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The freshwater planarian *Schmidtea mediterranea*: embryogenesis, stem cells and regeneration

Commentary

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Planarians have been used as a model to study development and regeneration for more than 200 years. Research on these animals has traditionally focused on surgical and pharmacological manipulations. Recently, the dissection of planarians has become more molecular in nature. The isolation of thousands of expressed sequence tags and the introduction of *in situ* hybridizations, immunocytology, and RNA-mediated gene interference has opened the door to gene discovery and to the study of gene function in planarians during development and regeneration. These advances promise to shed mechanistic insight into basic biological attributes such as regeneration and stem-cell regulation.

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Introduction

It is not generally known that before TH Morgan became indissolubly associated with *Drosophila melanogaster*, he produced a notable body of work on planarians (flatworms). From 1898 to1905, Morgan wrote a dozen incisive papers dealing with the biology of these organisms [1–12]. It is not too difficult to imagine how an animal that can regenerate complete individuals from minuscule body parts [1,13], and that possesses the uncanny ability to grow and 'de-grow' depending on food availability [14–19] could have so thoroughly intrigued and puzzled Morgan [20].

Planarians are, indeed, fascinating animals and in recent years, research on these organisms has begun to move away from mostly surgical and pharmacological studies into the realm of molecular biology and functional genomics [21,22]. The motivation for this significant methodological expansion is the same as it has been for over 200 years: to better understand the extraordinary biological attributes of planarians and to expand our knowledge of metazoan biology. For example, a small fragment removed from either flank of a planarian is capable of re-specifying its body midline to regain bilateral symmetry, while simultaneously preserving anteroposterior and dorsoventral polarities and resetting these axes to their appropriate positional values [1,23]. How this is accomplished is still not understood. Such plasticity illustrates the enormous capacity possessed by adult planarians to both maintain and regulate the form and function of their body and is in direct contrast with the rigidity displayed by the adult forms of other popular invertebrate model systems such as the fruitfly (*Drosophila melanogaster*) and the nematode worm (*Caenorhabditis elegans*).

The source of the plasticity and regenerative abilities of planarians is a dynamic population of adult, totipotent, somatic stem cells known as neoblasts. Neoblasts are attention grabbers: they are the only mitotically active cells in adult planarians and are capable of giving rise to all the cell types found in this organism [24–26], including the germ line [4]. The totipotentiality of the soma became patently clear to Morgan, who in 1902 observed how a planarian head fragment lacking any vestiges of the reproductive system could regenerate not only the missing trunk and tail, but also functional gonads from somatic tissues [4]. In other words, unlike *Drosophila* and *C. elegans*, planarians do not appear to segregate their germ-cell lineage during embryogenesis. The ability of neoblasts to generate both somatic and germ-cell lineages in adult tissues poses absorbing and still unanswered questions about the mechanisms by which totipotentiality and fate restriction are regulated in planarians.

In this *Commentary*, I discuss how the study of these and other planarian attributes are likely to complement and expand on current developmental biology research. I will also argue for the use of a particular species, namely *Schmidtea mediterranea*, to standardize these studies. Research into the developmental processes that are particularly accessible in the flatworm such as regeneration and stem cell activity will provide crucial insight into metazoan evolution, development, and the genetic mechanisms that generate diversity.

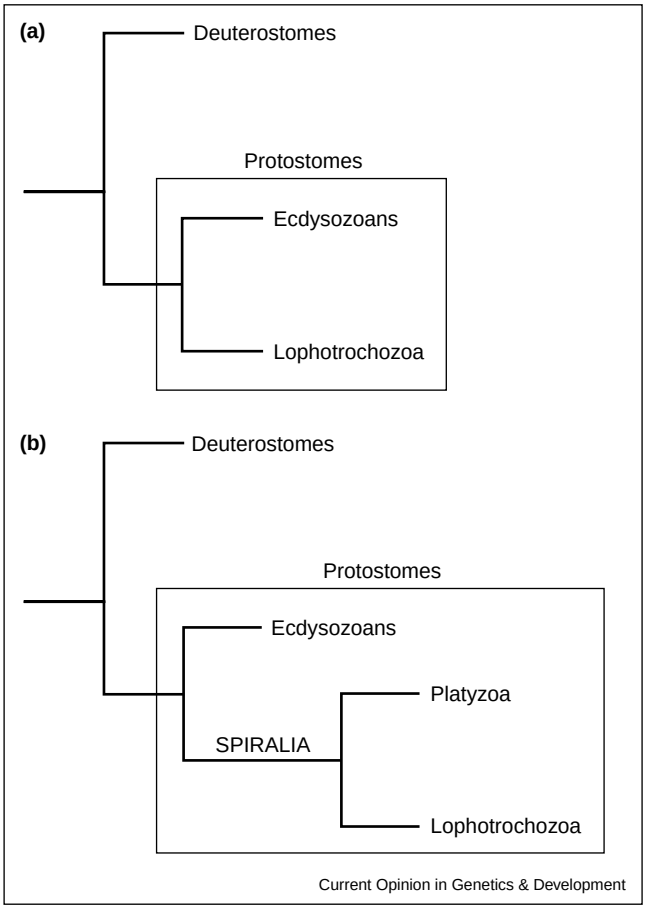
Are planarians lophotrochozoans?

Planarians, are members of the phylum Platyhelminthes, and share with vertebrates key traits such as bilateral symmetry, three germ layers — ectoderm, mesoderm and

endoderm — and dorsoventral and anteroposterior polarities. Planarians are also among the simplest bilaterians to display cephalization — that is, a complex and well-organized accumulation of neurons in their anterior region. These characteristics have attracted the attention of a long succession of zoologists, and the taxonomic history and classification of the flatworms have been issues of considerable controversy. On this topic, the great invertebrate biologist Libbie Hyman wrote, ''throughout the nineteenth century the number of arrangements published was about equal to the number of interested zoologists'' [27]. More recently, however, consensus is building to place planarians into a new taxonomic group known as the Lophotrochozoa [28,29], although some dissention still exists [30]. The Lophotrochozoa, by definition, is composed of a large group of monophylectic, protostome taxons that have either a lophophore (i.e. a set of tentacles surrounding the mouth used for feeding) or that develop through a free-swimming, ciliated (trochophore) larva [31]. In this scenario the Lophotrochozoa is a sister group to the Ecdysozoa — a group that includes arthropods and nematodes, among others (Figure 1) [29] — and the Deuterostomes (see the review by Tessmar and Arendt in this issue). It should be noted, however, that planarians lack a lophophore and that even though marine planarians such as the polyclads develop through a type of trochophore larva known as Müller's larva, this is not true of freshwater planarians, which, in fact, are direct developers (see below).

The diversity of developmental strategies and morphologies of free-living flatworms and other non-ecdysozoan protostomes makes it difficult to establish phyletic and interphyletic relationships using morphological characters alone. Presently, there is much acknowledged uncertainty about the intra-lophotrochozoan phylogenies [29]. For instance, on the basis of combined analyses of morphology and molecular data, it has been suggested that the acoelomate worms (including the Platyhelminthes) should be placed in an independent sister group to the Lophotrochozoa named the Platyzoa [32]. Because lophotrochozoans and platyzoans share a predominantly spiral mode of embryonic cleavage, it has been proposed that they be combined to create the superphylum Spiralia [33] (Figure 1). It is clear, therefore, that to create phyletic relationships of sufficient resolution and completeness, more molecular information on non-ecdysozoan protostomes is needed. Recently obtained cDNA sequence data from the freshwater planarian *Schmidtea mediterranea* [22] should help improve not only the reliability of intra-phyletic relationships in this group of animals but also general hypotheses of metazoan body-plan evolution.

Figure 1

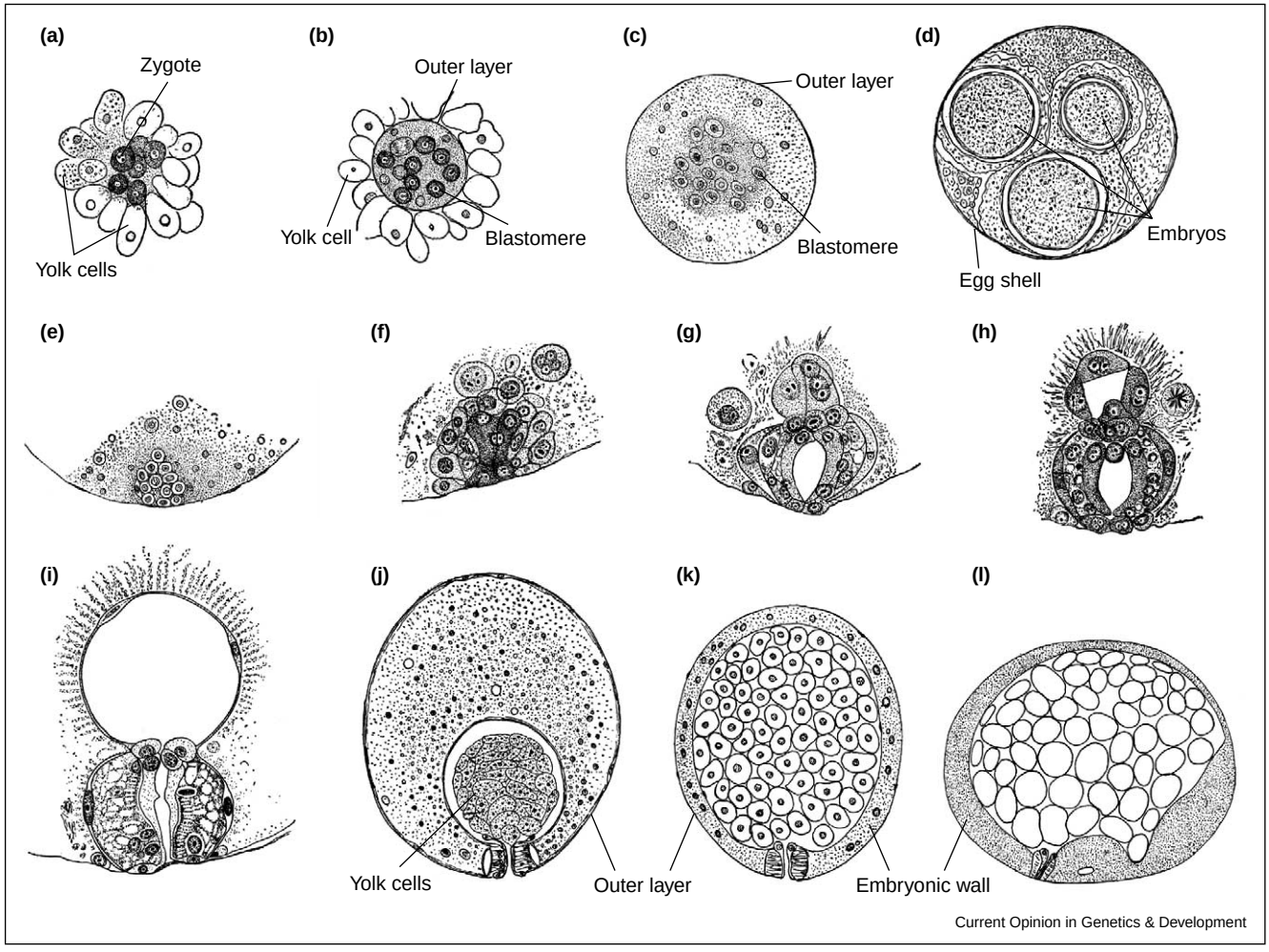


Two different assemblies of the metazoan tree of life. **(a)** The tripartite model of metazoan evolution [29]. **(b)** A different inference on the phyletic relationships of the non-ecdysozoan animals in which the acoelomate worms are grouped into the Platyzoa [32] to distinguish them from all other protostome worms (Lophotrochozoa). In this view, the Platyzoa and Lophotrochozoa are assembled into a 'super-phylum' named the Spiralia because of the predominantly spiral mode of cleavage of the embryos of these animals [33].

If not by spiral cleavage, how do the embryos of freshwater planarians develop?

Although much is known about the embryogenesis of other orders of flatworms such as the marine polyclads [34] and the acoels [35,36], little contemporary work has been done on the embryos of freshwater planarians [37–39]. To my knowledge, the last detailed studies of the highly derived embryogenesis of freshwater planarians were carried out by Metschnikoff (1883) [40], Ijima (1884) [41], Hallez (1887) [42], Korschelt and Heider (1895) [43], Mattiesen (1904) [44], and Fulinski (1916) [45]. Part of this body of work is shown in Figure 2. Unlike the typical quartet spiral cleavage of marine polyclads [34], and the modified duet cleavage of acoel flatworms [36], the pattern of cleavage in freshwater planarian embryos seems anarchic by comparison. The embryos are, in fact, ectolecithal (i.e. yolk cells reside outside rather than inside the embryo; Figure 2a,b) [20], and the *leitmotiv* of the early developmental stages appears to be the internalization of the yolk cells (Figure 2e–k).

Figure 2

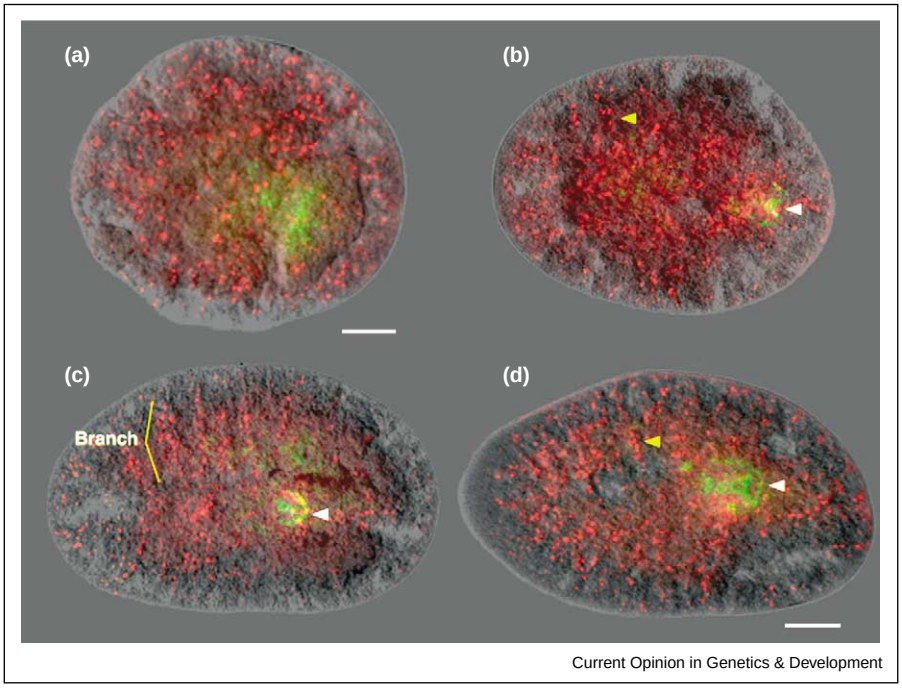


The embryogenesis of freshwater planarians. **(a)** A group of six zygotes surrounded by a mass of yolk cells (the capsule has been omitted for simplicity. See main text). **(b)** A single zygote surrounded by yolk cells displaying the blastomere-derived outer layer filled with dividing blastomeres. **(c)** A fully developed embryonic sphere. **(d)** A cross-section of an egg capsule showing three embryonic spheres surrounded by yolk cells. **(e–h)** Developmental stages of the temporary (embryonic) pharynx and intestine. In these images, the pharynx is towards the bottom of the panels and the intestine towards the top. **(i)** The differentiated temporary pharynx and intestine. **(j)** Initial steps in the internalization of yolk cells through the temporary pharynx. **(k)** An embryo with its temporary intestine distended and filled with yolk cells. **(l)** Initialization of embryo flattening, and resorption of the temporary pharynx and intestine. In images **(c–j)** the external yolk cells have been omitted for simplicity. **(a–c,e)** after Ijima [41]; **(d,j)** after Metschnikoff [40]; **(f–i)** after Mattiesen; **(k,l)** after Fulinski [45].

After fertilization, several zygotes (two to six) are encapsulated along with a mass of yolk cells (Figure 2a). As the fertilized egg divides, the cleavage progeny remain barely in contact with each other and some of the blastomeres begin to flatten to form a spherical, one-cell-thick outer layer that effectively surrounds the remaining blastomeres (Figure 2b,c). The outer layer and the blastomeres it encloses form what is known as an embryonic sphere [27]. The internal blastomeres continue to divide (Figure 2c), and as the embryonic sphere develops, some of the scattered blastomeres begin to aggregate near the outer layer (Figure 2e), where they differentiate into a temporary pharynx and a temporary intestine (Figure 2f–i). The temporary pharynx begins to ingest yolk cells (Figure 2j), filling and distending the temporary intestine until it is in close proximity to the outer layer (Figure 2k). The yolk internalization process transforms a spherical, hollow embryo into one that possesses three presumptive tissue layers: an outer cell layer; an embryonic digestive system filled with yolk; and a compacted layer of blastomeres, or embryonic wall [46] bounded externally by the outer layer and internally by the distended temporary intestine (Figure 2k,l).

At first glance, one could consider these morphogenic events akin to gastrulation. However, neither the outer

Figure 3



Fluorescent immunocytochemistry of *Schmidtea mediterranea* embryos. Four progressive stages of development after the resorption of the embryonic pharynx are shown. Embryos were stained with anti-phospho histone H3 antibody to visualize mitotic cells (red). Differentiated tissues such as the definitive pharynx were detected using an anti-phospho tyrosine antibody (green). In all panels, anterior is to the left. All views are ventral. The fluorescent images are superposed on the bright-field images (gray). **(a)** Initiation of proliferation of blastomeres in the embryonic wall. **(b)** Initial invasion and proliferation of cells from the embryonic wall into the yolk-cell compartment (yellow arrowhead) is accompanied by the initial stages of differentiation of the definitive pharynx (white arrowhead). **(c)** Branching of the intestine becomes apparent as rows of proliferating cells (yellow bracket) appear in the yolk-cell compartment. Pharynx differentiation is more advanced as evidenced by the appearance of its lumen (white arrowhead). **(d)** A flattened embryo with a well-developed pharynx (white arrowhead). At this stage, the embryos display negative phototaxis, yet no obvious differentiated photoreceptors can be observed. Note the absence of anti-phospho histone H3 signal at the anterior-most end of the animal. Scale bars are 100 μ in (a) and 200 μ in (b–d).

layer nor the distended embryonic intestine and its pharynx will form part of the embryo, as these tissues are resorbed. In fact, the entire animal develops directly from the embryonic wall, where the compacted blastomeres begin to proliferate rapidly (Figure 3a) and then invade and consume the yolk cells to form a digestive sac. As development proceeds, the permanent pharynx appears (Figure 3b) and branching of the digestive system begins (Figure 3b). Flattening of the embryo now becomes apparent (Figure 3c), the branching of the gastric system is more pronounced (Figure 3c), and the ventral epithelium differentiates cilia for eventual locomotion (not shown). One to two days later, the embryo is motile, with a well-developed pharynx (Figure 3d) and a branched gastric cavity (Figure 3d). Thus, it would appear that in the absence of a well-defined series of gastrulation events, the embryonic wall establishes anteroposterior and dorsoventral axes, and produces a central nervous system, mesenchymal tissue, and a digestive system. Unfortunately, nothing is yet known about the inductive processes that must be taking place in the embryonic wall to produce the definitive endoderm, mesoderm, and ectoderm of the freshwater planarian.

The embryos of *Schmidtea mediterranea* are accessible to molecular studies

To learn more about the embryogenesis of freshwater planarians, it is necessary to have ready access to large numbers of embryos and to develop the necessary techniques to study them at the molecular and cellular level. To accomplish this task, my laboratory has chosen to develop *S. mediterranea* as a species in which to carry out embryological and molecular developmental studies. There are several advantages to using *S. mediterranea* over other freshwater planarians, and these have been summarized recently elsewhere [20]. One such advantage, is that *S. mediterranea* exists as both sexual worms that reproduce as cross-fertilizing hermaphrodites and asexual worms that reproduce strictly by transverse fission [20].

Because the sexual strain is also capable of regeneration, it has been possible to establish clonal lines of this animal in the laboratory by serially amputating adult worms and

allowing the fragments to regenerate. Previous attempts to sexually propagate freshwater planarians in captivity resulted in either low fecundity or progeny that were infertile [47]. Recently, however, my laboratory has succeeded in breeding clonal lines of the sexual strain of *S. mediterranea* that produce fertile progeny, effectively overcoming existing limitations (visit http://planaria.neuro.utah.edu for a movie of an animal laying an egg). This is allowing us to generate inbred lines for genomic and genetic analyses, as well as to begin a detailed molecular and morphological characterization of the embryogenesis of this species (Figure 3).

Bilaterian development, regeneration and the embryos of *Schmidtea mediterranea*

As counterintuitive as it may sound, studying the highly modified embryogenesis of freshwater planarians is bound to expand our understanding of common regulatory processes operating during bilaterian development. By determining the molecular events leading to cell-determination and differentiation in the ectolecithal embryos of *S. mediterranea* (Figure 2), it should be possible to recognize plesiomorphic bilaterian features shared by the ecdysozoans, lophotrochozoans and deuterostomes. Such studies would help inform theories on the evolution of the bilateria [48]. Additionally, we also know *S. mediterranea* to possess genes that are absent in the genomes of *C. elegans* and *Drosophila*, but present in *Homo sapiens* [22]. Because it is likely that some of the genetic differences that exist between ecdysozoans and *S. mediterranea* will be manifested during embryogenesis, identifying common regulatory processes between planarians and deuterostomes should allow us to better define interphyletic relationships (see the review by Tessmar and Arendt in this issue). This is, in part, exemplified by the recent characterization of *nou darake* in planarians [21], a fibroblast growth factor receptor like protein found in humans but absent in *C. elegans* and *Drosophila*. This molecule plays a key role in regulating the inductive interactions involved in the formation of the central nervous system in planarians and in regulating the FGF pathway in vertebrates [21]. However, the role of *nou darake* during planarian embryogenesis is not known. Because planarians and vertebrates share *nou darake*, defining its expression pattern and function during the development of *S. mediterranea* is likely to provide new information not only on planarian but on deuterostome neurogenesis as well.

Adult planarians are also known to constitutively express genes such as *Otx*, *Otd*, and *Pax6* [49,50], which are known to be active only during embryogenesis in *C. elegans*, *Drosophila*, amphibians, chickens and mammals. However, dsRNA injections of *Otd* (Kiyokazu Agata, Sánchez Alvarado, unpublished data) or *Pax6* [49] fail to produce detectable regeneration phenotypes. The availability of embryos from the sexual biotype of *S. mediterranea* provides us with a unique scenario in which to compare and contrast the roles played by known developmental genes during embryogenesis and regeneration. For instance, would elimination by RNAi of developmental genes such as *Otx*, *Otd*, and *Pax6* in *S. mediterranea* embryos result in noticeable phenotypes? If such genes are required for development but not for regeneration, the regulatory elements governing the expression of these and other genes should provide us with mechanistic insight on the differences and similarities that may exist between development and regeneration.

Embryogenesis and the planarian stem cells

Any future studies of freshwater planarian embryogenesis will need to address the mechanism by which neoblasts arise during development, as these totipotent cells are maintained in the soma of the adult organism and give rise, post-embryonically, to the germ line. Neoblasts, therefore, embody a fundamental difference between the ontogeny of somatic and germ tissues in planarians and the ecdysozoa. For example, other than the gonads, the somatic tissues of flies and nematodes are entirely post-mitotic and not subjected to cell turnover and replacement. In planarians, on the other hand, somatic tissues are constantly being replaced by virtue of the proliferation and differentiation of neoblasts [26]. A dramatic example of tissue homeostasis at work in planarians is provided by their ability to grow and degrow, which occurs by the respective addition or elimination of cells without any noticeable changes in their form and function [18]. In addition, the totipotentiality of neoblasts and post-embryonic induction of the germ line in planarians [51], is in drastic contrast with the maternally supplied cytoplasmic determinants which segregate early in embryogenesis to give rise to the germ cells of *Drosophila* and *C. elegans*.

In planarians, therefore, and unlike nematodes and flies, neoblasts make both somatic and germ lineages immortal. This all points to the existence of a singular developmental mechanism capable of inducing genome stability in those cells of the freshwater planarian embryo that will eventually become neoblasts. This is evidenced in Figure 3. In adult planarians, the area in front of the photoreceptors is devoid of dividing neoblasts [26]. In the embryo, dividing cells are observed in the prospective anterior end (Figure 3b,c) followed by a progressive disappearance of dividing cells in this area of the developing flatworm (Figure 3c,d). A mechanistic appreciation of the embryonic specification of neoblasts will have repercussions in our understanding of how totipotentiality is determined and perpetuated in somatic and germ stem cells.

Conclusions

The molecular and genomic revolutions have greatly enhanced our understanding of ecdysozoan and deuterostome genetics and development. The same, however,

cannot be said of the non-ecdysozoan protostomes. Because of the evolutionary distance of flatworms from ecdysozoans and deuterostomes (Figure 1), the biology of *S. mediterranea* merits a closer look. Studying a non-ecdysozoan such as *S. mediterranea* in which gene function can be tested will facilitate comparative evolutionary and developmental studies. Thousands of non-redundant cDNAs have been obtained from *S. mediterranea* [22] and a sister species *Dugesia japonica* [21], and more are on the way. It is now possible to test gene function in clonal lines of planarians using RNA interference [52], and to label stem cells [26] and their differentiation progeny [53]. Therefore, the presence of totipotential stem cells, regeneration and somatic plasticity in *S. mediterranea* provides us with a unique opportunity to molecularly dissect biological attributes not saliently manifested in *Drosophila* and *C. elegans*.

Even though the molecular tools thus far developed are currently being applied to the study of regeneration in planarians [20], there is no reason to believe that they cannot be extended to explore embryogenesis in *S. mediterranea* (Figure 3). Access to *S. mediterranea* embryos expands the traditional experimental repertoire of planarians from a system dedicated to the study of regeneration into a model in which metazoan embryogenesis and regeneration can be functionally studied and compared to each other. More importantly, the development of techniques to rear viable and fertile offspring of *S. mediterranea* under laboratory conditions may allow us to introduce genetics to the study of these organisms. Therefore, when the phylogenetic position and the biological properties of planarians are considered as a whole, it becomes readily apparent that the study of *S. mediterranea* is likely to fill not only a major void in our understanding of phyletic relationships, but also help expand and complement ongoing investigations of metazoan developmental processes.

Update

Mineta *et al.* [54] have utilized a collection of ~3000 expressed sequence tags obtained from a close relative of *S. mediterranea* (*Dugesia japonica*) to investigate the evolutionary origins of the bilaterian central nervous system. From this collection of cDNAs, the authors identified 116 nervous-system related genes. Six of these genes were absent in *C. elegans* and/or *Drosophila*, yet all 116 planarian genes were represented in the *Homo sapiens* orfeome. These and other data allowed Mineta *et al.* to propose that the centralization of the nervous system in the bilateria did not occur solely by mutation and gene duplication, but also by gene loss and divergence. In this scenario, genes found in the human genome but absent in the Ecdysozoa are thus unlikely to be deuterostome innovations, but rather the result of gene conservation from an ancestor common to both the Lophotrochozoa and the Deuterostomes.

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