entire body. The perforated branchial sac is suspended in the pharyngeal

cavity leading into the oral siphon, the orifice through which food particles are driven into the underlying branchial sac for filtration and passing to the stomach for digestion (Fig. 1B). The endostyle, an elongate tube shaped organ, which is considered to be the ascidian homologue to the vertebrate thyroid gland (Ogasawara and Satoh, 1998), runs along the ventral side of the branchial sac, and the dorsal strand, a neural tissue, runs along its dorsal side. The viscera include the stomach, intestine, heart, and hermaphroditic gonad. The gonad and intestine are connected to gonoducts and a rectum respectively, which empty into the atrial cavity. The atrial siphon is used to expel gametes and fecal materials (Fig. 1B). The neural complex (NC), which consists of a single ganglion (brain) with an associated glandular organ (neural gland), lies in the apex between the two siphons and tapers into the dorsal strand on its posterior side. The brain is quite small, containing only a few hundred neurons and possibly some associated supporting cells (Millar, 1953; Bollner et al., 1992, 1993, 1995, 1997; Dahlberg et al., 2009). Paired nerve tracts exit the anterior and posterior ends of the brain and extend into the siphons, branchial sac, and viscera, dividing into multiple tracts and fibers along the way (Millar, 1953; Markman, 1958; Mackie et al., 2006).

Ciona adults grow rapidly and isometrically, eventually developing gonads and becoming gravid in only a few months (Berrill, 1947; Millar, 1952; Dybern et al. 1965; Peterson et al., 1995). During the growth phase, the NC and siphons show remarkable regenerative potential. However, the rate of regeneration declines continuously during the life cycle (Dahlberg et al., 2009; Auger et al, 2010). After about a 1.5 years, gamete production ceases, the tunic thickens and wrinkles, and the old animals eventually die (Fig. 1A). Regeneration capacity is severely compromised or can cease entirely during old age (Jeffery, 2012; 2014).

In summary, the *Ciona* life cycle exhibits three different periods with regard to regenerative capacity: 1) the embryonic/larval phase in which regeneration is absent, 2) the young and middle-age adult growth phase in which regeneration is extensive, and 3) old age in which regeneration is weak or non-existent (Fig. 1A). In this article, we focus on the second and third periods of the life cycle.

3. **Partial Body Regeneration**

Mingazzini (1891) and Loeb (1892) independently discovered the remarkable regenerative capacities of the *Ciona* NC and siphons. Subsequently, a detailed analysis showed that the NC could be completely replaced within about a month after ablation (Schultze, 1899). Many different investigators have now confirmed NC and oral siphon regeneration and contributed additional details about the processes (Sutton, 1953; Whittaker, 1975; Bollner et al. 1992, 1993, 1995, 1997; Dahlberg et al., 2009; Auger et al., 2010). The capacity for complete regeneration from body fragments has also been investigated. Hirschler (1914) separated adult body parts and recorded their capacity to regenerate an entire animal. He bisected animals into two parts by cutting across a horizontal plane immediately distal to the viscera and found that the proximal (basal) portion was capable of regenerating the distal portion, and thus a complete animal, but the distal portion could not regenerate the basal part and eventually died. Furthermore, if body pieces were separated at positions more distal to the border between the branchial sac and viscera, the basal parts, but not the distal parts, could regenerate complete animals. Using a similar approach, Jeffery (2015c) separated the body into three parts across the distal-proximal axis and showed that the middle part, which lacked the viscera, NC, or the siphons, but contained part of the branchial sac, also has the potential to replace the oral siphon. Therefore, in *Ciona* the

branchial sac appears to be a general regenerative center that is involved in the replacement of injured distal body parts, including the oral siphon.

The phenomenon of distal regeneration from proximal parts of the adult body was termed "partial body regeneration" (Jeffery, 2015b) to distinguish it from other types of regeneration displayed by ascidians: bipolar regeneration in *Clavellina* (Brien, 1968) and whole body regeneration in colonial ascidians (Freeman, 1964; Tiozzo et al., 2008; Brown and Swalla, 2012).

4. **The Oral Siphon Model**

Recent studies of *Ciona* regeneration have focused on the oral siphon as a model (Auger et al., 2010; Jeffery, 2012; 2015c). The oral siphon is a thin muscular tube covered by tunic on its outer side (Fig. 1C; 2A). The siphon tube is bounded by inner and outer epidermal epithelia, which cover a mesenchymal layer containing crisscrossing longitudinal and circular muscle bands (Fig. 2B), nerve tracts, and loosely organized cells embedded in a dense extracellular matrix. Different sensory organs are located at the distal and proximal ends of the oral siphon. The proximal end contains a ring of sensory tentacles, which can inter-digitate and close off the siphon aperture at its base. The distal rim contains pigmented sensory organs (oral siphon sensory organs or OPO) on its inner side, which lie in niches between extended lobes of siphon wall tissue (Dilly and Wolken, 1973) (Fig. 1C, 2A). Each OPO has three parts: a crypt of elongated epidermal cells, which are ciliated and rich in actin filaments, a cup-shaped layer of orange mesenchymal pigment cells, and a ganglion-like neural structure at its base (Fig. 2C-F). A yellow stripe of pigment cells, the inter-OPO pigment band, extends laterally along the siphon rim between each OPO. A ring of OPO connected by an inter-OPO pigment band also rims the atrial siphon (Fig. 2A), although there are no tentacles at its base. The OPO are thought to be

sensory organs (Dilly and Wolken, 1973; Auger et al., 2010), but their precise function is not understood.

The siphons are highly contractile organs (Mackie et al., 2006; Dahlberg et al., 2009). They have the capacity to shorten along their distal to proximal axis by contracting the longitudinal muscle bands and their lateral axis by contracting the circular muscle bands. Contractions can be elicited by water movements or by objects touching the surface of the body.

5. **Oral Siphon Regeneration**

Oral siphon regeneration has been separated into three steps (Auger et al., 2010). The first step is wound epidermis formation, which involves epidermal cell division to produce a new epithelium closing over the wound site (Fig. 3A). The wound epidermis forms within the first day after amputation. After the wound epidermis is formed, the OPO are replaced (Fig. 3B-D). The formation of the wound epidermis and the early steps in OPO replacement (see Section 5.1) occur before the beginning of siphon re-growth (Fig. 3A-C). Several days after amputation, a blastema of proliferating cells is formed proximal to the level of OPO replacement, the siphon grows outward (first replacing only the lobes and then showing isometric growth along the tube) (Fig. 3D-G), and new muscle bands and nerve tracts differentiate in the extending tube. A new ring of basal tentacles is also formed if the original ring was removed by siphon amputation. During the growth process, the neural ganglion at the base of each OPO is replaced, but the process involved is not completely understood. An inter-OPO pigment band is gradually re-formed during the period of siphon growth by differentiation of yellow pigment cells extending along the siphon rim from the lateral sides of each new OPO. Depending on the level of siphon amputation (at the distal tip, within the muscular tube, or at the base) and the age of the animal,

there are differences in the rate and details of oral siphon regeneration (Fig. 1C). The differences in regeneration of siphon parts after amputation at different levels is described below and summarized in Table 1.

5.1 Siphon Tip and Tube Regeneration

When the oral siphon is amputated at the tip (removing OPO and the inter-pigment band) or along the muscular tube (above a level approximating the tentacle bands) the OPO are replaced rapidly and with a high degree of precision (Table 1).

5.1.1. OPO replacement. The stages of OPO replacement during siphon tip or tube regeneration is shown in Figure 3AA-DD, H. First, orange pigment cells differentiate in a band of mesenchyme below the wound epidermis (Fig. 3AA, BB, H1). This region, called the OPO regeneration band, is a vertical stripe of loosely organized mesenchymal cells located between each longitudinal band of muscle fibers along the entire length of the siphon (Fig. 1C). There are as many OPO regeneration bands as OPO in the oral siphon. Second, the differentiated orange pigment cells migrate distally along the OPO regeneration band, line up along the wound epidermis, and condense into pigment spots (Fig. 3CC, DD, H2, 3). Third, the surface epithelium invaginates to form the ciliated epidermal crypt, and orange pigment spots surround the crypt, which becomes the orange cup of the OPO (Fig. 3H4, 5). At about the same time, the neural ganglion is re-formed at the base of the epidermal crypt, and the inter-OPO pigment band begins to be replaced.

Most animals in a natural population have a ring of eight OPO surrounding the siphon rim, although a small number of animals have rings of seven or nine OPO (Millar, 1953; Auger et al., 2010). There is a high degree of fidelity in re-patterning the number of OPO during both siphon tip and tube regeneration, as evidenced by their accurate replacement during multiple cycles of

oral siphon regeneration. Multiple cycles of regeneration are carried out by: 1) amputating the oral siphon along its tube, 2) allowing the siphon to re-grow, 3) amputating the regenerated siphon along its tube again, 4) allowing siphon to re-grow again, and so forth. The number of replaced OPO has been determined through four consecutive regeneration cycles (Auger et al., 2010). After three consecutive cycles, animals with 8 original OPO always formed exactly 8 new OPO, whereas animals with 7 or 9 original OPO always formed exactly 7 or 9 new OPO respectively. The response of the siphon to multiple amputations indicates that OPO replacement is a precisely controlled process. After the fourth consecutive amputation cycle, however, this there is no similarity between the numbers of original and new OPO, implying that pattern regulation has been affected.

The siphon tip and tube regeneration processes are similar, both beginning with the formation of a wound epidermis and subsequent replacement of the OPO and inter-OPO pigment band. However, since part of the underlying muscle, nerve, and OPO regeneration band are also removed, the tube regeneration process includes these tissues.

5.1.2. Short distance regeneration. The source of progenitor cells has been a major question in *Ciona* regenerative biology (Jeffery, 2015a, b). In principle, regeneration could be a short distance (local) process involving precursors from within the siphon tube itself, a long-distance process involving precursors from another part of the body, or a combination of both processes. Current evidence suggests that siphon tip regeneration involves a short distance process, whereas siphon tube regeneration involves both short and long distance processes (Table 1) (Auger et al., 2010; Jeffery, 2015c).

Two lines of evidence support a short distance process in siphon tube regeneration: UV irradiation and siphon explants (Auger et al., 2010) (Fig. 4). First, UV irradiation of the siphon

tube, which is carried out while shielding the remainder of the body, blocks OPO replacement and siphon regeneration following siphon amputation: if one side of the siphon is irradiated, OPO replacement occurs only on the non-UV irradiated side, whereas if both sides are irradiated OPO replacement is blocked on both sides (Fig. 4B, C). The UV irradiation results imply that siphon tube regeneration has a high degree of local autonomy. Autonomous replacement of the OPO by short distance regeneration is also supported by the destruction of a single OPO by microcautery, which quickly induces a new OPO to re-form in its place without affecting any other part of the siphon (Auger et al., 2010). Second, if the siphon is amputated at its distal tip and the tip and stump are cultured as explants, they show contrasting fates (Fig. 4G-I). The stump explants form OPO with fidelity in structure in number on their distal but not their proximal ends. The tip explants, which already have OPO on their distal ends, do not form new OPO on their proximal ends. Thus, OPO regeneration in culture respects the original polarity of the siphon. The UV irradiation and explant experiments suggest that the oral siphon responds to injury by activating a local source of stem or progenitors cells for OPO replacement.

Short distance regeneration is independent of cell proliferation (Table 1). This has been demonstrated by the inability of OPO to be labeled with the cell proliferation marker EdU and the inability of the cell division inhibitors colchicine and nocodazole to affect OPO replacement (Jeffery, 2015c). Accordingly, local stem or progenitor cells must differentiate directly into new OPO, which explains their rapid replacement during siphon tube regeneration.

5.1.3. Long distance regeneration. Long distance regeneration, which occurs during siphon tube and base (see below) regeneration, but not during siphon tip regeneration (Table 1), involves the activity of progenitor cells originating from outside of the siphon. As OPO replacement is occurring at the distal end of the regenerating siphon, a blastema appears in the

proximal region of the siphon stump (Fig. 3J, K). The presence of a blastema indicates that long distance regeneration occurs by epimorphosis. Blastema formation involves the proliferation and migration of progenitor cells from the branchial sac (see Section 7), which are involved in the replacement of muscle and nerves in the regenerated siphon tube.

During the first few days following amputation, there are no muscle cells in the regenerating siphon and it is not able to contract. Muscle differentiation begins after blastema formation. The first muscle cells to be replaced are circular muscle fibers, which begin to differentiate and extend from a germinal area in the OPO regeneration band. The circular muscle bands are formed *de novo* by the differentiation of progenitor cells in the blastema since the original muscle cells (and any satellite cells associated with them) are completely removed when the siphon is amputated. In contrast, the longitundinal muscle bands are replaced by the outgrowth of preexisting longitudinal muscle fibers that were severed during the amputation process. As a consequence of new muscle differentiation, the regenerating siphon eventually regains the ability to contract. Considering the large number of muscle fibers in the siphon, a significant part of siphon regenerative activity must be expended on their replacement after injury.

The oral siphon is rich in nerve fibers, which ramify and radiate distally from a large nerve tract beginning in the NC. When the oral siphon is amputated these siphon nerve fibers are also severed. As observed in transgenic *Ciona* with GFP staining throughout the nervous system, new neurons extend distally into the blastema from the severed tips of the pre-existing fibers, eventually contacting the rim of the regenerating siphon (Auger et al., 2010). The replacement of siphon nerves and OPO is independent of the NC: siphon nerves invade the blastema and OPO are replaced after NC ablation or severing its neural connections, although regeneration occurs

more slowly without the NC (Auger et al., 2010). Replacement of siphon axons by distal extension is possible because of cell bodies are present in the nerve tracks of the oral siphon (Dahlberg et al., 2009). Presumably nerves are directly involved the *Ciona* siphon regeneration, as they are in the regenerating vertebrate limb (Brockes, 1987), but this is yet to be demonstrated experimentally.

5.2 Siphon Base Regeneration

When the oral siphon is severed at its base other parts of the body must be involved in its replacement, implying a long distance regeneration process. Accordingly, siphon base regeneration occurs more slowly than siphon tip or tube regeneration (Table 1). Moreover, OPO replacement does not show fidelity: multiple OPO are reformed even after a single cycle of base amputation, suggesting that patterning mechanisms are changed (Auger et al., 2010). The ring of tentacles is also removed and subsequently replaced during siphon base regeneration. Little is known about tentacle regeneration except that it involves cell division (Jeffery, 2015c).

During the longer interval required for base regeneration, large numbers of orange precursor cells differentiate in the OPO regeneration band and form thick lines along the wound epidermis (Fig. 3I1-2). The thick lines of pigment cells then condense into pigment spots; however, multiple spots and OPO develop in the place of a single original OPO (Fig. 3I3-5). For each original OPO, two or three OPO can be formed (Fig. 3L, M). Thus, the regenerating siphon of animals subjected to base amputation can show two (16) or three (24) times the normal number of OPO. The multiple OPO are packed very close along the margin of the siphon without increasing its girth (Auger et al., 2010).

Interestingly, if animals with multiple OPO produced by base regeneration are amputated a second time, but along the siphon tube rather than the base, there is a tendency to reproduce the

same number of (multiple) OPO developed in the previous cycle, rather than the original number of OPO (Jeffery, unpublished). This suggests that there is a "memory" of pattern involved in OPO replacement. The basis for this "memory" of pre-existing pattern is currently unknown, but could involve structural cues in the siphon tube, possibly the number and/or width of new OPO regeneration bands, which is under the influence of siphon base amputation.

6. **Adult Stem Cells**

The progenitor cells involved in animal regeneration are produced by pluripotent stem cells (Weissman, 2000; Voog and Jones, 2010; Sanchez Alvarado and Yamanaka, 2014). In some cases, these stem cells are located near the sites of regenerative activities. Examples are the stem cells involved in replacement of the hair follicles in the epidermis of mammals, stem cells that replenish cells lining the stomach, and the satellite stem cells that replace injured vertebrate striated muscle cells. In other cases, stem cells are located in niches distant from the sites where the progenitor cells derived from them ultimately function. Examples are the hematopoietic stem cell niches in the pancreas and the bone marrow of mammals. The *Ciona* regeneration studies discussed above suggest that stem cells located both within (short distance regeneration) and outside (long distance regeneration) the siphon may be involved in the replacement of the oral siphon.

6.1 Multiple Stem Cells

The location of *Ciona* adult stem cells has been addressed in *Ciona* using the stem cell markers alkaline phosphatase (AP) and PIWI (Jeffery, 2015c). Pluripotent stem cells display active AP on their surfaces, which can be detected using appropriate substrates of this enzyme. AP has been used as a stem cell marker in a variety of animals (Riekstina et al., 2009), including

ascidians (Akhmanieva et al., 2007). The *piwi* gene is expressed specifically in totipotent stem cells (germ cells and gametes) and pluripotent adult stem cells throughout the animal kingdom (Cox et al., 1998; Seipel et al., 2004; Palalkodeti et al., 2008) and has also been used to detect stem cells in ascidians (Brown et al., 2009; Rinkevich et al., 2010; Suganaga et al., 2010; Kawamura and Sunanaga, 2011).

Adult stem cell niches have been detected in four locations in the *Ciona* body according to the expression of the AP and PIWI markers (Fig. 5A, C). First, stem cells are present in groove-like structures in the stomach and intestinal epithelium (Fig. 5A). These stem cells replenish the gut epithelial cells, which undergo continuous turnover during the digestion process (Ermak, 1975a; 1976a; 1981). Second, stem cells are detected in the basal stalk or stolon, the structure that attaches the body to the substrate (Fig. 5A). The function of basal stalk stem cells is unknown, however, they may be involved in forming a body growth zone. Third, stem cells are present in the transverse vessels of the branchial sac where they are concentrated in lymph nodes (Fig. 5A, C). Lymph nodes containing stem cells are also localized in the vessels lining the pharynx, the endostyle, and the atrial cavity. Stem cells in lymph nodes are involved in the renewal of coelomic hemoblast cells (Ermak, 1975b; 1976b), which are dispersed throughout the animal and have many different functions (De Leo et al., 1987; Satoh, 1994). In colonial ascidians, coelomic hemoblasts differentiate into body wall muscle (Sugino et al., 2007). Fourth, small clusters of stem cells are present in the siphon walls, where they are localized within or near the OPO regeneration bands (Fig. 5B). As mentioned above, orange pigment cells of the OPO and circular muscle fibers begin to differentiate in the OPO regeneration bands. Thus, the *Ciona* body contains multiple stem cell niches, and those of the branchial sac are especially prominent (Fig. 5A).

6.2. Branchial Sac Stem Cells

The branchial sac stem cells respond to siphon amputation by dividing to produce progenitor cells. The response of branchial sac stem cells to siphon injury was determined by subjecting *Ciona* adults to short pulses of EdU after oral siphon amputation (Jeffery, 2015c). The results showed that cell proliferation occurred almost exclusively in the transverse vessels of the branchial sac (Fig. 5D). Furthermore, double labeling with EdU and the AP stem cell marker demonstrated that stem cells in the lymph nodes were induced to proliferate as a response to siphon injury (Fig. 5D inset). Lower levels of cell proliferation were also noted in the stomach and intestine during the short EdU pulses (Fig. 5D), however, their levels were unchanged after amputation, suggesting that they represent a constitutive level of cell renewal not associated with siphon injury. EdU pulse labeling also confirmed that cell proliferation is not involved in OPO replacement after the siphon tube is amputated. Thus, the branchial sac appears to be the major adult stem cell niche involved in long distance regeneration.

7. **Stem and Progenitor Cell Mobilization and Deployment**

The branchial sac contains two different cell types that are deployed in long distance oral siphon regeneration: AP/PIWI stained stem cells and EdU labeled progenitor cells. After a delay of a few days, progenitor cells are mobilized in the branchial sac and migrate into the siphon stump, where they form a regeneration blastema. This has been demonstrated by EdU pulse-chase experiments (Jeffery, 2015c). In these experiments, branchial sac cells labeled with EdU during a short pulse beginning after oral siphon amputation were subsequently detected in the blastema during the chase (Fig. 6B). If the same pulse-chase regime is applied, but the siphon is not amputated, the EdU labeled cells are not chased into the siphon and remain in the branchial

sac, demonstrating that siphon injury triggers the mobilization and deployment of branchial sac progenitor cells (Fig. 6A).

It remains to be determined whether cells from the branchial sac are solely responsible for blastema formation or if they join other progenitor cells that already present in the siphon stump. However, it is likely that the local contribution of proliferating cells to the blastema is minimal. Two lines of evidence support the possibility that long distance migration is responsible for most, if not all, of the progenitor cells in the regeneration blastema (Jeffery, 2015c). First, dividing cells are not revealed in the siphon (with the exception of the wound epidermis) by EdU labeling during the first few days after amputation. Second, differentiation of orange pigment cells and their incorporation into nascent OPO occurs without cell proliferation.

Techniques are not yet available to directly follow cell migration from the branchial sac into the siphon. Accordingly, the evidence for long distance stem cell migration comes has been obtained by branchial sac transplantation experiments (Fig. 7) (Jeffery, 2015c). In these experiments, branchial sacs of small EdU labeled animals are isolated and transplanted into the branchial sacs of larger unlabeled animals, and then the distribution of EdU labeled donor cells is determined after oral siphon amputation of the unlabeled hosts (Fig. 7A). As a response to siphon amputation, EdU labeled cells in the donor branchial sac migrate into the host regeneration blastema (Fig. 7C). This migration is a specific response to amputation: if the host siphon is not amputated then EdU labeled donor cells do not appear in the siphon. These experiments suggest that progenitor cells originating in the branchial sac migrate distally to form the regeneration blastema.

Unexpectedly, stem cells of the branchial sac also appear in the regenerating siphon (Jeffery, 2015c). In contrast to progenitor cell migration, the stem cells migrate into the siphon

early after amputation, particularly when siphon amputation occurs at the base (Fig. 6F). After base regeneration, branchial sac stem cells, which continue to be detectable by AP labeling, invade the regenerating siphon and differentiate directly into orange pigment cells of the OPO without prior cell division (Fig. 6C-F). The branchial sac stem cells can be detected in the new OPO because they still express stem cell markers for a short time after their differentiation into orange pigment cells. This is in contrast to the situation for siphon tip and tube regeneration, in which small clusters of stem cells located in the OPO regeneration bands (Fig. 5B), and not the branchial sac stem cells, give rise to OPO components (Table 1).

In summary, two sources of stem cells are involved in oral siphon regeneration (Fig. 6F). When the siphon is amputated at its tip, a source within the siphon is mobilized and deployed for short distance regeneration. When the siphon is amputated along its tube both the local and long distance (branchial sac based) sources are involved in regeneration: OPO are derived from the local source and the blastema is derived from the long distance source. When the siphon is amputated at its base only the long distance source is involved in regeneration: OPO are directly formed from branchial sac stem cells and the blastema is derived from progenitor cells that also originate in the branchial sac. The local clusters of stem cells normally present in the OPO regeneration bands are likely to be replenished by the invasion of branchial sac stem cells during long distance regeneration.

8. **Aging and Oral Siphon Regeneration**

The regenerative abilities of *Ciona* decline with age. Regenerative aging of the NC and oral siphon have been observed in both natural marine environments and laboratory cultures (Dahlberg et al., 2009; Auger et al., 2010; Jeffery, 2012; 2015c). In wild collected adults,

regeneration capacity during the life cycle was evaluated using size (e. g. distal to proximal length) as a proxy for age. Under favorable conditions, wild *Ciona* adults grow continuously during a life span of about 1-1.5 years (Berrill, 1947; Millar, 1952; Dybern et al, 1965; Peterson et al., 1995). NC ablation or oral siphon amputation of different sized animals resulted in the discovery of an inverse relationship between body length (age) and the rate of regeneration (Dahlberg et al., 2009; Auger et al., 2010): larger (older) animals regenerate much more slowly than smaller (younger) animals (Fig. 8A). A similar decline in regeneration rate as a function of age also occurs in laboratory cultures in which animals of precisely known age are grown from fertilized eggs (Jeffery, 2015c).

Despite a decrease in the rate of regeneration during the adult life cycle, the regenerative processes involved, for example OPO replacement, are still completed with accuracy. This situation changes as animals reach an age threshold, when the capacity for regeneration disappears (Jeffery, 2012). Oral siphon regeneration is compromised in the oldest animals collected in the wild or cultured in the laboratory (Jeffery, 2012; 2015c). The old animals show some morphological and reproductive abnormalities relative to their younger counterparts. They exhibit a thickened and withered tunic, a reduction in gamete production, and have malformed and larger OPO. Moreover, the pharynx and siphons of these animals appear to be inflamed due to the overproduction of orange pigment cells, a condition that is possibly related to stress (Parrinello et al., 2010). When the siphons of old animals are amputated at any position, the rate of regeneration is either very slow or it does not occur at all, even after a month or more of observation (Fig. 8B).

Even though there is no net siphon growth after amputation in old animals, OPO replacement still occurs, although it shows several differences from normal replacement in

younger animals (Jeffery, 2012). First, when the oral siphons of old animals are amputated in the tube, OPO replacement occurs, but multiple OPO are formed in the place of the original single OPO. Thus, siphon tube regeneration in old animals resembles siphon base regeneration in younger animals. This difference could be due to the abundance of differentiated orange pigment cells both in the siphons and elsewhere in the body of old animals. Second, OPO replacement is arrested when old animals are subjected to an additional cycle of siphon amputation. After two consecutive cycles of amputation, large numbers of orange pigment cells differentiate in the siphon stump, and the pigment cell masses line up along the wound epidermis, which seems to be formed normally, but the orange pigment cells but do not condense into distinct pigment spots or form OPO. Instead, they arrest in lines along the siphon rim and later mix with similar lines of yellow pigment responsible for inter-OPO pigment band formation (Fig. 8B, C). Lastly, when the amputated siphon stumps of old animals are excised and cultured as explants *in vitro,* the OPO regeneration bands overproduce orange pigment cells, but they do not move distally to form OPO, as occurs in siphon explants derived from young animals (Fig. 4I). Therefore, although orange pigment cells still differentiate, and in fact are over-produced, OPO replacement is defective, suggesting that aging disrupts morphogenetic processes in old animals.

The formation of a blastema of proliferating cells plays an important role in oral siphon regeneration, contributing the precursors of new muscle and nerve cells during siphon growth (Auger et al., 2010). As described above, the blastema is formed by stem cells that migrate from the branchial sac early during regeneration and reform the OPO without undergoing cell division and progenitor cells that proliferate in the branchial sac and migrate into the blastema later during regeneration (Jeffery, 2015c). Although OPO replacement can occur (albeit defectively)

in old animals, they do not develop a blastema of proliferating cells (Fig. 8D) (Jeffery, 2012). Accordingly, old animals do not replace muscle cells and regain the ability for siphon contraction. There is no information available about the effects of age on nerve cell replacement.

The absence of a blastema in old animals suggests that defects might occur in the production or migration of proliferating cells. Branchial sac stem cells have been compared in young and old animals following oral siphon amputation by EdU pulse labeling and expression of AP and PIWI stem cell markers (Jeffery, 2015c). The structure of the branchial sac and distribution of proliferating cells appears to be abnormal in old animals (Millar, 1952; Jeffery, 2015c). Furthermore, the branchial sac of old animals shows much lower levels of AP and PIWI labeled adult stem cells compared to their younger counterparts. Thus, a reduction in stem cell number, the failure of stem cells to produce progenitor cells, and/or the inability of progenitor cells to migrate into the injured siphons may be responsible for regenerative aging. It is also possible that the injured siphon stumps of old animals do not produce a normal signal for the attraction of progenitor cells to the regeneration blastema.

Based on all the information currently available about siphon regeneration, stem cells, and aging, a model has been suggested to explain the gradual decline and eventual abrupt cessation of regenerative capacity of the *Ciona* oral siphon (Jeffery, 2015c). The model states (1) that all of the branchial sac stem cells that are used in growth (and potentially in regeneration) are produced early during the adult life cycle, (2) after a maximal number of stem cells is reached they slowly lose potency or disappear as animals age, and (3) during old age, no additional stem cells are available for further growth or, if necessary, regeneration. A test of this model would entail the experimental reduction and elevation of stem cell number, and approaches to accomplish this are currently under development.

9. **Concluding Remarks and Perspectives**

We suggest that new animal models are needed to study the relationship between regeneration and aging. As well as being able to reveal the principles underlying regenerative during aging, these models should also help us understand how and why regeneration has been lost during the evolution of some animals, including ourselves. Ultimately, using information gleaned from these models, we might find ways to reverse the loss of regenerative potential that is imposed during aging and evolution. *Ciona intestinalis* is an animal that fills the need for a model in regenerative aging.

Although many avenues of regenerative biology research need to be explored further in *Ciona*, in recent years there has been progress in deciphering the underpinnings of regeneration. In *Ciona* it is possible to study regeneration in a small part of the animal while also observing related changes in the entire animal, thus allowing both short and long distance regeneration processes to be analyzed. This approach led to the discovery of the *Ciona* branchial sac stem cell niche and its long distance contribution to the regenerating siphon (Jeffery, 2015c).

Most of the insights described in this article are derived from experiments on oral siphon regeneration, however, *Ciona* also promises to be an excellent model to study the regeneration capacity of other organs, such as the brain and heart, and these studies should be vigorously pursued in the future. Although some vertebrates, namely amphibians, show partial brain regeneration (Endo et al., 2007), no chordate group other than the tunicates can regenerate an entire brain after its complete removal. Thus, insights about vertebrate brain regeneration may be gained from studying the capacity for complete brain regeneration in *Ciona*.

We have described recent results showing that adult stem cells are instrumental in *Ciona* regeneration and aging. Similar properties of stem cells have been observed in other animals (e. g. Conboy and Rando, 2005; Sharpless and Schatten, 2009; Waterstrat and Van Zant, 2009). It is remarkable that all stages in the life and function of adult stem cells, from their initial formation during metamorphosis (or perhaps even during embryogenesis) to their possible depletion or decay during old age, are accessible for study in *Ciona*. This attribute is likely to have important impacts on understanding the life history of adult stem cells.

Molecular analysis of *Ciona* regeneration is still being developed. This approach will be fostered by the existence of molecular and genomic tools that have been pioneered for the studying the development of this organism (Stolfi and Christiaen, 2012). Thus, it will be imperative to take advantage of these tools in future studies to reveal the molecular basis of regenerative aging.

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References

Akhmadieva, A.V., Shukalyuk, A.I., Aleksandrova, Y.N., Isaeva, V.V., 2007. Stem cells in asexual reproduction of the colonial ascidian *Botryllus tubaratus* (Tunica: Ascidiacea). Russ. J. Mar. Biol. 33, 181–186.

Auger, H., Sasakura, Y., Joly, J-S., Jeffery, W. R., 2010. Regeneration of oral siphon pigment organs in the ascidian *Ciona intestinalis*. Dev. Biol. 339, 374–389.

Bely, A. E., 2010. Evolutionary loss of animal regeneration: Pattern and process. Integr. Comp. Biol. 50, 515–527.

Berrill, N. J., 1947., The development and growth of *Ciona*. J. Mar. Biol. Assoc. UK 26, 616–625.

Berrill, N. J., 1951., Regeneration and budding in tunicates. Biol. Rev. 26:456–475.

Bollner, T., Beesley, P. W., Thorndike, M., 1992. Pattern of substance P- and cholecystokinin-like immunoreactivity during regeneration of the neural complex in the ascidian *Ciona intestinalis*. J. Comp. Neurol. 325, 572–580.

Bollner, T., Beesley, P. W., Thorndike, M. C., 1993. Distribution of GABA-like immunoreactivity during postmetamorphic development and regeneration of the nervous system in the ascidian *Ciona intestinalis*. Cell Tissue Res. 272, 553–561.

Bollner. T., Howalt, S., Thorndyke, M. C., Beesley, P. W., 1995. Regeneration and post-metamorphic development of the central nervous system in the protochordate *Ciona intestinalis*: A study with monoclonal antibodies. Cell Tissue Res. 279, 421–432.

Bollner, T., Beesley, P. W., Thorndike, M. C., 1997. Investigation of the contribution from peripheral GnRH-like immunoreactive ‘neuroblasts’ to the regenerating central nervous system

in the protochordate *Ciona intestinalis*. Proc. R. Soc. Lond. B. 264, 1117–1123.

Bourlat, S. J., Juliusdottir, T., Lowe, C. J., Freeman, R., Aronowicz, J., Kirschner, M., Lander, E. S., Thorndyke, M., Nakano, M., Kohn, A. B., Heyland, A., Moroz, L. L., Copley, R. R., Telford, M. J., 2006. Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. Nature 444, 85–88.

Brien, P. 1968., Blastogenesis and morphogenesis. Adv. Morphogen. 7: 151-203.

Brockes, J. P., 1987. The nerve dependence of amphibian limb regeneration. J. Exp. Biol. 132, 79–91.

Brockes, J.P., Kumar, A., 2008. Comparative aspects of animal regeneration. Annu. Rev. Cell Dev. Biol. 24, 525–549.

Brown, F. D., Swalla, B. J., 2012., Evolution and development of budding by stem cells: Ascidian coloniality as a case study. Dev. Biol. 369, 151–162.

Brown, F. D., Keeling, E. L., Le, A. D., Swalla, B.J. 2009., Whole body regeneration in a colonial ascidian, *Botrylloides violaceus*. J. Exp. Zool. (Mol. Dev. Evol.) 312B, 885–900.

Cirino, P. A., Toscano, D., Caramiello, A., Macina, A., Miraglia,V., Monte, A., 2002. Laboratory culture of the ascidian *Ciona intestinalis* (L.): "a model system for molecular developmental biology research." Mar. Mod. Elec. Rec. http://www.mbl.edu/BiologicalBulletin/MMER/cirino/CirTit. html

Conboy, I. M., Rando, T. A., 2005. Aging, stem cells, and tissue regeneration. Lessons from muscle. Cell Cycle 4, 407-410.

Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D., Lin, H., 1998. A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. 12, 3715–3727.

Dahlberg, C., Auger, H., Dupont, S., Sasakura, Y., Thorndyke,M., Joly, J-S., 2009. Refining the model system of central nervous system regeneration in *Ciona intestinalis*. PLoS One 4:e4458.

Delsuc, F., Brinkmann, H., Chourrout, D., Phillipe, H., 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439, 965–968.

De Leo, G., Parrinello, N., Di Bella, M.A., 1987. Fine structure of blood system in *Ciona intestinalis* (Tunicata). Vessel and hemocytes in pharyngeal wall. Arch. Biol. 98, 35-52.

Dilly, P. N., Wolken, J. J., 1973. Studies on the receptors of *Ciona intestinalis*: IV. The ocellus in the adult. Micron 4, 11–29.

Dybern, B. I., 1965. The life cycle of *Ciona intestinalis* (L.) f. typical; in relation to environmental temperature. Oikos 16, 109–131.

Endo, T., Yoshino, J., Kado, K., Tochinai, S. , 2007. Brain regeneration in anuran amphibians. Dev. Growth Diff. 49, 121-129.

Ermak, T. H., 1975a. Cell proliferation in the digestive tract of *Styela clava* (Urochordata): as revealed by autoradiography with tritiated thymidine. J. Exp. Zool. 194, 449–466.

Ermak, T. H., 1975b. An autoradiographic demonstration of blood cell renewal in *Styela clava* (Urochordata: Ascidiacea). Experientia 31, 837–838.

Ermak, T. H., 1976a. Cell migration kinetics in the stomach of *Styela clava* (Urochordata; Ascidiacea). J. Exp. Zool. 197, 339–346.

Ermak, T. H., 1976b. The hematogenic tissues of tunicates. In: *Phylogeny of thymus and bone marrow–bursa cells*. Wright, R. K., Cooper, E. L. (eds), Elsevier/North Holland, Amsterdam, The Netherlands. pp. 45–56.

Ermak, T. H., 1981. A comparison of cell proliferation patterns in the digestive tract of

ascidians. J. Exp. Zool. 217, 325–339.

Freeman, G., 1964. The role of blood cells in the process of asexual reproduction in the tunicate *Perophora*. J. Exp. Zool. 156, 157–184.

Givan, J. E., Olson, W. N., Hall, B. K., 2002. Hind-limb regeneration in the dwarf African clawed frog, *Hymenochirus boettgeri* (Anura: Pipidae). J. Herpetol. 36, 537-543.

Hall, P. A., Watt, F. M., 1989. Stem cells: the generation and maintenance of cellular diversity. Development 106, 619-633.

Hirschler, J. 1914. Uber die Restitutions-und Involutionsvorlange bei operierten Exemplaren von *Ciona intestinalis* Flem. (Teil I) nebst Bemurkungen uber den Wert des Negativen fur das Potenzproblem. Arch. Mikr. Anat. 85, 205–227.

Illingworth, C. M., Barker, A. T., 1980. Measurement of electrical currents emerging during the regeneration of amputated finger tips in children. Clin. Phys. Physiol. Meas. 1, 87-89.

Jeffery, W. R. 2001., Determinants of cell and positional fate in ascidian embryos. Int. Rev. Cytol. 203, 3–62.

Jeffery, W. R., 2012. Siphon regeneration capacity is compromised during aging in the ascidian *Ciona intestinalis*. Mech. Ageing Dev. 133, 629–636.

Jeffery, W. R., 2015a. The tunicate *Ciona*: a model system for understanding the relationship between regeneration and aging. Invert. Repro. Dev. 59, 17-22.

Jeffery, W. R., 2015b. Closing the wounds. One hundred and twenty five years of regenerative biology in the ascidian *Ciona intestinalis*. Genesis 53, 48-65.

Jeffery, W. R., 2015c. Distal regeneration involves the age dependent activity of branchial sac stem cells in the ascidian *Ciona intestinalis*. Regeneration 1, doi: 10.1002/reg2.26

Joly, J.-S., Kano, S., Matsuoka, T., Auger, H., Hirayama, K., Satoh, N., Awazu, S.,

Legendre, L., Sasakura, Y. 2007. Culture of *Ciona intestinalis* in closed systems. Dev. Dyn. 236, 1832–1840.

Kawamura, K., Sunanaga, T., 2011. Role of Vasa, Piwi, and Myc-expressing coelomic cells in gonad regeneration of the colonial tunicate, *Botryllus primigenus*. Mech. Dev. 128, 457–470.

Kourakis, M. J., Smith, W. C., 2015. An organismal perspective on *C. intestinalis* development, origins and diversification. eLife 4, e06024. DOI: 10.7554/eLife.06024

Loeb, J. 1892. Unter such ungen zur physiologischen morphologie. II. Wurzburg.

Mackie, G. O., Burighel, P., Caicci, F., Manni, L., 2006. Innervation of ascidian siphons and their responses to stimulation. Can. J. Zool. 84, 1146–1162.

Markman, B., 1958. On the peripheral nervous system of ascidians. Acta Zool. 34, 13–18.

Millar, R. H., 1952. The annual growth and reproductive cycle in four ascidians. J. Mar. Biol. Assoc. UK 31, 41–61.

Millar, R. H., 1953. Ciona. In: Colman JS, (Ed). LMBC memoirs on typical British marine plants and animals. Liverpool University, Liverpool, UK. pp 1–123.

Mingazzini, P., 1891. Sulla rigenerazione nei Tunicati. Boll. Dell. Soc. Natur. Napoli 5, 76–79.

Mizell, M., 1968. Limb regeneration: Induction in the newborn opossum. Science 161, 283-286.

Ogasawara, M., Satoh, N. 1998. Isolation and characterization of endostyle-specific genes in the ascidian *Ciona intestinalis*. Biol. Bull. 195, 60-69.

Palakodeti, D., Smielewska, M., Lu, Y.C., Yeo, G.W., Graveley, B. R., 2008. The PIWI proteins SMEDWI-2 and SMEDWI-3 are required for stem cell function and piRNA expression

in planarians. RNA 14, 1174–1186.

Parrinello N, Vizzini, A., Salerno, G., Sanfratello, M.A., Cammarata, M., Arizza, V., Vazzana, M., Parrinello, D. 2010. Inflamed adult pharynx tissues and swimming larva of *Ciona intestinalis* share CiTNFalpha-producing cells. Cell. Tiss. Res. 341, 299–311.

Peterson, J.K., Chou, O., Thor, P., 1995. Growth and energetics in the ascidian *Ciona intestinalis*. Mar. Ecol. Prog. Ser. 120, 175–184.

Poss, K.D., 2010., Advances in understanding tissue regenerative capacity and mechanisms in animals. Nature Rev. Genet. 11, 710–722.

Reed, M. J., Koike, T., Puolakkainenen, P. 2003. Wound repair in aging. Meth. Mol. Med. 78, 217-237.

Riekstina, U., Cakstina, I., Parfejevs, V., Hoogdujn, M., Jankovskis, G., Mulznieks, I., Muceniece, R., Ancans, J. 2009. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. Stem Cell Rev. 5, 378–786.

Rinkevich, B., Shlemberg, Z., Fishelson, L. 1995. Whole body protochordate regeneration from totipotent blood cells. Proc. Natl. Acad. Sci. USA 92, 7695–7699.

Rinkevich, Y., Rosner, A., Rabinowitz, C., Lapidot, Z., Moiseeva, E., Rinkevich, B., 2010. Piwi positive cells that line the vasculature epithelium underlie whole body regeneration in a basal chordate. Dev. Biol. 345, 94–104.

Sanchez Alvarado, A., Yamanaka, S., 2014. Rethinking differentiation: stem cells, regeneration, and plasticity. Cell 157, 110-119.

Sasakura, Y., 2007. Germline transgenesis and insertional mutagenesis in the ascidian *Ciona intestinalis*. Dev. Dyn. 236, 1758–1767.

Satoh, N., 1994. Developmental biology of ascidians. Cambridge University Press, Cambridge UK. pp 1–234.

Satoh, N., 2013. Developmental Genomics of Ascidians. Wiley-Blackwell, Hoboken, NJ. pp. 1-216.

Schultze, L. S., 1899. Die Regeneration des Ganglions von *Ciona intestinalis* L und uber das Verhaltneiss der Regeneration und Knospung zur Keimenblatterlehre. Jena Z. f. Naturwiss. 33, 263–344.

Seifert, A. W., Voss, S. R., 2013. Revisiting the relationship between regenerative ability and aging. BMC Biol. 11:2 doi: 10.1186/1741-7007-11-2

Seipel, K., Yanze, N., Schmid, V., 2004. The germ cell line and somatic stem cell gene Cniwi in the jellyfish *Podocoryne carnea*. Int. J. Dev. Biol. 48, 1–7.

Sharpless, N. E., Schatten, G., 2009. Stem cell aging. J. Gerontol. A. Biol. Sci, Med. Sci 64A, 202-204.

Sousounis, K., Badour, J. A., Tsonis, P. A., 2014. Aging and regeneration in vertebrates. Curr. Top. Dev. Biol. 108, 217-246.

Stolfi, A., Christiaen, L., 2012. Genetic and genomic toolkit of the chordate *Ciona intestinalis*. Genetics 192, 55-66.

Sugino, Y., Matsumura, M., Kawamura, K., 2007. Body muscle-cell differentiation from coelomic stem cells in colonial tunicates. Zool. Sci., 24, 542–546.

Sunanaga, T., Inubushi, H. , Kawamura, K. 2010. Piwi-expressing hemoblasts serve as germline stem cells during postembryonic germ cell specification in colonial ascidian, *Botryllus primigenus*. Dev. Growth Diff. 52, 603–614.

Sutton, M. F., 1953. The regeneration of the siphons of *Ciona intestinalis* L. J. Mar. Biol.

Assoc. UK 32, 249–286.

Takamura, K., Fujimura, M., Yamaguchi, Y. 2002. Primordial germ cells originate from the endodermal strand cells in the ascidian *Ciona intestinalis*. Dev. Genes Evol. 212, 11-18.

Tiozzo, S., Brown, F. D., De Tomaso, A. W., 2008. Regeneration and stem cells in ascidians. In: Bosch, T. C. G. (Ed). Stem Cells: From Hydra to Man, Chapter 6. Springer, New York. pp 95–112.

Voog, J., Jones, D. L., 2010. Stem cells and the niche: a dynamic duo. Cell Stem Cell 6, 103-115.

Waterstrat, A., Van Zant, G., 2009. Effects of aging on hematopoietic stem and progenitor cells. Curr. Opin. Immunol. 21, 408-413.

Weissman, I. L., 2000. Stem cells: Units of development, units of regeneration, and units in evolution. Cell 100, 157-168.

Whittaker, J. R., 1975. Siphon regeneration in *Ciona*. Nature 255, 224–225.

Figure Legends

Figure 1. *Ciona intestinalis.* A. Life cycle. B. Body organization. C. Oral siphon structure showing the approximate locations (red horizontal lines) of amputation at the tip, along the tube, and at the base in regeneration studies. OS: oral siphon. AS: atrial siphon. NC: neural complex. BS: branchial sac. V: Viscera. CM: circular muscle band. LM: longitudinal muscle band. PB: Inter OPO pigment band. OPO: oral siphon pigment organ. TB: tentacle band. C: Modified from Auger et al. (2010).

Figure 2. Oral siphon pigment organ structure. A. The distal portion of an adult showing the oral siphon (OS), atrial siphon (AS), and a longitudinal muscle band (LMB). B. A phalloidin stained oral siphon showing bands of circular muscle (CM) and longitudinal muscle (LM). C. The oral siphon rim with an oral siphon pigment organ (OPO) and the inter-OPO pigment band (PB). D. A drawing showing an oral siphon pigment organ in section. The inner side of the oral siphon is on the right. PC: yellow pigment cells. OC: Orange pigment cup. RC: neuronal receptor cells in the epidermal crypt. E. A section through the distal rim of an OS showing the structure of an oral siphon pigment organ. F. A fluorescent micrograph of an oral siphon from a transgenic animal with a GFP labeled neural ganglion (NG) located at the base of the OPO (outlined). From Auger et al. (2010).

Figure 3. Oral siphon regeneration. A-G (including AA-DD). Oral siphon regeneration and oral siphon pigment organ regeneration showing the first two steps in the absence of siphon growth (A-C) and showing the third step involving growth (D-G). The time span from A to G is

about a month. AA-DD are magnifications of the siphon rim in A-D respectively showing the wound epidermis (WE) and orange pigment cell differentiation. OPO: oral siphon pigment organ. PB: inter OPO pigment band. OPL: line of orange pigment cells. OPS. Orange pigment spot. H, I. Diagram showing oral pigment organ replacement after siphon amputation at thetip/tube (H) or base (I). J, K. Siphon tube regeneration showing (J) neural ganglion (NG) replacement and (K) the blastema of proliferating cells. The diagonal dashed lines represent the approximate location of amputations. L, M. Siphon base regeneration showing multiple oral siphon pigment organ replacement. M. Enlargement of the siphon rim showing duplicated and triplicated oral siphon pigment organs. From Auger et al. (2010).

Figure 4. Local source of progenitor cells for oral siphon pigment organ regeneration demonstrated by oral siphon ultraviolet irradiation and explant experiments. Top. Ultraviolet (UV) irradiation. A, D. Normal oral siphon pigment organ replacement after UV irradiation of a shielded control animal prior to siphon tube amputation. B, E. No oral siphon pigment organ replacement after UV irradiation of both sides of the oral siphon prior to siphon tube amputation. C, F. No oral siphon pigment organ regeneration on the left side of an animal after UV irradiation of the left side of the oral siphon prior to siphon tube amputation. Black rectangles indicate the shielded areas during UV irradiation (downward pointing arrows). Vertical lines indicate the amputation planes. Bottom. Explant cultures. G. Diagram of the two-step procedure used to obtain explants of regenerating siphons. The first amputation (1) yields an oral siphon tip explant and the second amputation (2) an oral siphon mid-section explant. H. A siphon mid-section explant after 6 days in culture showing orange pigment cell accumulation in oral siphon pigment organ regeneration bands (arrowhead). Arrow shows the exposed siphon tentacles. I.

Explants after 9 days in culture showing an oral siphon distal explant containing the original oral siphon pigment organs on its distal margin (top) and an oral siphon mid-section (bottom) explant with oral siphon pigment organ formation on its distal (arrowhead) but not its proximal side. From Auger et al. (2010).

Figure 5. Adult stem cell localization. A. A young adult showing the distribution of AP labeled stem cells. B. A portion of an oral siphon (OS) showing clusters of AP labeled stem cells (arrows). C. A section through the transverse vessel of the branchial sac showing PIWI labeled stem cells (arrows). D. EdU pulse labeling following oral siphon amputation shows cell proliferation primarily in the transverse vessels (TV) of the branchial sac (BS). The OS stump is outlined by dashed lines. Insert: higher magnification of D showing proliferating cells in a lymph node (LN). CG: central ganglion (neural complex). AS: atrial siphon. E: endostyle. R: rectum. I: intestine. S: stomach. H: heart. Sk: basal stalk. OPO: oral siphon pigment organ. From Jeffery (2015c).

Figure 6. Stem and progenitor cell contributions to the oral siphon regeneration blastema. A, B. The results of a pulse chase EdU labeling experiment. After a 2 day EdU pulse followed by a 4 day chase, animals show strong labeling in the oral siphon after amputation the tube (B) but not if there is no amputation (A). C. AP labeled stem cells in the oral siphon of a regenerating animal. Horizontal line indicates the original plane of amputation. D, E. Oral siphon pigment organ of a regenerating animal showing pigment cells labeled with alkaline phosphatase (D) but not EdU (E). F. Diagram illustrating temporal differences in appearance of non-proliferating branchial sac stem cells (filled circles) and proliferating (open circles)

branchial sac progenitor cells in the siphon regeneration blastema. OS: oral siphon. AS: atrial siphon. NC: neural complex. E: endostyle. BS: branchial sac. TV: transverse vessels. R: rectum. I: intestine. S: stomach. H: heart. Sk: basal stalk. Left. A non-regenerating animal is shown with non-proliferating stem cells localized in the TV of the branchial sac. Middle. A regenerating animal (1−3 h post-amputation) is shown with non-proliferating stem cells in the distal regeneration blastema. Right. A regenerating animal (4+ days post-amputation) is shown with branchial sac stem cells in the transverse vessels and the regeneration blastema and proliferation of progenitor cells in the transverse vessels. Some of the branchial sac stem cells have migrated into the regenerating siphon and differentiated into oral siphon pigment organs. Dashed line: site of amputation: Open star shaped structures: original oral siphon pigment organs. Closed star shaped structures: new oral siphon pigment organs. From Jeffery (2015c).

Figure 7. Branchial sac transplantation. A. Diagram showing the transplantation of a donor branchial sac from a small EdU labeled animal into the host branchial sac (BS) of a large unlabeled animal. The horizontal lines indicate the sites of oral siphon (OS) amputation in the donor prior to BS transplantation and the host after transplantation. T: transplant. B. A chimeric host animal containing the (red-stained) EdU labeled donor branchial sac. C. EdU labeling (circle) in the regenerating oral siphon of a host animal containing a transplanted donor branchial sac. CNS: NC. From Jeffery (2015c).

Figure 8. Aging and oral siphon regeneration. A. The time required for oral siphon pigment organ replacement increases during the life cycle. A: From Auger et al. (2010). B. Lack of oral siphon growth in an old animal subjected to two consecutive cycles of amputation.

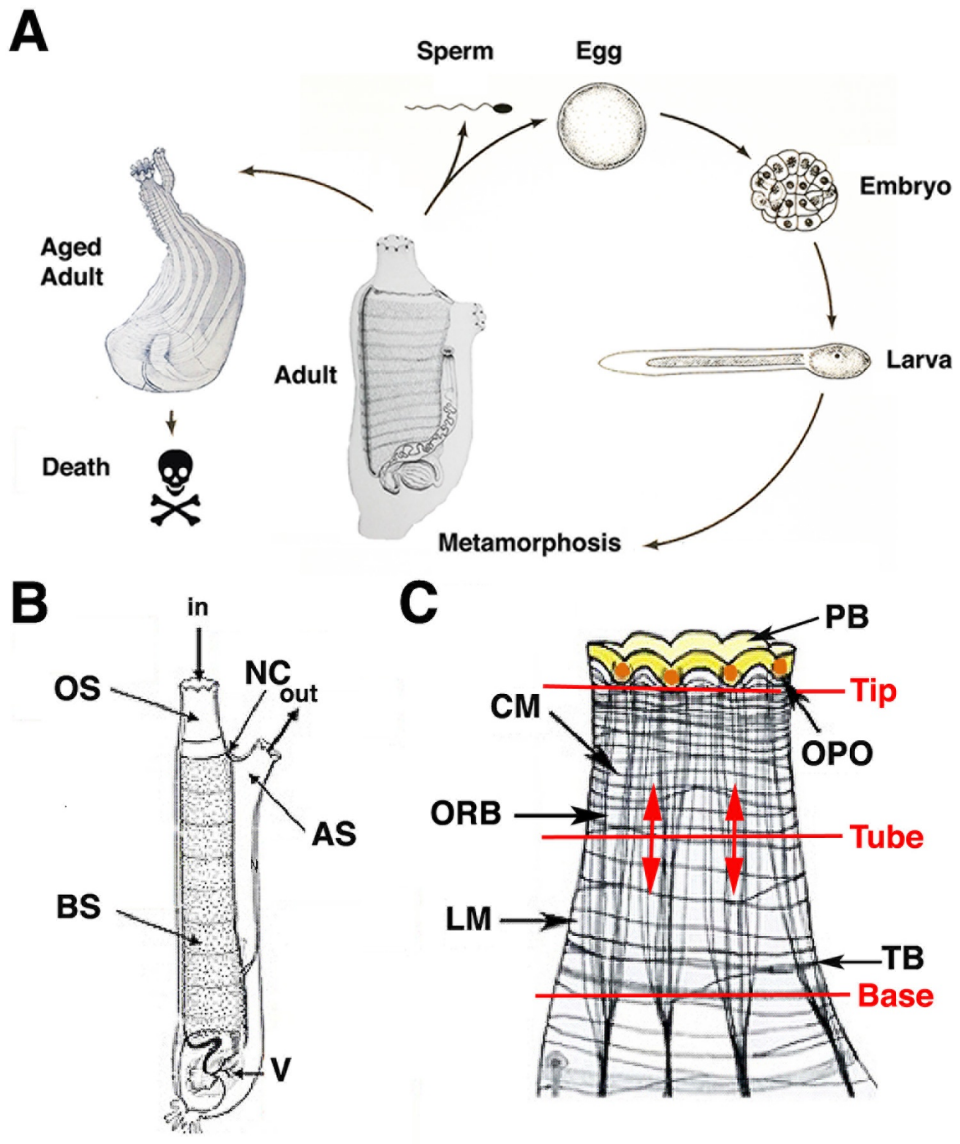
C. Oral siphon pigment organ (OPO) replacement has been arrested at an intermediate stage as a line of mixed orange and yellow pigment cells (arrows). D. Cell proliferation determined by phosphohistone H3 antibody staining in the regeneration blastema of middle aged animals and in the siphon stump of old animals. The number of proliferating cells is significantly higher in the region above the siphon amputation plane (A----A) compared to the region below it in middle age animals but not in old animals. Asterisks represent significant differences. N is shown below each bar. B-D: From Jeffery (2012). E, F. Lymph node cells stained with PIWI stem cell marker in transverse vessels (TV) in the branchial sac of middle age (E) but not old (F) animals. E, F: Fom Jeffery (2015c).

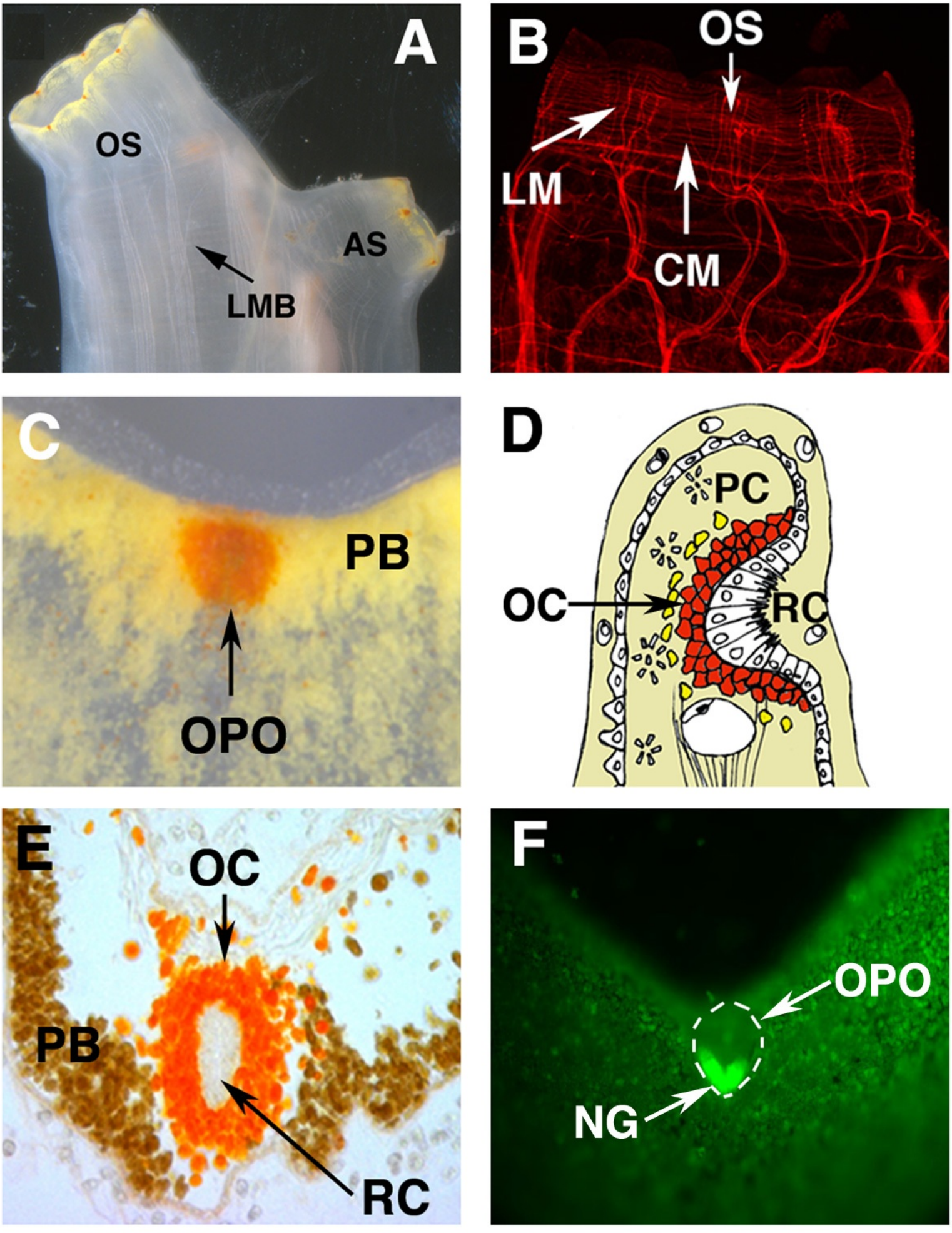
Table 1. Characteristics of regeneration after amputation at different levels of the oral siphon

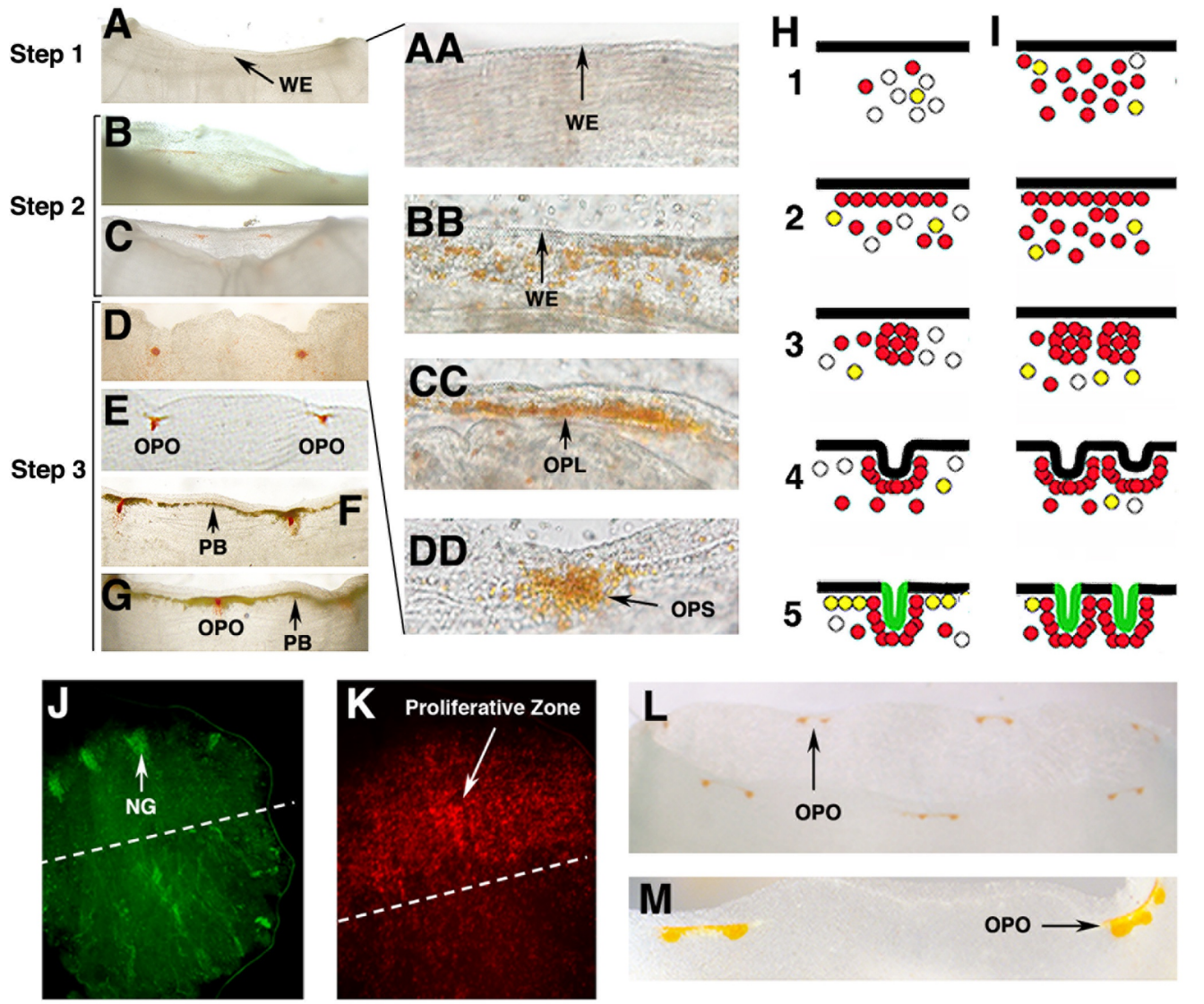
Amputation Level	Regeneration Rate	Replacement Type	Source	Blastema	Cell Division
Tip	Fast	Short distance	SSC	No	No
Tube	Fast	Short distance	SSC	No	No
		Long distance	BCSC	Yes	No
		Long distance	BSPC	Yes	Yes
Base	Slow	Long distance	BCSC	Yes	No
		Long distance	BSPC	Yes	Yes

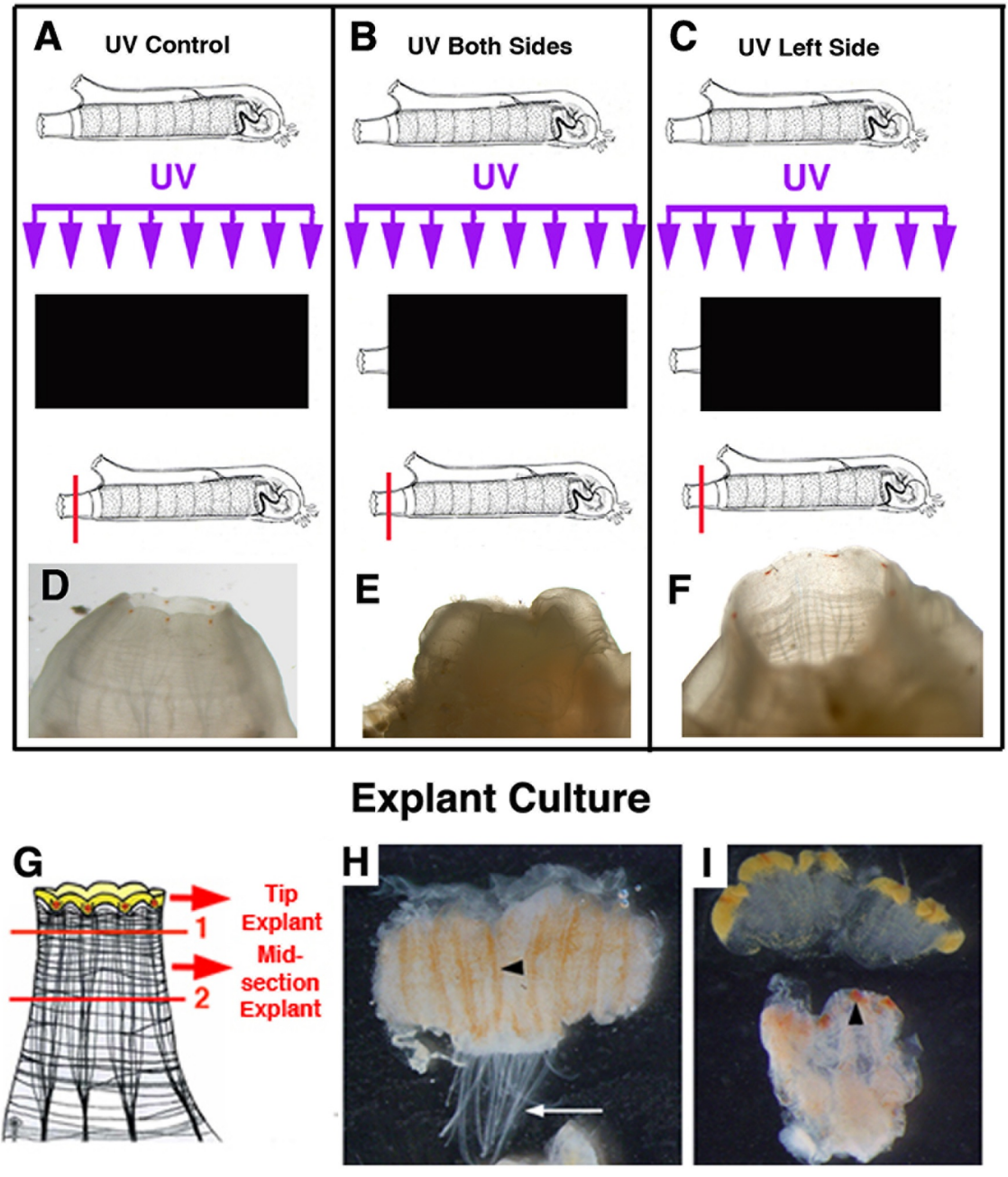
SSC: local siphon stem cells. BCSC: branchial sac stem cells. BSPC: branchial sac progenitor cells.

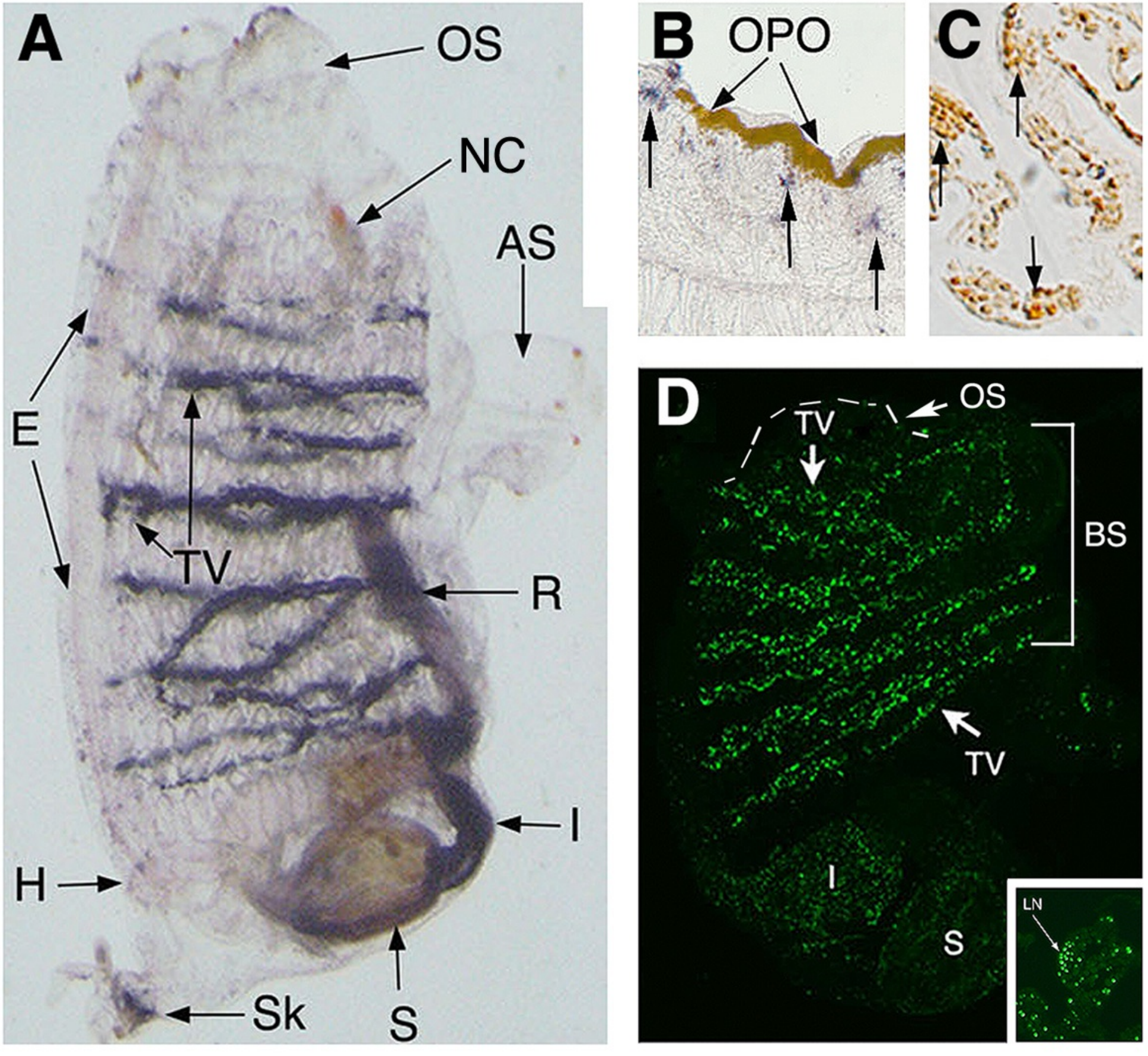
Data compiled from Auger et al (2010) and Jeffery (2015c).

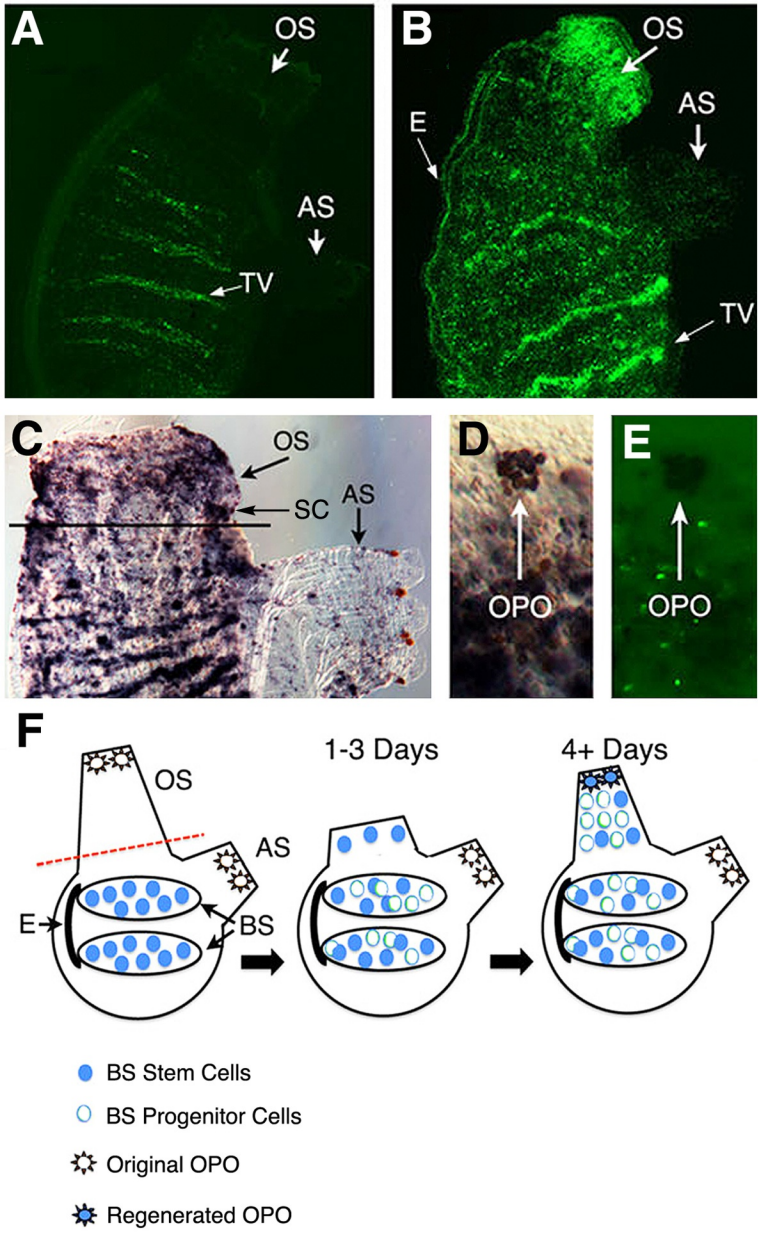

A
Sperm
Egg
Embryo
Larva
Metamorphosis
Adult
Aged
Adult
Death
B
in
OS
NC
out
AS
BS
V
C
PB
Tip
CM
OPO
ORB
Tube
LM
TB
Base

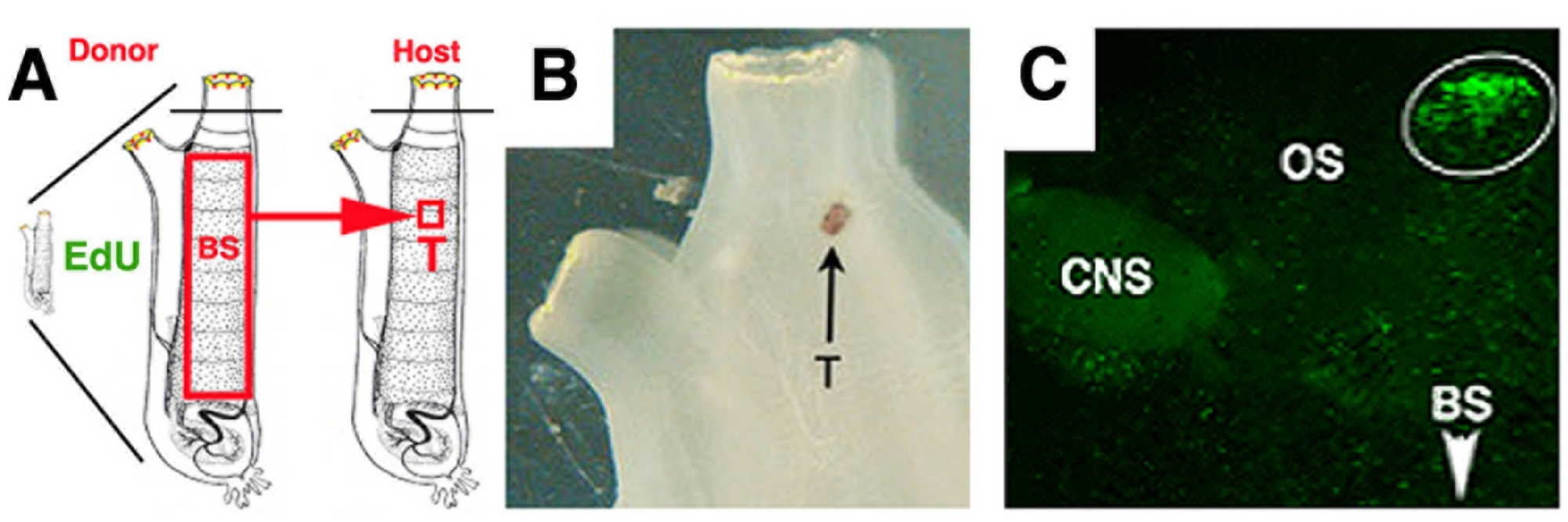

A
OS
AS
LMB
B
OS
LM
CM
C
PB
OPO
D
PC
OC
RC
E
OC
PB
RC
F
OPO
NG

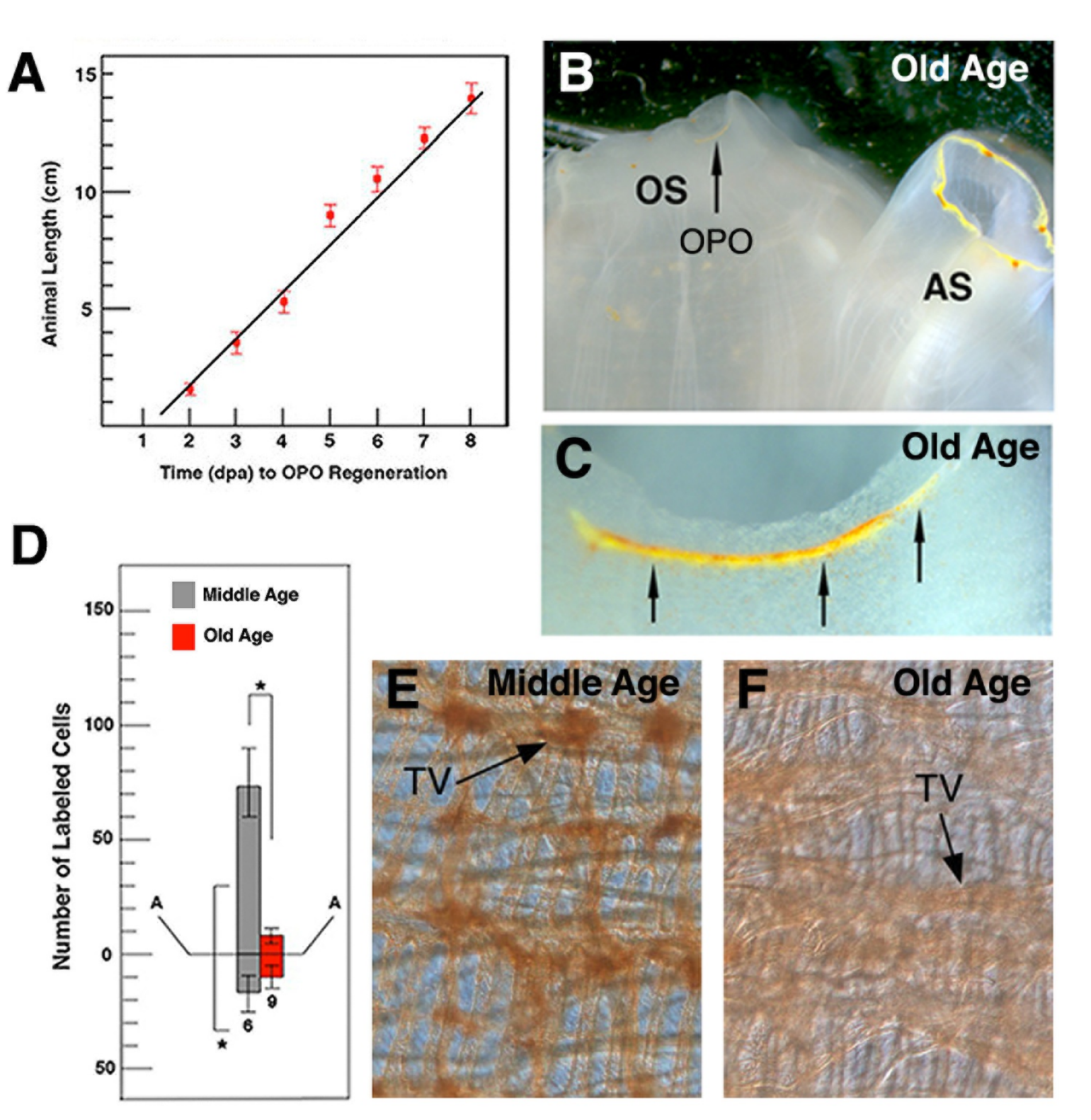

Step 1
Step 2
Step 3
A
WE
B
C
D
E
OPO
OPO
F
PB
G
OPO
PB
AA
WE
BB
WE
CC
OPL
DD
OPS
H
I
1
2
3
4
5
J
NG
K
Proliferative Zone
L
OPO
M
OPO


UV Irradiation
A
UV Control
B
UV Both Sides
C
UV Left Side
UV
UV
UV
D
E
F
Explant Culture
G
Tip Explant
1
Mid-section Explant
2
H
I


A
OS
NC
AS
E
TV
R
I
H
Sk
S
B
OPO
C
D
OS
TV
BS
TV
I
S
LN


A
OS
AS
TV
B
OS
AS
E
TV
C
OS
SC
AS
D
OPO
E
OPO
F
OS
AS
E
BS
1-3 Days
4+ Days
BS Stem Cells
BS Progenitor Cells
Original OPO
Regenerated OPO


A
Donor
Host
EdU
BS
T
B
T
C
OS
CNS
BS


A
Animal Length (cm)
15
10
5
1
2
3
4
5
6
7
8
Time (dpa) to OPO Regeneration
B
Old Age
OS
OPO
AS
C
Old Age
D
Middle Age
Old Age
Number of Labeled Cells
150
100
50
0
50
A
A
6
9
E
Middle Age
TV
F
Old Age
TV