[Frontiers in Bioscience 13, 6374-6394, May 1, 2008]

Stem cells and regeneration in planarians

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1. ABSTRACT

Understanding stem cells is a major goal of current research because of its potential medical applications. Although great advances have been made, such as the culturing and differentiation of embryonic stem cells and reprogramming of cell fates, many basic questions remain unanswered. Describing the mechanisms underlying regeneration will help to understand the biology of stem cells and therefore to control their behavior. While regeneration is being studied in a variety of models, the planarian is particularly noteworthy. In this model system a fragment as small as 1/279 of the animal can regenerate completely within a few weeks. These animals can also grow and degrow—specifically degenerating certain tissues—according to environmental conditions, thus demonstrating a complete control of their stem cell dynamics. However, one of the most interesting aspects of the planarian model system is the presence of a unique type of stem cell that can differentiate into all cell types found in the organism, including the germ line. This represents a simple, extremely powerful, and accessible stem cell system in which to address a variety of important questions. In the last ten years, molecular, cellular, and bioinformatics tools have been established for use in this model, making it ideally placed for in vivo analysis of stem cells in their natural environment without ethical complications.

2. STEM CELLS AND REGENERATION

Stems cells are cells that retain the ability to self-renew through mitotic cell division and can differentiate into a diverse range of specialized cell types. The enormous potential of these cells to be used to treat diseases that were thought to be incurable has captured widespread attention, and as a consequence, understanding the biology of these cells has become one of the most important challenges in modern biomedical research. Nevertheless, the promise of a panacea is still far from becoming reality and many important questions still remain to be answered, despite the magnitude of the ongoing effort. Although to date less attention has been paid to regeneration, we believe that understanding this process will provide significant insights into stem cell behavior. Elucidating the general principles underlying regeneration through research using different model animals with varying levels of complexity will help us to clarify the networks controlling stem cell dynamics, and such studies have already contributed a great deal of data to the nascent field of regenerative medicine. In this review, we will provide an overview of current understanding of regeneration, paying particular attention to the role of stem cells and the use of planarians as a model system.

2.1. Stem cells

Despite technical advances in stem cell research, the nature of these cells is still not fully understood. One of the major breakthroughs in this field, and in biology in general, was the description of the embryonic stem (ES) cell (1, 2). The potential medical applications of ES cells became apparent from their ability to survive for long periods as a pure undifferentiated population, even after several passages in culture, and their subsequent capacity to contribute to tissues in adult mice, including the germ line (3), and to differentiate into a wide variety of cell types in culture (4). Such cells could clearly provide an essentially unlimited source of cell types for a variety of applications. The possibility of medical applications of these cells come a step closer with the isolation of human ES (hES) cells (5). However, ES cells are not the only multipotent cells that have been described. Adult stem (AS) cell systems have been described in both invertebrates and vertebrates, including the ovary and testis of *Caenorhabditis elegans,* ovary and testis, midgut and malpighian tubules of *Drosophila* and AS cells from the bone marrow, hair follicle, intestine, brain and testis of mammals (6, 7). In all cases, except midgut and malpighian tubules of *Drosophila,* stem cells are located in a stem cell niche, a microenvironment composed of specialized tissue surrounding the stem cells. The niche produces factors that control stem cell fate and proliferation. These factors include BMP and Wnt signals, and their associated pathways are known to control self-renewal and differentiation of stem cell populations (6). AS cells are able to generate a few cell types and are responsible for organ-specific homeostasis and regeneration, but their potential for self renewal is limited. Fetal stem (FS) cells are more plastic than AS cells but with even less self-renewal potential, meaning that they are not as good as ES cells for cell transplantation procedures (8). Even though the use of AS and FS presents fewer ethical problems, their limited capacity for self renewal makes them less suitable for use in clinical research.

Since the initial use of cultures with serum and feeder cells, advances have been made in identifying the factors responsible for ES cell maintenance. Leukemia inhibitory factor (LIF) was found to be able to substitute the effect of feeder cells (9), and more recently it has been shown that BMP4 in the presence of LIF can replace the serum requirement (10). In the case of LIF, the effect is mediated by the transcription factor STAT3, which was already known to play a role in the maintenance of pluripotency (11), while BMP4 acts through SMAD-mediated activation of helix-loop-helix Id factors. However, the main role in stem cell maintenance is played by other transcription factors, namely Oct 3/4 (12, 13) and Nanog (14, 15, 16). These two transcription factors, together with Sox 2 (17), seem to be the earliest-expressed set of genes implicated in maintenance of pluripotency (reviewed in 18). They are thought to act together with other transcription factors to establish the pluripotent transcriptional or genomic ground state, the point from which all other expression patterns are generated during development. This establishes a transcription-factor hierarchy, such that these genes maintain the cells in a pluripotent state by interacting with other factors and processes. Some of these factors and processes have already been identified and the relationships between these prime transcription factors and others seem to control the specific steps involved in differentiation; for instance, the ratio of Oct-4/Cdx2 influences the first overt lineage decision (19). However, the process is still far from fully understood, and studies are underway to establish a regulatory network involved in these differentiation steps, e.g. through the analysis of transcription factor binding sites in the mouse (20) and human (21) genomes.

Research is also underway into the epigenetic profile and chromatin dynamics that allow the conservation of expression profiles in a pluripotent state. ES-cell chromatin seems to be in a transcriptionally permissive state, with extensive acetylated histone modifications, presenting high genomic plasticity. The role of proteins that are important in the conservation of gene expression, such as the polycomb group proteins, has also been studied in these cells, and they have been found to actively influence the control of gene expression. Furthermore, many efforts have been made to relate data on mouse ES cells to insights from hES cells in order to come closer to developing final therapeutic applications. Although problems have arisen with the recognition that there are differences between the two cell types, such as the lack of involvement of LIF and STAT3 in the maintenance of pluripotency, the expression in hES cells of Oct-3/4 and Nanog suggests that the importance of these transcription factors is conserved between human and mouse systems. However, despite the undoubted importance of these studies, it is also necessary to pay attention to findings in other models, as these could help to generate a more general description of stem cell biology

Stem cells are currently being studied in a variety of different models, but in most of those models the stem cells are more directly involved regenerative processes. Sponges have totipotent stem cells, the archaeocytes that give rise to germ cells for sexual reproduction and gemmule cells for asexual reproduction. The archaeocytes are also the origin of the differentiation pathways to the main terminally differentiated cells, such as epithelial, contractile and skeletal-cells (22). Like all cnidarian polyps, hydra has two cell layers, and their cells differentiate from three distinct stem cell populations. The interstitial stem cells, which are fast-cycling multipotent stem cells, are uniformly distributed along the body column but absent from the head and foot regions. They differentiate into different cell types depending on their position and exhibit great cell plasticity. Ectodermal and endodermal epithelial cells are also continuously cycling in the body column and play a morphogenetic role during regeneration (23). Despite the presence of stem cells in hydra, transdifferentiation has also been observed (24). In annelids, segmentally iterated populations of cells (called neoblasts as in planarians) are a primary if not the exclusive source of somatic cells within regenerated tissue. In annelids, there are also independent germline and somatic stem cell lineages (25). Some insects have the capacity to regenerate appendages during molting. The cells that are

responsible for regenerating the lost appendages come from either the epidermis or imaginal disc cells (26). Echinoderm regeneration can be considered a physiological phenomen, as it is common to all classes and is observed in both larval and adult stages. Regeneration is extensively employed to reconstruct external parts and internal organs; it is also part of the life cycle as an indispensable complement to the programme of asexual reproduction. The cells involved in this process are derived both from dedifferentiating myocytes and from undifferentiated amebocytes and coelomocytes (27).

2.2. Regeneration across the animal kingdom

Regeneration is a process that appears in nature in different forms and at different levels of complexity. Regenerative phenomena range from the simplest processes in highly complex animals, such as the healing of human skin following minor injury, to the most amazing potential for regeneration seen in basal bilaterians like the flatworms (28). However, aside from considering the regeneration process itself, it is also interesting to look at the distribution of regenerative capacity throughout the tree of life. Animals with such amazing capabilities are widely distributed in many different phyla, and this, coupled with the differences apparent among species in the same phyla raises interesting questions not only about the biological process but also in terms of its evolutionary implications.

In invertebrates we find two main models of regeneration, namely hydra and planarians. These two systems have a long history in regeneration research and many laboratories are currently using them to analyze the signaling pathways and molecular networks involved in this process. However, these are not the only invertebrate organisms of interest in regeneration research, and other models like Macrostomum (Platyhelminthes, Rhapditophora) and Convoluta (Acoelomorpha, Acoela) are also used. Furthermore, many other invertebrates are known to display regenerative capacities, for example arthropods, annelids, nemerteans, and some deuterostome invertebrates like echinoderms and ascidians. Although there are fewer examples of regeneration in vertebrates, a number of important exceptions exist. The most widely used models are amphibians, namely newts, axolotl, and salamanders, followed by zebrafish (29) and the more phylogenetically distant mammals. In these models, the regenerative potential may not be comparable to invertebrates, but neither is the complexity of the process.

Regenerative capacity is always based on the same kind of basic processes present throughout almost all of the animal kingdom. However, these processes can act in different ways, giving rise to different mechanisms of regeneration. It is difficult to classify the regenerative processes present in nature, because many of them overlap with tissue homeostasis, if there are in fact any differences between them. For the purposes of this review, we will use the classical concepts based on the different dynamics of the process and will not consider the mechanistic significance of the processes in and of themselves. Classically, regenerative events were divided into two mechanisms, morphallaxis, and epimorphosis (30). These concepts refer to the organization of the different sub processes that we find in regeneration: pattern respecification, tissue regrowth, and others. Morphallaxis refers to regeneration through substantial remodeling and re-establishment of patterns within pre-existing tissue, with little new growth. Hydra regeneration is a classic example of this process. Epimorphosis, by contrast, occurs when lost parts are built anew through the growth of new correctly patterned tissues. The most typical examples are tail and limb regeneration in newts.

Besides the basic differences underlying this classification, the processes differ in other ways, for example in the origin and identity of the blastema-forming cells. They can come from differentiated cells that de-differentiate or change their cell fate to form this new tissue, or from local stem cells that divide to form the blastema. Also, blastema-forming cells can be derived from stem cells located far from the target and that migrate until they reach the regenerating area and then begin to divide. The presence or absence of mitotic activity within the blastema and the presence or absence of the blastema itself can also vary; even the wound-healing process after injury can be different (31-34). Thus, even though the signaling pathways involved in regeneration do not differ substantially among the huge range of organisms that display regenerative capacity, the process itself is complex.

2.3. Making use of regeneration

Neurodegenerative diseases, diabetes, chronic liver diseases, heart disease, and muscle diseases are only a few of the conditions on the long list for which a potential solution could lie in the use of stem cell therapy (35). Most approaches seek to reproduce a regenerative process by introducing stem cells or committed cells capable of replacing the damaged or lost structures or cell types (36). Other approaches use the nursing concept, which consists of the introduction of cells that instead of replacing old tissues produce factors that help the host cells to recover and survive by themselves, in this way obtaining a healthy tissue (37). All these approaches take advantage of research into ES cell differentiation (reviewed in 4). Reprogramming of a cell's fate is a way to overcome the ethical dilemmas associated with research using ES cells and some advances have been made in reprograming somatic gene activity by fusing the nuclei of these cells with nuclei from pluripotent cells (reviewed in 38). However, although great advances have been made in recent years in terms of the possible therapeutic applications of ES cells, we still do not have a clear understanding of ES cell biology.

There continue to be technical problems relating to the application of stem-cell therapies, mainly caused by the inability to guarantee normal behavior of the cells once transplanted into the host and the associated risk for abnormal growth and tumor formation. This is because we are still some way from fully understanding the processes that take place during regeneration and because we are attempting to induce regeneration in tissues that normally have little or no regenerative capacity. Better understanding of the cells and molecules involved in regeneration will

facilitate the development of new therapeutic techniques and ensure safe application of those that are already being developed. However, to achieve this understanding it is not enough to study the dynamics of ES cell cultures, because this may not reflect the *in vivo* process and the results obtained may not on there own be suitable for clinical applications. In addition, the complexity of the organs and tissues of the models used in regenerative medicine, mainly mice, makes it difficult to design experiments aimed at understanding how cells communicate and behave during regeneration in terms of information received and sent by a cell, integration into the new environment, and control of cell death and survival. These basic but important issues are more amenable in a system that permits more direct *in vivo* observation of cells. A model of regeneration such as the planarian meets these requirements.

3. PLANARIA, A REAL REGENERATING MODEL SYSTEM

The term planarian is used here for freshwater, free-living triclads. Since planarians possess remarkable regenerative capacities and a constant pool of stem cells, their use in stem cell and regeneration research is both appropriate and lacking in ethical problems. The planarian offers a model in which stem cells can be studied in their natural environment. Furthermore, they are easy to breed in the laboratory and amenable to molecular techniques.

3.1. Historical overview

Already during the 18th and 19th century there was growing interest in the ability of planarians to regenerate (30, 39-41, see reference 42 for a more in-depth review of early planarian research). The research was focused mainly on the extensive ability of the planarian to regenerate, along with its capacity to maintain or reverse polarity (heteromorphosis) during regeneration, to grow and "degrow", to undergo fission and to change its reproductive strategy from asexual to sexual and *vice versa* (reviewed in 42).

Scientists were attracted by the regenerative power of the planarian and marveled at them with statements such as those of Dalyell (1814) that they could "almost be called immortal under the edge of the knife" or Harriet Randolph (1897) that "a piece only large enough to be seen with the naked eye will develop into a perfect whole." Also, Thomas H Morgan carried out exhaustive work on planarian regeneration. He defined the process of morphallaxis during regeneration (see section 2. 2), at that time referring to "morpholaxis" (30), and studied polarity, focusing on the occurrence of heteromorphic heads and tails in regenerating anterior and posterior fragments, respectively (30, 43, 44). Morgan also showed that a piece as small as 1/279 of a planarian is able to remake an entire new organism, albeit with delayed or imperfect regeneration (45). The only planarian tissues that are incapable of regenerating are the pharynx and the region in front of the eyes, due to a lack of mitotically active stem cells, known in planarians as neoblasts (41, 45, 46). Morgan further showed that a sexually reproducing planarian was able to reform the entire reproductive system from a head piece lacking the germ cells, and this led him suggest that the germ cells arise from the neoblasts (47). W Curtis made a similar proposal based on his observation of the accumulation of "formative cells", as he called the neoblasts, in the tissue where the gonads would differentiate. This formed part of his observations of the morphological changes when the planarian *Girardia tigrina* (previously known as *Planaria maculata*) changed reproduction strategy from asexual to sexual and thus formed an entire complex reproductive system (48). He also described the amazing ability of the planarian to reproduce asexually by architomy, a process of fission giving rise to two planarian pieces that then followed the principles of regeneration to obtain two identical organisms, an observation that was actually already described by H Randolph in 1897, but without referring to the process of fission as "asexual reproduction." She studied the regenerative capacity of the planarian and saw that some of the animals would undergo fission by themselves (41).

3.2. Planarian anatomy

Planarians are free-living freshwater flatworms from the phylum Platyhelminthes (Order Tricladida, Class Turbellaria) belonging to the lophotrocozoan clade of the Protostomes (49, 50). As the name implies, they are dorsoventrally flattened bilaterians that lack a coelom, circulatory, respiratory, and skeletal system. Characteristic of the triclads is the three-branched digestive system that is connected to the pharynx (the mouth opening) in the middle of the ventral side of the planarian, but that is blind-ended at the opposite end and thus lacks an anus (Figure 1A, C). The loose connective tissue between the body wall musculature and the gut is called the parenchyma (or mesenchyme) and contains diverse cell types such as epidermal gland cells, fixed parenchymal cells, which connect different cell types and tissues, pigment cells that give the planarians a brownish color, and the neoblasts. In the anterior part, a pair of eyes, consisting of photoreceptor cells and pigment cells, can be distinguished on the dorsal side of the planarian. The axons of the photoreceptor cells project to the brain, which is bilobed and located on the ventral side of the animal, and which is further connected to the two ventral nerve cords that run longitudinally, each following the lateral sides of the planarian body and fusing in the tail (reviewed in 51). The animal is unsegmented, although the digestive system and the nervous system show some iteration. The body lacks circulatory systems (acoelomate) and diffusion of factors such as oxygen and nutrients takes place over short distances in the compact body tissue. The animal moves through a combination of muscle movements, slime secretion, and the use of the ciliated ventral epidermal tissue and adhesive glands, representing one of the largest organisms to employ ciliary locomotion. Planarians can reach lengths of up to 5 cm (reviewed in 51).

3. 3. Planarian biology

Planarians are usually hermaphroditic and most often possess the ability to reproduce both asexually and sexually (Figure 1B, 2) (48, reviewed in 52). In the period of asexual reproduction, planarians do not have mature

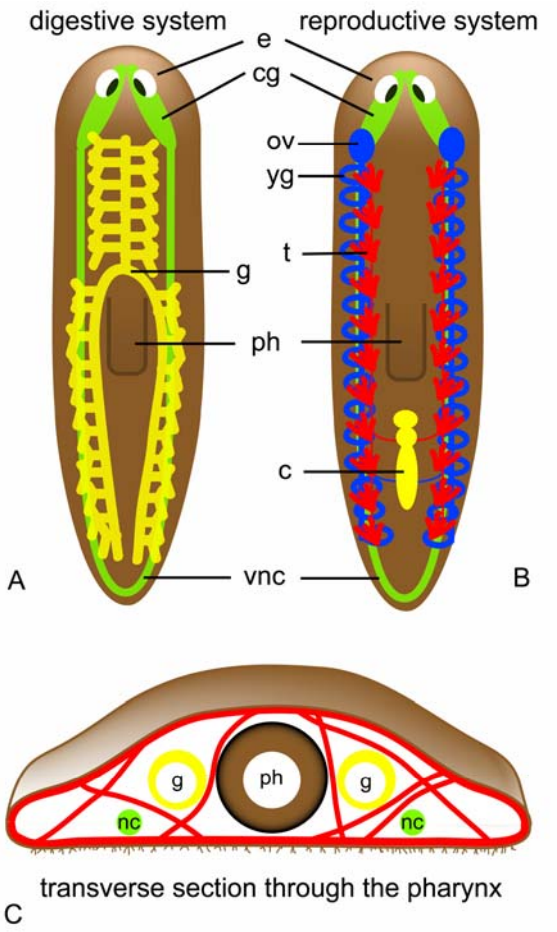

Figure 1. Overview of planarian anatomy. (A, B) Dorsal view of a planarian; anterior is to the top. The ventrally located nervous system is indicated in green with the cephalic ganglia (cg) connected to two lateral nerve cords (vnc). A pair of eyes (e) can be found in the head region. (A) The blind-ended three-branched digestive system (in yellow, g) is connected to the pharynx (ph), which is situated on the ventral side of the planarian and is used for eating and for expelling waste material. (B) Most sexually reproducing planarians are hermaphroditic, and the size and position of the reproductive organs can differ between the species. In the planarian *Schmidtea mediterranea*, the testes (in red, t) are located on the dorsal side in two bilateral rows. A pair of ovaries (in blue, ov) is situated ventrally in the anterior part of the planarian and connected to the ventral oviducts, which in turn are connected to the yolk glands (in blue, yg). Both the testes and the oviducts lead to the copulatory apparatus (in yellow, c), which is located posterior to the pharynx. (C) Transverse section of an asexual planarian at the level of the pharynx (ph). Dorsal is to the top and characterized by the brownish pigmented epithelium. The ventral side is covered with cilia used for locomotion. On each side of the pharynx (ph) a gut branch (g) can be found. Two lateral nerve cords (nc) are located ventrally in relation to the gut. A loose connective tissue, the mesenchyme or parenchyma (in white), and transverse and longitudinal muscles (in red) surround the organs.

gonads and these must be formed *de novo* when the animal sexualizes (48, 53, 54). However, recent molecular data suggest that the asexual organism does in fact possess primordial germ cells (54-56). In contrast, the reproductive organs degenerate when planarians shift to asexual reproduction, and during regeneration and starvation (48, 57-60). The two reproductive strategies are a consequence of an adaptation to external circumstances, especially temperature and availability of food (61, reviewed in 52). Within the free-living flatworms, a clear correlation can be seen between asexual reproduction and the ability to regenerate (reviewed in 62). The mechanisms underlying the remaking of new tissues following the two processes are thought to be similar (63), but we can only speculate as to which of these processes were the first to appear during evolution. When there are environmental changes the planarian can shift to sexual reproduction. Differentiation of the gonads and of the copulatory apparatus takes place, and fertilization normally occurs by cross-copulation between the two hermaphroditic planarians. An ectolecithal polyembryonic egg capsule is produced (the yolk cells are located outside the egg) and attached to a surface, and after 10 days to several weeks, depending on the planarian species and the temperature, the juveniles hatch (52).

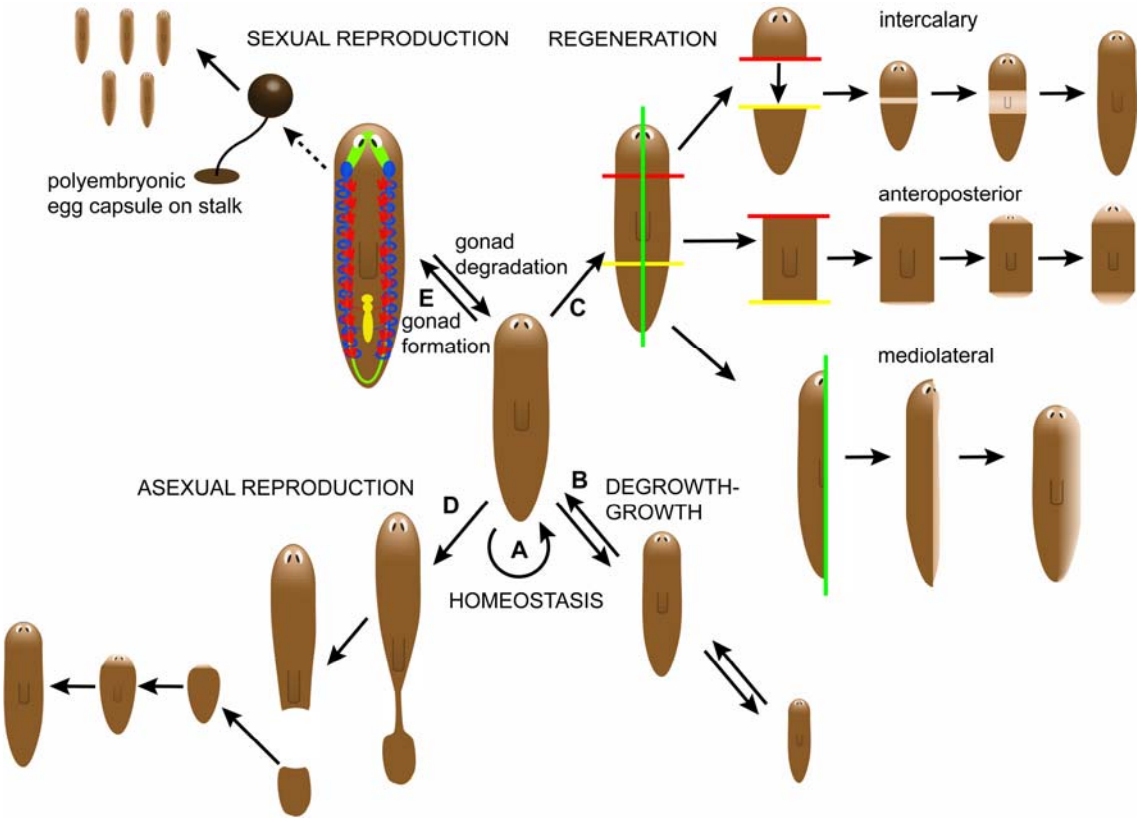

Figure 2. Regenerative, reproductive and homeostatic capacity of planarians. (A) Tissue homeostasis. A continuous process of renewal of all planarian cells occurs in which an equilibrium exists between cell proliferation and cell death. (B) Degrowth-growth. The planarian is very plastic and upon starvation there is a reduction in the total body cell number (degrowth) caused by an increase in the rate of cell death compared with that of cell proliferation. After feeding, the planarian will grow in size and cell number will increase again (growth) through a shift in the equilibrium to favor mitosis over cell death (see also Figure 3A). (C) Regeneration. Practically any imaginable amputation of the planarian can give rise to two individual animals. A sagittal cut induces the formation of a lateral non-pigmented blastema along the length of the planarian body. The missing eye differentiates and the remaining organs are remodeled, giving rise to an entirely regenerated planarian that is smaller than the original animal. Following transverse cuts, the middle fragment forms both an anterior and a posterior blastema. The head, including the eyes and the brain, is regenerated in the anterior blastema and the tail in the posterior blastema. A process of remodeling takes place in the middle fragment, adjusting the organs to the new smaller size of the planarian. When two fragments with different positional values are joined together, intercalary regeneration reforms the missing tissues between the two regions and gives rise to a single planarian. (D, E) Reproduction. Many planarian species can alternate between asexual and sexual reproduction, depending on factors such as temperature and nutrient availability. (D) Asexual reproduction takes place by fission in the posterior part of the planarian. Two fragments are obtained, a head-pharynx fragment and a tail fragment. Both pieces will regenerate the missing body parts and give rise to two planarians. When the planarian sexualizes, the reproductive structures are formed *de novo*. (E) Sexual reproduction takes place by cross-fertilization between two hermaphroditic planarians (dotted arrow) and a polyembryonic egg capsule is produced. The egg capsule is set apart from the planarian body and attached to a surface by means of a stalk. Hatching occurs after 10 days to several weeks depending on the planarian species. When the planarian converts to asexual reproduction there is a degradation of the gonads.

Planarians also show high plasticity in the control of morphology and cell turnover. Tissue homeostasis takes place continuously to renew all cell types, but apart from this, degrowth of the planarian by reducing the total body cell number is a natural consequence of restricted food availability (59, 64). When food becomes available again, the planarian will re-grow by increasing the cell number (Figure 2, 3A) (64). Growth and degrowth represents a dynamic equilibrium between cell proliferation, autophagy and cell death (57, 59). During growth there is more cell proliferation than cell death, leading to an increase in the total number of cells in the body. In contrast, during degrowth the equilibrium goes towards cell death so that the total number of cells in the planarian body will decrease and the planarian will get smaller (64, 65). It has been suggested that a re-juvenilization takes place during the degrowth-growth process (61, 66). This has also been suggested for the regeneration process and for asexual reproduction (61, 66), suggesting that planarians may be able to "escape" the aging process in many different ways.

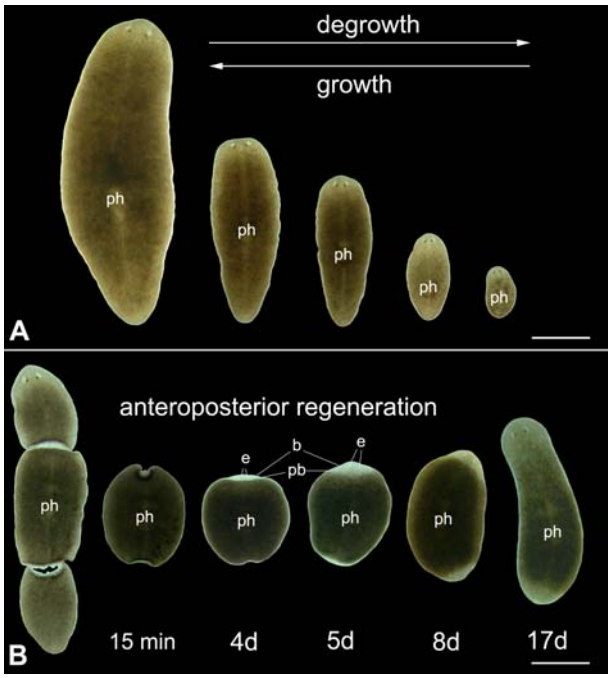

Figure 3. The degrowth-growth (A) and regeneration (B) process in the planarian *Schmidtea mediterranea*. Dorsal view, anterior is to the top. (A) When food is limited, the planarian can reduce many times in size. During degrowth a reduction in the total body cell number occurs since the rate of cell death is higher than the rate of cell proliferation. The planarian will grow in size and cell number again upon feeding since the rate of mitosis exceeds that of cell death. (B) Anterior and posterior regeneration in *S. mediterranea* after a transverse cut, at 19˚C. Transverse amputation of the planarian is carried out at two levels, pre- and post-pharyngeal, resulting in three fragments: a head fragment, a middle fragment including the pharynx (ph), and a tail fragment. During regeneration of the middle fragment, wound closure of both the anterior and posterior wounds can be observed almost immediately (15 min). In the first few days of regeneration, a white blastema (b) is formed made up of undifferentiated cells, and at 3-4 days of regeneration small eyespots (e) appear in the anterior blastema (4d). Following their initial appearance in the blastema, the eyespots grow to their normal size by aggregation of newly differentiated cells (5d, 8d). Also, the brain has regenerated in the anterior part and pigment cells start to be visible in the blastema region. In the posterior part, the missing organs such as the ventral nerve cords and the digestive system have been reformed. After some weeks of regeneration, the planarian is fully regenerated, although smaller than the original organism (17d). Remodeling of the existing tissue has occurred, e.g. the size of the pharynx has been adjusted to the dimensions of the new organism. Growth of the planarian will occur upon feeding. b, blastema; e, eyes; d, days; pb, post-blastema; ph, pharynx. Scalebar: 4mm

The lifespan of turbellarians is mostly known from laboratory cultures (61). Thus, in Barcelona we have maintained a clonal line from a single asexual *S. mediterranea* animal for 3 years. Hundreds of fissions have already taken place, giving rise to thousands of individuals that are literally identical at a genomic level.

4. REGENERATION IN PLANARIANS

4.1. The process

The process of regeneration in planarians does not strictly follow either morphallaxis or epimorphosis. A mixed morphallactic-epimorphic model was proposed (67) that would accomplish the process via two steps: a first morphallactic one, where an initial pattern is established within a narrow area of blastema and post-blastema cells, and a second step in which this small pre-pattern is amplified and refined by production of new cells (epimorphosis) (Figure 4). We can find a similar process occurring in amphibian models of limb regeneration, where it has been suggested that proximal and distal identities may be established very early in the blastema (68, 69), and in recent models of proximodistal pattern formation during vertebrate limb development, an early and non-progressive specification of the proximodistal progenitor cells has led to the proposal of an early specification model (70, 71). In both systems—planarian head regeneration and vertebrate limb regeneration and development—there is an early determination of an axial pre-pattern that is subsequently refined by the incorporation of new cells. There is also another kind of regeneration in planarians, intercalary regeneration, which takes place when two planarian pieces from different positions are placed in direct contact (72).

4.1.1. Wound healing

The very first process that takes place after amputation or fission is the closure of the wound. This is a very rapid process that occurs within the first 30 minutes after amputation (Figure 3B). Contact between the old dorsal and ventral epidermis is facilitated by muscle contraction (73-75). An epidermal layer then covers the wound, and after muscle relaxation this layer becomes

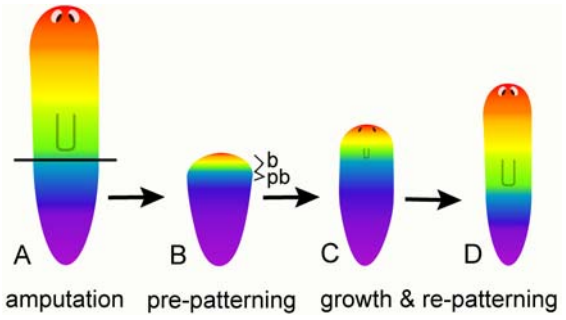

Figure 4. Summary of the morphallactic-epimorphic model of pattern formation, reformation and growth during planarian regeneration. (A) Axial division of the planarian into head (red), prepharyngeal (yellow), pharyngeal (green), and postpharyngeal (blue) areas, and tail (purple). The black line indicates amputation of the planarian in the postpharyngeal region. (B) After amputation, an early morphallactic phase pre-patterns the anterior structures in the small newly formed blastema (b) (as indicated with the red, yellow and green colors) and in the post-blastema (pb), which corresponds to the old tissue close to the wound. (C) A combined morphallactic-epimorphic phase with cell proliferation and differentiation transforms the early structures into visible morphological patterns with the appearance of the eyespots and the pharynx. Remodeling is also necessary to adjust the planarian body to the new smaller proportions. (D) After some weeks the regeneration process is completed by extensive growth and re-patterning, resulting in a smaller planarian but with the correct proportions, as indicated by the different colors.

thinner (for a detailed explanation see 76).

The molecular nature of the signaling responsible for the initiation of the regeneration process, however, still remains unknown. The important role of the mesenchymal-epidermal interaction in other systems (77-80) has led to the idea that the induction of regeneration starts in the epithelium (74, 81, 82). Even though in vertebrates this is known to play a key role, no conclusive data have been obtained in planarians. It has also been proposed that in planarians direct contact between dorsal and ventral epidermal cells is important for triggering regeneration (83-85). Grafting experiments involving inversion of the dorsoventral (DV) axis of the donor tissue with respect to the host planarian induced new organizing centers in each of the confronted pieces, leading to the generation of multiple new planarian axes (Figure 5) (83-87); however, this effect was not observed with inversion of the anteroposterior (AP) axis, normal intercalary regeneration repairs the change. These observations indicate that DV interaction and tissue confrontation with different positional values are two distinct scenarios in which regeneration is activated.

4.1.2. Blastema formation

After wound closure there is a peak of mitotic activity in the neoblasts that mainly occurs in the old tissue adjacent to the blastema, called the postblastema. In this way, the cells that are going to form the new tissue are generated. These cells migrate into the blastema area, where they stop dividing (67, 88, 89). The origin and location of this mitotic activity has been a topic of discussion. Some results point towards a local origin and short migration mainly by random spreading. A small area of 300 microns below the blastema was observed to be sufficient to produce the cells necessary for the new tissue (for detailed experiments see 90, 91). Also the ability of this animal to undergo intercalary regeneration was shown to be equal from both sides (92). Other studies have demonstrated that neoblast cells from the tail are able to form the blastema tissue of the other side of the animal, covering the distance in between. This was recognized using partial-irradiation experiments, utilizing the fact that neoblasts are known to be the only proliferative cells and they are sensitive to irradiation (93). By partial irradiation and then anterior amputation, it was observed that, after a delay of several days, the non-irradiated neoblasts had crossed the irradiated tissue and produced a normal anterior blastema (for more detail, see references 88, 94, 95). However, molecular studies labeling the neoblasts with PCNA have shown that there is no directional migration after this treatment (96) and the phenomenon could also be explained by passive spreading due to cell division. This could also explain the cell turnover observed in areas of the planarian that are known not to have mitotic activity, mainly the area in front of the eyes and the pharynx. Despite these findings, it is still not completely clear whether or not these cells are able to undergo directed migration. Although neoblasts have generally been considered to be responsible for forming the blastema, the evidence is indirect, and in many models of regeneration the blastema cells, or the cells that participate in regeneration, come from dedifferentiation of somatic cells. The main experiments in support of dedifferentiation in planarians were done by Gremigni and co-workers (97, 98). They saw that in a species with different chromosome numbers in somatic and germ cells, the germ cells could contribute to blastema formation and differentiate into new structures. By analyzing cell ploidy, they found that germ cells could dedifferentiate and form different cell types. However, these results could also be interpreted as transdetermination of partially determined stem cells, the germ line.

Several classical observations, along with the results of more recent experiments, suggest that the blastema is formed by neoblasts. Thus, irradiation causes the loss of cell renewal, regenerative capabilities, and finally death. This lethal phenotype can be rescued by injection of a neoblast-enriched cell suspension (99). In contrast, injection of a cell suspension enriched in differentiated cells did not rescue the phenotype and no mitosis was observed (99). Recent experiments involving BrdU-labeling of neoblasts has confirmed these observations (46).

4.1.3. Patterning

Patterning during planarian regeneration occurs soon after wound healing (Figure 3B, 4). This was shown by grafting experiments that took advantage of the ability of the head and the pharynx, once determined, to inhibit the

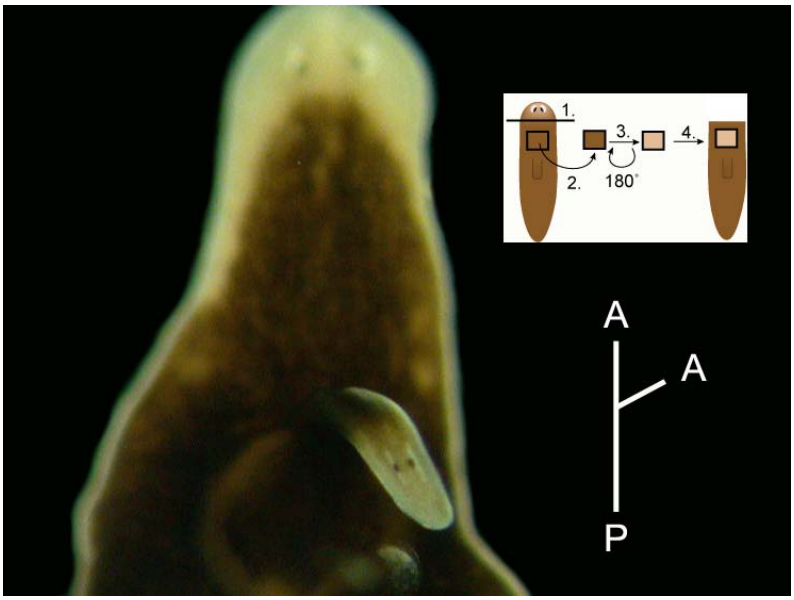

Figure 5. Planarian grafting experiment. To perform the transplant, the head of the planarian is first amputated to prevent the animal from moving (1.). In the example shown, a square of tissue is then dissected from the prepharyngeal area (2.), rotated 180° in the dorsoventral plane (3.) and replaced inside the wound such that the anteroposterior axis is unaltered (4.). This causes confrontation of the dorsal and ventral tissues between the planarian body and the fragment. The end result is the generation of a planarian (with a newly regenerated head) with another smaller planarian growing out from it. Both animals have the same anteroposterior orientation, but their dorsal sides are turned against each other. Many of these monster planarians were created in the early planarian regeneration studies (86, 87). A, anterior; P, posterior.

formation of another head and pharynx, respectively (34, 75,). These experiments showed that determination occurred very rapidly, within the first 24 hours in the case of the head and up to 36 hours for the pharynx, in both cases at a temperature of 17ºC. Since this process is so rapid, it therefore also occurs in an extremely narrow piece of tissue, because at 24 hours of regeneration the blastema is hardly visible. These classic experiments are now supported by descriptions of the gene expression patterns for markers of cell differentiation, such as Otx, Pax-6 and other neural markers (reviewed in 100, 101). Also, X-ray irradiation experiments have shown that even without proliferation, blastema formation and repatterning of the tissue is still possible, indicating that repatterning is independent of proliferation. Besides the repatterning of the blastema there is also a remodeling of the old tissue that allows it to adapt to the new proportions. This process has been studied by analysis of changes during regeneration in the expression of different genes, such as the Hox genes. These studies indicated that within two days the positional values are reset to adapt the whole body to the new situation (34). A position-dependent commitment of neoblasts outside the blastema has also been described (102); this could be due to the requirement for new cells to repattern the old tissue. Analysis of cintillo, a gene expressed in the sensory neurons, also revealed that body size is readapted (103). The number of neurons expressing this gene is proportional to the size of the animals, this number of cells changes during regeneration to adapt the quantity of cells expressing cintillo to the new size. To achieve this reorganization of the old tissue without disturbing the normal function of the organs, the balance between cell death and proliferation is controlled (58). Autophagy plays an important role in this equilibrium and allows the cellular material to be recovered as well as helping to repattern the differentiated cells, as recently demonstrated by the analysis on *Gtdap-1* (58-51) All these data are consistent with an epimorphic-morphallactic model of regeneration in planarians (Figure 4). Related experiments were carried out by Le Moigne in the 1970s (104, 105). By treating young animals with Actinomycin D they saw that RNA synthesis did not affect regeneration, whereas inhibiting protein synthesis with cycloheximide did affect the process. The capacity to regenerate in the presence of Actinomycin D decreased during growth and was lost in the adult; however, this capacity was recovered after a round of amputations (106). These findings suggested the presence of a stable population of mRNA that could sustain the protein expression needed, maybe due to the chromatoid bodies, another characteristic of undifferentiated cells (see below).

Recent results showed an important conservation in the mechanisms involved in pattern formation and maintenance in planarians. The functional characterization of elements belonging to two conserved metazoan pathways, Wnt and BMP, revealed that both play a pivotal role in planaria in AP patterning in the case of Wnt, and DV patterning in the case of BMP (107-113).

4.2. The key: Neoblasts

Clearly the neoblast is of particular interest due to its indispensable role in the process of planarian regeneration. The term neoblast was first for the putative

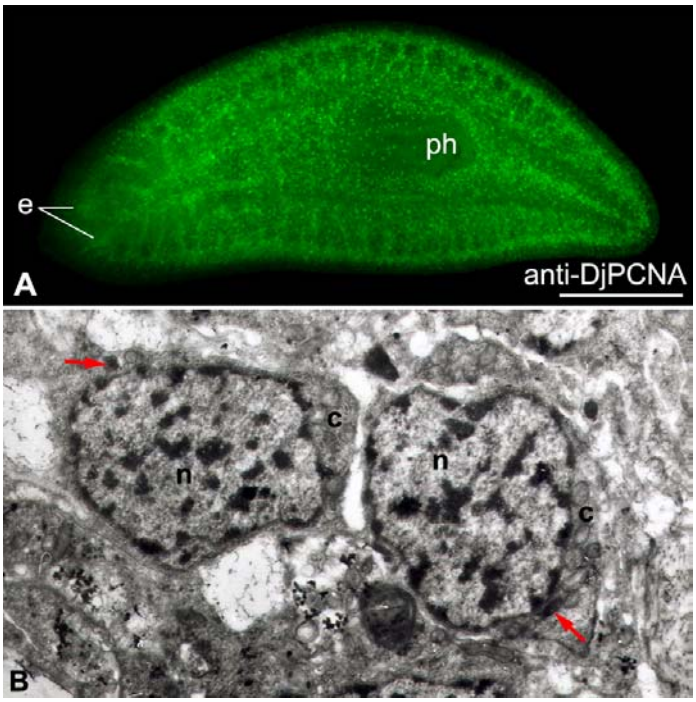

Figure 6. Neoblast distribution and morphology. (A) Whole-mount immunostaining with the antibody anti-DjPCNA in the planarian *Schmidtea mediterranea*. PCNA (proliferating cell nuclear antigen) is a marker of planarian neoblasts (96). The neoblasts can be observed throughout the mesenchyme in between the multiple branches of the digestive system, but not in the pharynx (ph) or in front of the eyes (e). Ventral view; anterior to the left. Scalebar: 2 mm. (B) Electron-microscopy image of two neoblasts. The cells measure 6-10 µm and are characterized by their high nucleocytoplasmic ratio and by the presence of chromatoid bodies (red arrows). n, nucleus ; c, cytoplasm.

stem cells participating in Annelid regeneration, where they are thought to give rise only to the mesodermal tissue (114, 115). Later it was used to describe the pluripotent planarian stem cells (88, 116).

The importance of neoblasts in the formation of the blastema and thus the process of regeneration was discovered through experiments involving partial irradiation (88, 94, 95), grafting experiments with nuclear and chromosomal markers (91, 99) and by labeling of the neoblasts with BrdU (46). In addition to their role in blastema formation, their status as mitotically active, pluripotent cells means that they can differentiate into all planarian cell types, including the germ cells in sexual organisms (99).

4.2.1. Neoblast structure

Neoblasts are small ovoid cells (6-10 µm) with a large nucleus containing a small nucleolus and a high nucleocytoplasmic ratio (Figure 6B). The cytoplasm is strongly basophilic, rich in free ribosomes and with few mitochondria, and contains characteristic chromatoid bodies in close proximity to the nucleus (reviewed in 117, 118). The chromatoid bodies are RNA processing centers that decrease in number during differentiation and finally disappear (118, 119). They most probably correspond to the polar and germinal granules in *Drosophila* and *C. elegans* or to the processing bodies in mammalian cells (reviewed in 120). Chromatoid bodies are also present in the planarian germ cells, which, together with the neoblasts, are the only mitotic cells described in the planarian (54).

4.2.2. Neoblast distribution and subpopulations

A number of neoblast markers have been described based on analysis of the molecular fingerprint of planarian cells (Table 1) (detailed reviewed in 121-134). These markers have been used to examine the distribution of the neoblasts in the planarian body and show that they are found throughout the mesenchyme except for the pharynx and the area in front of the eyes (Figure 6A) (46, 95, 130). A number of studies suggest that the neoblasts constitute 20-30% of the total cell population (46, 64, 65, 130, 132). Various experiments have now shown that they can be divided into subpopulations according to their distribution along the AP, DV, and mediolateral axes. Although they are generally distributed throughout the mesenchyme, their specific distribution pattern varies between planarian species. Baguñà (131) reported that *Schmidtea mediterranea* (previously known as *Dugesia mediterranea*) has a higher density of neoblasts at the anterior and posterior ends than in the middle region where the pharynx is located. A similar distribution with a higher number of neoblasts in the pre- and postpharyngeal regions and a minimum in the pharyngeal area was described by Brøndsted and Brøndsted (133) in planarians with different regeneration capacities along the AP axis, suggesting that the regeneration capacity of a planarian is not entirely dependent on neoblast distribution. Baguñà (65, 130)

Table 1. Markers of neoblast cells in the planarian (Order Tricladida)

Gene (g)/ Protein (p)/ Labeling (l)	Gene Product	Gene (Planarian Species)	Expression (E) and Functional (F) Features	Ref
BrdU-incorporation (l)		(Ph, Gd, Smed)	Incorporation in proliferative cells, the neoblasts	46
Chomatoid body component (g/p)	DEAD box RNA helicase gene of the RCK/p54/Me31B family	*Djcbc-1*/DjCBC-1 (Dj)	**(E)** Neoblasts, cell bodies in neurons of the brain, germ cells	122
Bruno (g/p)	RNA-binding protein	*bruli*/Bruli (Smed)	**(E)** Neoblasts and central nervous system **(F)** Neoblast maintenance	123
Anti-H3Pser10 (p)	Anti-histone H3 phosphorylated serine 10	(Smed)	**(E)** Mitotic cells, the neoblasts	46
Anti-H4-K20me1 (p)	Anti-Histone H4 monomethyl-K20	(Smed)	**(E)** Neoblasts, probably in early S-phase	123
Innexin (g)	Gap–junctional proteins	*Smedinx-2* and *-11* (Smed)	**(E)** Neoblasts **(F)** *smedinx-11* required for tissue regeneration, homeostasis and neoblast maintenance.	124
MCM2 (g)	Minichromosome maintenance protein, a DNA replication factor	*DjMCM2* (Dj)	**(E)** Neoblasts	125
PCNA (g/p)	Proliferating cell nuclear antigen, an auxiliary protein to DNA polymerase δ and associated with DNA replication	*Djpcna*/ DjPCNA (Dj)	**(E)** Proliferative cells, neoblasts and germ cells	96
Piwi (g/p)	Member of the PIWI/Argonaute family, a component of the RISC complex in the RNA-interference pathway	*Djpiwi-1* and *-4* (Dj) *Smedwi-1* and *-2* (Smed)	**(E)** Neoblasts, germ cells and at the level of the cephalic ganglia. *Djpiwi-1* marks a neoblast subpopulation along the midline **(F)** *Smedwi-2* required for neoblast differentiation.	122 123 126 127
Pumilio (g)	PUF protein, RNA binding protein	*DjPum* (Dj)	**(E)** Neoblasts and at the level of the cephalic ganglia **(F)** Neoblast maintenance	128
Vasa (g)	An ATP-dependent RNA helicase with DEAD box, component of the chromatoid body	*DjvlgA*, *Djvlg-B* (Dj)	**(E)** Both in germ cells. DjvlgA also in neoblasts and in central nervous system	129

Species abbreviations: Dj, *Dugesia japonica*; Gd, *Girardia dorotocephala*; Ph, *Phagocata* sp.; Smed, *Schmidtea mediterranea.*

counted mitotic cells by staining with lactic-acetic orcein and described an AP gradient in the number of neoblasts undergoing mitosis, with more dividing cells in the pre-pharyngeal area than in the posterior part of the planarian. Recent molecular studies analyzing histone H3 (phosphorylated -S10), a conserved marker of mitosis (46), showed a similar distribution of mitotic cells in *S. mediterranea* (124). Knockdown of *smedinx-11,* which encodes an innexin gap-junctional protein expressed in neoblasts, revealed a striking loss of mitotic activity in neoblasts that occurred in an anterior to posterior direction (125). The authors of that study suggested that smedinx-11 gap junctions regulate the movement of small molecules that control the maintenance, migration and differentiation of the progeny of proliferative neoblasts. Along the DV axis, mitotic neoblasts are distributed in the dorsal and ventral parts of the mesenchyme, as shown by anti-H3P labeling of the mitotic neoblasts, suggesting that the neoblasts migrate to these areas before division (46). In the planarian species *D. japonica* three neoblast markers *DjMCM2*, *Djpcna* and *DjPiwi-1* (Table 1), have been used to define two spatially distinct neoblast populations in the dorsal mesenchyme with a mediolateral distribution: two lateral *DjMCM2* and *Djpcna*-positive domains and a domain containing *DjMCM2, Djpcna* and *DjPiwi-1*-positive neoblasts along the midline (96, 125, 126). Also, most of the genes described as expressed in neoblasts, such as *bruno*, *Djcbc-1*, *piwi*, *pumilio* and *vasa*, are found to be expressed around the brain (122, 123, 128, 129, 134,) (see Table 1). Interestingly this expression domain seems to be irradiation tolerant and could correspond to either non-proliferating determined nerve cells or already differentiated neurons (122, 123, 128 and E. Saló, unpublished data). Even though no actual stem cell niche has been described in planarians, these findings indicate that, in addition to distribution throughout the parenchyma, neoblasts tend to accumulate in domains along the midline and lateral regions in *D. japonica*.

Cytological studies have also been used to divide the neoblasts into subpopulations based on ultrastructural features and cell cycle distribution. By looking at electron microscopy images of neoblasts, Higuchi and co-workers (119) defined a type A and type B neoblast on the basis of the size of the cell, the chromatin structure and the number of chromatoid bodies. Type A neoblasts are larger (9.6 ± 2.9 μm) with more chromatoid bodies (4.4 ± 2.1) than type B neoblasts (size, 6.2 ± 1.4 μm; number of chromatoid bodies, 2.1 ± 0.9); type A neoblasts are also rich in euchromatin, whereas type B neoblasts are rich in heterochromatin. The nucleus-to-cell ratio of type A is smaller than that of type B. Fluorescence-activated cell sorting (FACS) showed that type A cells belonged to irradiation-sensitive dividing cells, whereas type B cells were irradiation-sensitive non-dividing cells (119, 132). The proportion of type A neoblasts including some differentiating neoblasts, type B neoblast, and an X-ray insensitive fraction made up of differentiated cells was estimated at 1:2:6 (132), which is consistent with an earlier estimation of neoblasts representing 20-30% of the cell population (64). Those authors also described a population of differentiating cells with chromatoid bodies and a few organelles that continued to divide (119).

4.2.3. Neoblast division and differentiation

Feeding and regeneration are two physiological

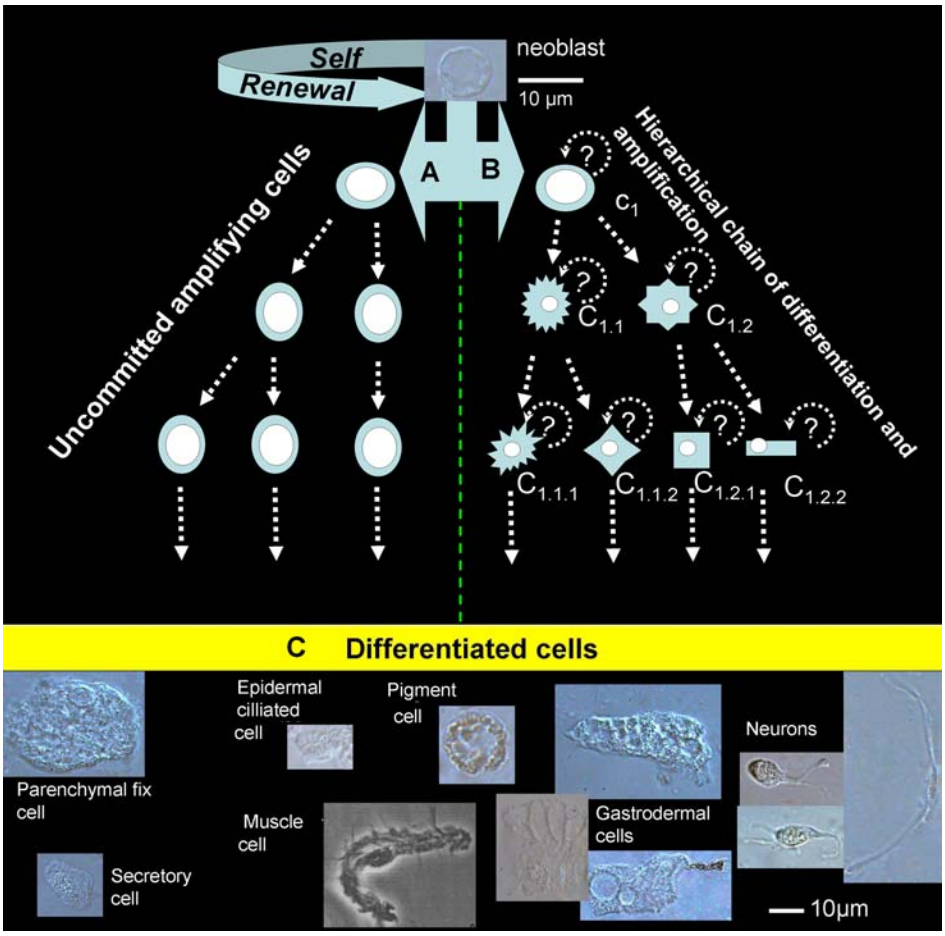

Figure 7. Diagram showing putative neoblast dynamics. Although relatively little is known about this process, self-renewal must take place to avoid depletion of the neoblast pool. However, the division patterns of the neoblasts are unclear. Also the determination and differentiation pathways of the neoblasts are unknown, as are the steps through which neoblast potential is restricted. A number of hypotheses can be proposed to explain this process: (A) A series of proliferation steps may be followed by direct determination. Cell determination would be regulated by signals from the surrounding environment that provide positional information and thus a specific differentiation program. This process would be similar to other regulatory systems based on a "niche microenvironment". (B) Alternatively, different determination states could be organized in a hierarchical chain of gradual differentiation (c_n represents the different commitment steps). These possibilities represent opposite ends of a spectrum of possibilities, and more complex regulation systems involving a mixture of the processes shown in this figure are clearly possible. (C) Some examples of neoblast-derived differentiated cells are shown.

processes that increase the rate of neoblast division. Two peaks have been described in the time course of mitosis after feeding or amputation of the planarian, with the first maximum at 4-12 hours and a second higher maximum at 2-4 days at 17ºC (65, 67, 135). This early first mitotic peak led to the proposal of the existence of a neoblast population in G2 phase ready to enter mitosis (65, 67, 135). However, labeling of neoblasts with BrdU combined with detection of mitotic cells with anti-H3P to study the length of the G2 phase cast doubt on this hypothesis. The median length of the G2 phase was described to be around 6 hours, fast enough to account for the first mitotic maximum (46). Interestingly, recent studies comparing BrdU labeling of neoblasts with the expression pattern of the genes *pcna* and *nanos* in *D. japonica* showed no overlap between the dorsal domains of clusters of *pcna* and *nanos*-positive cells and BrdU-labeled neoblasts (54, 96). These dorsal clusters of cells are believed to correspond to a subpopulation of neoblasts, the germline stem cells. The authors of those studies suggested that these cells might be in cell cycle arrest, supporting the earlier hypothesis that there was a G2 population. Furthermore, in the platyhelminths *Macrostomum* sp a population of slow-cycling neoblasts arrested in G2 has recently been described (136).

Neoblasts are pluripotent and can differentiate into all the planarian cell types. The division pattern and differentiation pathway of the neoblast, however, is still an open question, and it is not clear whether they divide asymmetrically to generate a neoblast and a differentiated cell (Figure 7B) or whether they divide symmetrically, giving rise only to pluripotent cells or only to differentiated

cells depending on the stimuli from the surrounding cells (Figure 7A). Also, the differentiation pathways and the potential of the differentiating neoblasts are unknown. It is not clear whether differentiating neoblasts lose their potential gradually, changing from being pluripotent to multipotent to unipotent (Figure 7B). At least 20 types of differentiated planarian cells have been described (64), some of which are shown in Figure 7C.

Culturing of neoblasts could be a useful tool with which to analyze the factors that regulate the division and differentiation of these cells. Many attempts have been made to culture neoblasts (reviewed in 137), and the most successful have managed to keep the cells alive for up to 6 weeks, during which mitosis was detected during the first 7 days (138).

4.2.4. In search of unique neoblast markers

Probably the most common way to separate and enrich live neoblasts is currently by filtration through a series of nylon filters with a progressively smaller mesh size or by FACS (119, 132). Since no specific surface markers of neoblasts have been described, FACS has to be carried out indirectly by labeling the DNA and cytoplasm of all of the cells with commonly used fluorochromes such as Hoechst 33342 or DAPI to label DNA and calcein AM to label the cytoplasm. When looking, for instance, at DNA concentration based on Hoechst33342/DAPI fluorescence intensity *versus* side scatter, which indicates the amount and type of cytoplasmic granules, at least the dividing neoblasts can be easily separated because they contain twice the amount of DNA. However, this only allows separation of a portion of the total neoblast population. To be able to separate dividing as well as non-dividing live neoblasts by FACS different approaches could be used. Antibodies to unique neoblast cell-surface markers need to be developed, or alternatively, a transgenic planarian in which the promoter of a gene expressed exclusively in the neoblasts directs expression of a fluorescent marker could be generated to allow FACS separation of labeled cells.

Various differential-screening approaches have been used in an effort to identify specific neoblast markers. What many of these approaches have in common is the comparison of wild-type planarians with planarians in which neoblasts have been depleted. Using an oligonucleotide microarray chip (the Dj600 chip) Rossi and coworkers compared wild-type planarians with X-ray irradiated planarians, and in this way they identified putative neoblast-specific genes involved in chromatin modeling, RNA metabolism and transcription, including previously identified genes expressed in neoblasts (Table 1). They further compared these two samples with a sample of planarians exposed to non-lethal low-dose irradiation and identified genes that are important for neoblast cytoprotection, proliferation and migration (139). A similar comparison was carried out in *Macrostomum lignano* (Platyhelminthes, Rhapditophora). Pfister and co-workers (140) made a cDNA library by suppressive subtractive hybridization between cDNA from wild-type *Macrostomum* and cDNA from irradiated animals or planarians treated with hydroxyurea (HU). HU treatment arrests the cell cycle in early S phase and results in neoblast depletion. In that study, no genes with homology to known stem cell or proliferation markers were found, but 54.3% of the genes had no known homologues and could, therefore, contain novel stem cell genes. A recent proteomic study comparing protein extracts from irradiated and non-irradiated organisms and from *smedwi-2* knock-down planarians (127) *versus* control organisms has also revealed candidate genes that may be important for neoblast maintenance or differentiation (Enrique Fernandez-Taboada, Josep Abril, Emili Saló, work in progress). Finally an RNAi screen was carried out with 1065 genes selected from two cDNA libraries derived from 2 to 3-day old blastemas, which are therefore rich in neoblasts, and from planarian heads (141). Of these genes, 22.5% showed external phenotypes when inhibiting the gene. These phenotypes allowed the division of gene function into homeostasis, neoblast proliferation, and blastema differentiation and patterning during regeneration.

Many of the described neoblast markers, such as *piwi, vasa* and *pcna*, which are also expressed in the planarian germline, coincide with known markers of stem cell systems in other organisms such as the germline stem cells of *Drosophila* and mammals. Some markers of pluripotency that have been described in murine embryonic stem cells, such as *oct3/4, sox2* and *nanog*, have not been found in planarians, perhaps because they are rapidly evolving genes. The numerous screens that are currently being undertaken in planarians are very likely to uncover previously unidentified neoblast markers that could be very useful for future stem cell research in general.

4.3. Regeneration *versus* development

Most studies of planarians have been carried out in adult organisms, and only a few have addressed the embryology of the planarian. This is explained by the high regenerative capacity and easy accessibility of the adult planarian; nevertheless, the planarian offers a unique opportunity to compare the molecular and cellular mechanisms used during development and regeneration. A major barrier to research into planarian embryology is the limited access to embryos, as they are surrounded by a massive population of yolk cells, which are in turn covered with a chitinous, dark egg shell (142-144). Therefore it is practically impossible to follow their development *in vivo* and the embryos must be fixed before histological and molecular studies can be carried out.

4.3.1. Neoblasts *versus* blastomeres

Early planarian embryology shows fundamental differences in comparison with the ancestral spiral cleavage pattern described in Platyhelminthes (reviewed in 145). An interesting aspect of planarian embryology is the high degree of similarity between the final stages of development and regeneration of the posterior parts of the planarian, including the pharynx and tail region. The direct development of the triclads into juveniles lacking germ layers and without clear organ primordia facilitates comparison of the mechanisms involved in embryogenesis and regeneration (144). Of course, the starting point is different—the blastomeres are the cell source used during

development, while the neoblasts are used during regeneration. Also, the cells surrounding these cell types and the stimuli that they receive are different. The blastomeres are surrounded by yolk cells and the neoblasts by the regenerating tissue and differentiated cells. However, from around stage 4-5 of development (out of the 8 stages described (144); Le Moigne divided embryogenesis into 7 stages (142)), massive cell differentiation takes place, and it is from this stage onwards that striking parallels can be found between embryogenesis and regeneration (144). Furthermore, it has been proposed that the blastomeres give rise to neoblasts around this stage of development (143, 146) and there is an ongoing discussion about the relationship between these two cell types. Morphologically, the blastomeres are much larger than the neoblasts. They measure 25 μm in diameter and contain a nucleus with uncondensed chromatin. The large cytoplasm is rich in free ribosomes and endoplasmic reticulum and contains 2 or 3 large vesicles. Electron-dense particles are found near the nuclear membrane, and these could be interpreted as chromatoid bodies (144, 147) (for neoblast morphology see section 4.2.1). Since different cell types can be distinguished with molecular markers earlier than stage 5 (146, 147), is the blastomere the real totipotent stem cell of the planarian? It could be speculated that neoblasts are actually determined pluripotent stem cells or possibly that the neoblast population consists of various determined pluripotent or multipotent cell types. Attempts have been made to culture blastomeres, but until now without success (146). To date, only a few studies have compared the molecular mechanisms involved in embryogenesis and regeneration (55, 148).

5. WHAT CAN WE DO WITH PLANARIANS?

Although the first 100 years of research into planarian regeneration has provided extensive insight into the nature of the cells responsible for regeneration and the mechanism by which it takes place, many questions still remain unanswered, such as the division pattern of the neoblasts, the pathways of neoblast determination and differentiation, and the relationship between germ cells and the neoblasts, to name just a few.

5.1. Current knowledge

Although the absence of molecular tools meant that for many years our understanding of planarians was restricted to morphological and indirect observations, many advances were nevertheless made in addressing the cell biology and biochemistry of regeneration in these organisms. More recently, this knowledge has been extended through the efforts of several groups to develop molecular and cellular protocols that would allow further elucidation of the molecular basis of regeneration and stem-cell biology in this system. A large number of genes have been cloned and their expression patterns described, and in a number of cases functional data have also been obtained. Genes have been identified that can be used as markers of neoblasts (Table 1) or differentiated cell types. However, more specific markers of different cell types are needed in order to follow those cells through the process of regeneration and development. The description of genes already known to be important in classical models, such as *piwi* (127), *bruno* (123) and *nanos* (54, 55, 56), has gone some way to improving our understanding of neoblasts, as has the analysis of other known functional characteristics of undifferentiated cells, such as the presence of chromatoid bodies. However, much work still remains if we are to develop a more accurate description of neoblast dynamics, proliferation and differentiation. One hope is that improved methods for neoblast culture in combination with molecular tools will allow greater insights to be gained into the control of neoblast dynamics.

Our understanding of the process of regeneration has been increased substantially by studies using planaria as a model system. However, a clear overall vision of how this process is controlled is still lacking, and future research will be needed to extend and consolidate current knowledge. It is also time to review old topics in the field and revisit them using the technology and experience that we have now; this includes the factors triggering wound healing and regeneration.

5. 2. Available tools

Many things have changed since research into planarians began. The potential of molecular biology and biochemistry has reached unexpected levels, and the availability of bioinformatics tools with which to exploit the data obtained is growing exponentially. Not surprisingly, these technical improvements have had an impact of planarian research. It is now possible to follow the expression pattern of a gene by *in situ* hybridization in the whole animal, in sections or in dissociated cells (125,149, 150,), and also to locate and follow the product of those genes in the same tissues by immunostaining (151, 152). Automated protocols have been developed that allow these techniques to be employed in high-throughput studies (141). Tools for functional analysis are also available, and loss-of-function studies utilizing a variety of approaches are now standard techniques in many labs. These techniques rely on the introduction of a gene-specific double-stranded RNA (dsRNA) probe into the planarian cells. The probe will interact with the endogenous messenger RNA of the gene and with elements of the RNA interference pathway such as Dicer and Piwi, and cause degradation of the mRNA, thereby eliminating the gene transcript. The dsRNA probe can be introduced by injection, feeding or soaking (153-155), and a high-throughput screen has even been undertaken (141), leading to a number of interesting findings (Figure 8 A-G). Gain-of-function approaches have also been successfully applied in *G. tigrina* using the 3xP3 opsin promoter from *Drosophila* (Figure 8 H-L) (59, 158), and this is now being applied to other planarian species. A search for planarian promoters for ubiquitous and cell-specific genes will extend the possibilities of this technique.

Other well-established approaches are also being introduced in planarian research. These include proteomics and microarrays, both of which are being facilitated by the planarian genome project (159). The 800MB planarian genome has been sequenced and an assembly of the 90,000 contigs is currently being carried out at the Washington

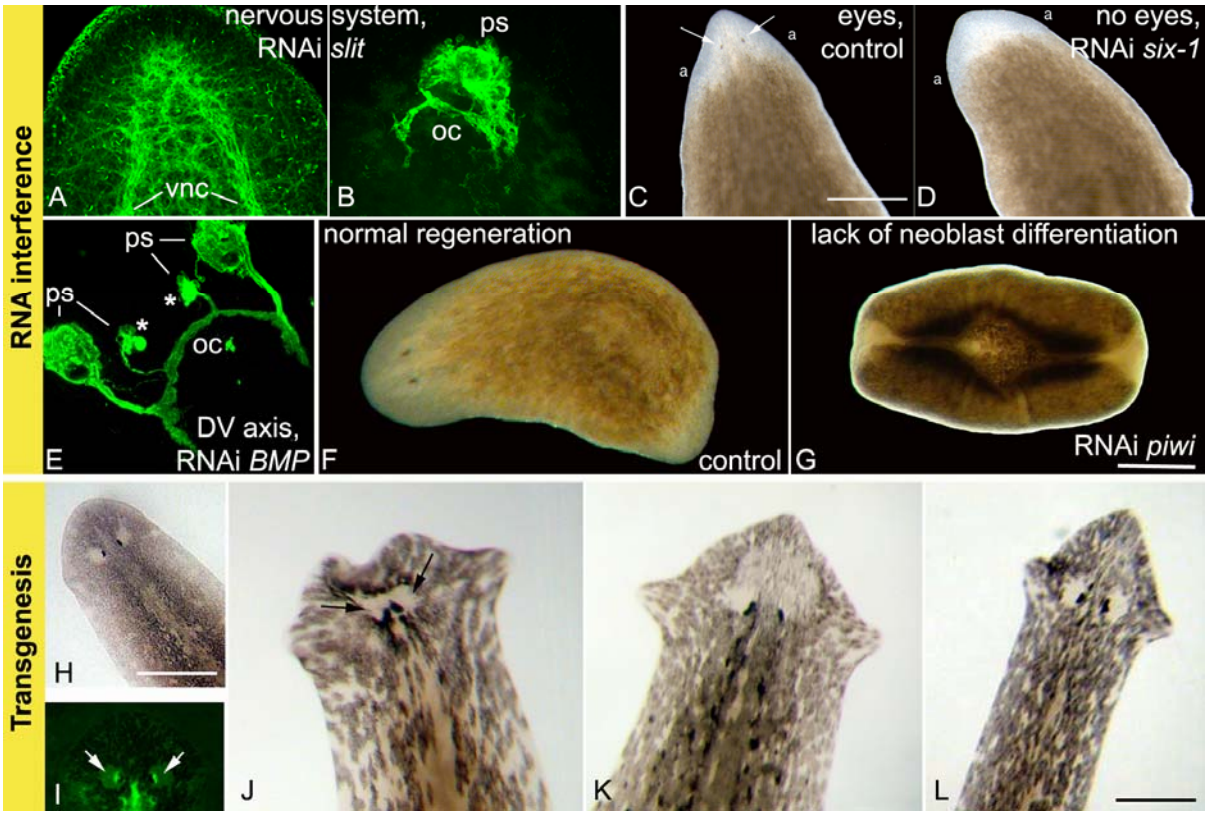

Figure 8. A selection of RNA interference (RNAi) and transgenic phenotypes in planarians. (A-G) RNAi experiments in *Schmidtea mediterranea*. (H-L) Transgenesis in *Girardia tigrina*. (A-B) RNAi of *Smed-slit*, a planarian homologue of the slit family of axon guidance cues. Whole-mount immunostaining with anti-tubulin (A) and anti-VC-1 (B) are shown in 13-day regenerating planarians. (A) Knock-down of *Smed-slit* causes collapse of the regenerated ventral nervous cords (vnc) at the midline (156). (B) Also, the newly differentiated eyes are fused at the midline in *Smed-slit* RNAi-treated animals (156). (C-D) Inhibition of planarian eye regeneration by RNAi of *sine oculis/six-1*, a member of the *six* gene family (157, Kay Eckelt and Emili Saló unpublished data). Bright-field images showing dorsal views of live regenerating planarian heads. (C) Control organisms regenerate the head with differentiated eyes (arrows). (D) Knock-down of *Smedsix-1*, a *six-1* ortholog in *S. mediterranea*. Although the head regenerates normally with a complete brain (not shown) and auricles (a), no eye differentiation can be observed in the white blastema (Kay Eckelt and Emili Saló, unpublished data). (E) Inhibition of *Smed-BMP*, a planarian member of the bone morphogenetic protein (BMP) family, disturbs the dorsoventral (DV) axis. Whole-mount immunostaining with anti-VC-1 in an intact organism 30 days after injection of dsRNA against *Smed-BMP*. Apart from disturbing the DV axis, the formation of extra eyes (*) was observed (107). (F, G) Inhibition of neoblast differentiation by RNAi for *smedwi-2*, a planarian member of the PIWI/Argonaute family. Bright-field images of live 10-day regenerating planarians. (F) Dorsal view of a control organism showing a normal regeneration pattern with the formation of a head with eyes anteriorly and a tail posteriorly. (G) *smedwi-2* is expressed in the neoblasts (not shown). Upon inhibition of *smedwi-2* by injection of dsRNA and pre- and post-pharyngeal amputation of the planarian, a small blastema is formed, and eye differentiation takes place. Soon after, there is a strong regression in the tissues all around the edges, giving the planarian a curly shaped body as shown (ventral view of the planarian). It was suggested that *smedwi-2* is important for the differentiation of the neoblasts (127). (H, I) Transgenic *G. tigrina* generated by electroporation with a 3xP3-EGFP Hermes vector construct (dorsal view, anterior to the top). (H) Bright-field image of the planarian head. (I) Fluorescence image of the transgenic planarian head. Strong EGFP expression is observed in the photoreceptor cells of both eyes (arrows), which is where the 3xP3 opsin promoter is active (158). (J-L) Gain-of-function mutant for *Gtdap-1* generated by electroporation of *G. tigrina* with a Hermes construct containing 3xP3-EGFP-3xP3-Gtdap-1 (59). *Gtdap-1* expression is under the control of Pax6, which activates the 3xP3 promoter (158). *Gtdap-1* is an ortholog of human dead-associated-protein-1. In planarians it has been proposed to play a role in autophagy during remodeling (59). Bright-field images of the planarian head (dorsal view, anterior is to the top). (J) Cell death in and around the eyes of the head region was observed (arrows). (K) After 4-5 days, the damaged tissue is recovered, as can be seen by the brighter less-pigmented tissue. The lack of differentiated eye cells and thus an inactive 3xP3 promoter makes regeneration possible. (L) Complete differentiation of the missing tissue including the eyes has taken place. The 3xP3 promoter and thus the cell-death pathway will be reactivated and the process starts all over again (J to L) (59). a, auricles; oc, optic chiasm; ps, photosensitive cells. Scale bars: (C, D) 1 mm, (F, G) 1.5 mm, (H, I) 2 mm, (J-L) 0.75 mm.

University Genome Sequencing Center (http://genome.wustl.edu/). The genome sequencing project has provided a powerful tool with which to extend research in planarian biology, making almost all of the existing approaches faster and easier, and introducing the possibility of modeling and prediction of genes and domain structures through the use of bioinformatics.

6. PERSPECTIVES

Now that we have entered the so-called post-genomic era, planarian studies have also reached new levels of development. The arrival of new molecular tools and high-throughput methods can now be used to exploit the knowledge acquired during many years of work on regeneration and stem-cell behavior. The outcome of this research will be of use in regenerative medicine and will provide real opportunities for the development of new therapeutic approaches. Examples have already been described of planarian genes involved in the correct organization of regenerating neural tissue (101). Also, since planarians share genes with humans that are not present in the ecdysozoans *Caenorhabditis elegans* and *Drosophila* (159, 160), they offer a good alternative model in which to address unanswered questions. In addition to the obvious medical applications, planarians also offer an excellent model in which to address fundamental biological questions and ultimately develop a more integrated understanding of the regulation and function of stem cells.

7. ACKNOWLEDGEMENTS

Mette Handberg-Thorsager, Enrique Fernández contributed equally to this manuscript. We would like to thank Dr R. Romero, Dr. J. Baguñà and Dr. F. Cebrià for providing literature; Dr. K. Watanabe and Dr. H. Orii for providing VC-1 and DjPCNA antibodies; Dr. J. Baguñà for providing the electron microscopy image for Figure 6; Dr. F. Cebrià, Dr. R. Romero, M. D. Molina, D. Felix, Dra. C. González and K. Eckelt for providing images for Figures 7 and 8; L. Camps and members of the Saló lab for critical reading of the manuscript; Dr. R. Romero and Dr. A. Cardona for discussion of planarian embryology; M. Hilbrant for suggestions to Figure 2; and Dr. I. Patten for editorial advice. This work was supported by grants BCM-2002-03992 and BFU-2005-00422 from the Ministerio de Educación y Ciencia, Spain, and grant 2005SGR00769 from AGAUR (Generalitat de Catalunya, Spain). E.F.T. received an FPI fellowship from the Ministerio de Ciencia y Cultura and M.H-T received an FPU fellowship from the Ministerio de Educación y Ciencia.

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Abbreviations: AP: anteroposterior, AS: adult stem, BMP: bone morphogenetic protein, Dj: *Dugesia japonica,* DV: dorsoventral, ES:embryonic stem, FE: fetal embryonic, Gd: *Girardia dorotocephala,* hES: human embryonic stem, Ph: *Phagocata* sp., Smed: *Schmidtea mediterranea*

Key Words: Regeneration, Stem cell, Planarian, Neoblast, Review

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