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Planarians, a tale of stem cells

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Abstract. Planarians possess amazing abilities to regulate tissue homeostasis and regenerate missing body parts. These features reside on the presence of a population of pluripotent/totipotent stem cells, the neoblasts, which are considered as the only planarian cells able to proliferate in the asexual strains. Neoblast distribution has been identified by mapping the cells incorporating bromodeoxyuridine, analyzing mitotic figures and using cell proliferation markers. Recently identified molecular markers specifically label subgroups of neoblasts, revealing thus the heterogeneity of the planarian stem cell population. Therefore, the apparent totipotency of neoblasts probably reflects the composite activities of multiple stem cell types. First steps have been undertaken to understand how neoblasts and differentiated cells communicate with each other to adapt the self-renewal and differentiation rates of neoblasts to the demands of the body. Moreover, the introduction of molecular resource database on planarians now paves the way to renewed strategies to understand planarian regeneration and stem cell-related issues. (Part of a Multi-author Review)

Keywords. Planarians, neoblasts, somatic stem cells, germ stem cells, X-ray, RNA interference (RNAi), microarray, neoblast sub-populations.

Introduction

Although the phenomenon of regeneration has been known to scientists for over 250 years, students of a biology class are always astonished to see how a hydra or a planaria can be divided in several pieces each of them capable rebuilding a new organism, just right! Even though the complex molecular mechanisms underpinning this process remain largely unknown, it is obvious that fascinating issues such as patterning, tissue polarity and control of size play a role. Biologists are actively focused on ascertaining molecular mechanisms in a variety of model systems that differ in complexity and phylogenetic position and share more or less marked regeneration capabilities. Among those systems ranging from sponges to vertebrates, freshwater planarians (Fig. 1) – simple tripoblastic organisms with bilateral symmetry and cephalization – are a classic model for the study of regeneration [1–7]. In the molecular age this model has been strongly re-proposed as a flexible system to address genetic questions [8–10]. Regeneration in planarians is more than restoration of body parts after a traumatic amputation, and several planarian species choose regeneration as an asexual reproduction strategy. Planarians are indeed able to spontaneously transverse-fission, generating two genetically identical fragments that will regenerate their respective missing structures. No morphological or molecular differences between fissioning and traumatic regeneration have been identified to date. Planarian regeneration potentiality is extreme: every body piece of at least 10000 cells except for the most anterior part of the head and the pharynx is able to rebuild an entire organism [11, 12]. This means that tail fragments can reform a head including a new brain and sensory organs; heads can rebuild a tail and a new pharynx; while central body fragments can reform both a new tail and a new head (Fig. 2). Amazingly,

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lateral fragments can define a new midline for restoring bilateral symmetry. Such terrific potentiality relies on the presence in the planarian body of a population of adult stem cells, the so-called neoblasts. Neoblasts are considered to be totipotent stem cells on the basis of the evidence that the injection of neoblast-enriched cell suspensions into lethally irradiated planarians (i.e. devoid of proliferating cells) results in restoration of the viability and regenerative abilities of the recipient animals [13]. Since their first description, neoblasts have been extensively studied in different planarian species by classical and molecular approaches. The aim of this review is to deal with the different findings on neoblast biology and to suggest future directions for uncovering the secrets of these fascinating cells.

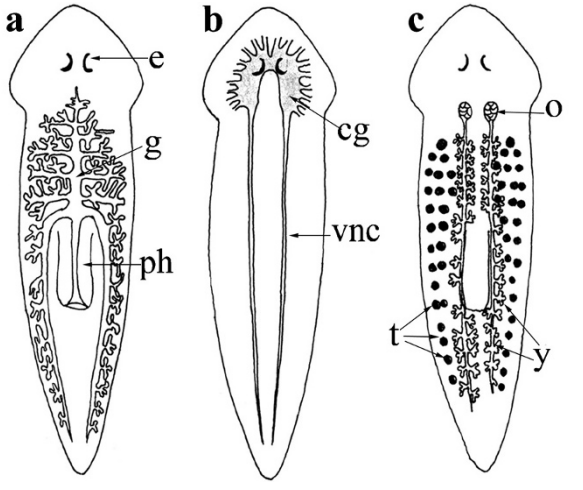

Figure 1. Anatomy of a planaria. (*a*) Digestive system. (*b*) Nervous system. (*c*) Gonads. g, gut; ph, pharynx; cg, cephalic ganglia; vnc, ventral nerve cords; e, eyes; t, testes, o, ovaries; y, yolk glands.

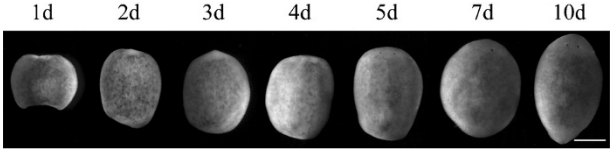

Figure 2. Regeneration in *Dugesia japonica.* Animals have been transected anterior and posterior to the pharynx (bidirectional regenerants). Scale bar, 500 μm. Planarian regeneration is a complex phenomenon resulting from concomitant cell proliferation, *de novo* differentiation and old tissue remodeling. Recent description of the regeneration process can be found in [5] and [50].

Morphological criteria and cellular aspects that define neoblasts

The term 'neoblast' refers to a heterogeneous population of cells characterized by certain morphological features. Neoblasts are small cells (5–10 μm in diameter) with a high nucleo-cytoplasmic ratio which are distributed throughout the planarian parenchyma (Fig. 3a), a mesenchymal tissue with multiple functions [5, 14]. The thin rim of ribosome-rich cytoplasm that surrounds the nucleus is undifferentiated and shows no cellular organelle but free ribosomes and a few mitochondria (Fig. 3b) [15]. Typical structures that are frequently observable in the neoblast cytoplasm are the so-called chromatoid bodies (Fig. 3b), electron-dense aggregates often adjacent to the nuclear envelope and frequently surrounded by mitochondria. The planarian chromatoid bodies resemble the germline granules found in *Drosophila* and *Xenopus* embryos and are today interpreted as ribonuclear particles containing masked transcripts [16] that allow neoblasts to quickly respond to environmental stimuli. Little is known about the nature of transcripts accumulated in the chromatoid bodies, and the planarian homologue (*Djnos*) of the *Drosophila Nanos* gene is the only RNA so far localized in this structure [17]. However, *Djnos* expression is restricted to a neoblast subpopulation (see below), suggesting that the chromatoid body composition could change depending on the neoblast subtype or on the level of cell determination-differentiation [18].

Neoblasts are continuously recruited to replace aged differentiated cells. As a consequence, along with neoblasts, the neoblast progeny (i.e. daughter cells at the early stages of cell lineage, committed cells or cells at early stages of differentiation whose morphological features do not significantly differ from those of neoblasts) can be also detected in the parenchymal tissue. The neoblast population is also defined by a behavioral parameter: the ability to divide. In fact, with the exception of spermatogonia and oogonia, all the cells that exhibit mitotic division in the planarian body are defined as neoblasts. Therefore, neoblasts are considered as the only dividing cells in asexual specimens. As a consequence of this definition, neoblasts are the only cells destroyed by X- or gamma irradiation [19–27]. Recently, a combined approach of X-ray irradiation and fluorescent-activated cell sorting confirmed that the only cells destroyed by irradiation have a neoblast-like morphology and express neoblast markers [28].

In intact planarians the analysis of mitotic figures [29, 30], BrdU incorporation [30] and expression of molecular markers of cell proliferation [21, 23] demonstrates the exclusive parenchymal distribution of neoblasts, which are actually absent in the most anterior part of the head and in the pharynx. The data obtained using these different methods provide evidence of some dissimilarity in the distribution of these cells. In *Dugesia japonica,* the expression of two

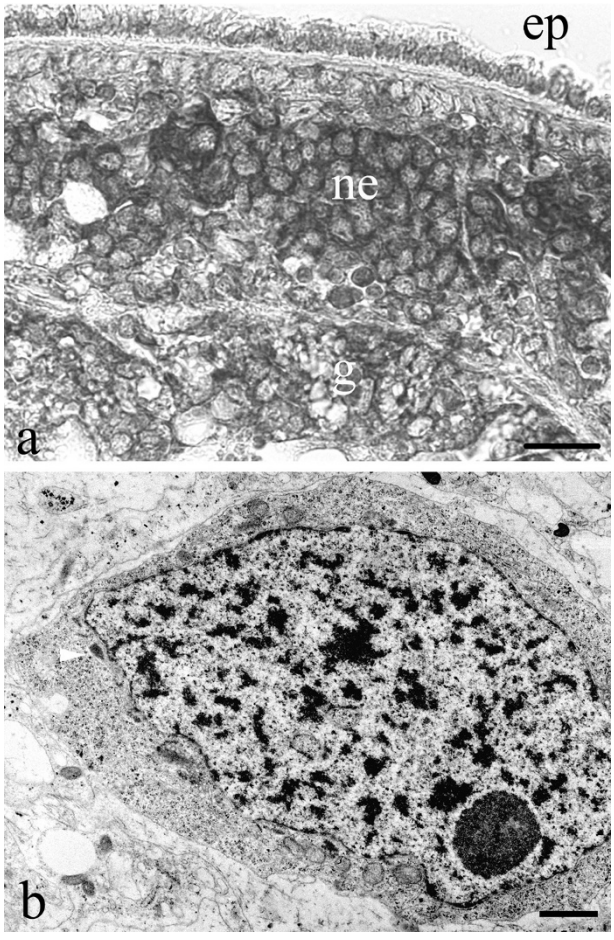

Figure 3. Neoblasts. (*a*) Light microscopy of a methylene blue and toluidine blue-stained cross-section of an intact planarian. Neoblasts (*ne*) are visualized as small cells (5–10 μm) intensely stained in the cytoplasm and located in the parenchyma between the epidermis (*ep*) and the gut cells (*g*). Scale bar, 30 μm. (*b*) Electron microscopy of a neoblast showing undifferentiated cytoplasm rich in ribosomes. *Arrowhead* indicates a chromatoid body. Scale bar, 1 μm.

proliferation-related molecular markers (*DjMCM2* [23] and *DjPCNA* [21]) revealed, in addition to labeled cells with a dispersed distribution, clusters of labeled cells distributed in the dorso-lateral parenchyma and along the midline (Fig. 4a, b, f, g). Whereas the accumulation of MCM2/PCNA-positive cells along the midline at the post-pharyngeal level may correspond to the principal region of parenchymal accumulation (between the two posterior gut branches), the cells clustered along the midline in the pre-pharyngeal region do not correspond to any parenchymal tissue accumulation. On the contrary, these cells reside in a thin region located between the anterior branch of the gut and the epidermis (Fig. 4f). Twenty-three hours after feeding with BrdU, no comparable accumulation of BrdU-positive cells was detected in the dorso-lateral parenchyma or along the midline of *Schmidtea mediterranea*, *Phagocata* sp. and *Girardia dorotocephala* [30]. Moreover, Orii and co-workers provided evidence that in *D. japonica* clustered PCNA-positive neoblasts were not labelled after 6 days of BrdU exposure, although the dispersed PCNA-positive neoblasts of the dorsal and ventral parenchyma were BrdU-labelled [21]. On the basis of this finding, these Authors suggest that the clustered PCNA-positive neoblasts may have a very long cell cycle or that their cell cycle may be arrested. In our opinion this latter hypothesis is the most probable. Indeed, continuous BrdU-labelling experiments demonstrate that the slow-cycling population of neoblasts is not large in *S. mediterranea* [30]. Therefore, the differences observed in neoblast distribution might not reflect species-specific differences but more likely the presence of distinct neoblast populations with different proliferative capabilities. Consequently, the neoblasts can be divided into at least two distinct categories: i) dispersed and actively proliferating, or ii) clustered along the pre-pharyngeal midline and lateral lines and not proliferating (unable to incorporate BrdU).

The molecular age and the identification of distinct neoblast subpopulations

The first tangible evidence for the presence of neoblast subpopulations came up with the identification of a *D. japonica* homologue (*DjPiwi-1*) of the *Drosophila Piwi* gene. *DjPiwi-1* is expressed in a group of small cells preferentially distributed along the dorsal midline anterior to the pharynx (Fig. 4c). *DjPiwi-1* positive cells match the neoblast definition, as in fact they show a neoblast-like morphology and are susceptible to X-ray irradiation [31]. However, *DjPiwi-1*-positive cells do not directly participate in the regeneration process. The role of *DjPiwi-1*-positive cells has not yet been clarified, opening several hypotheses. One possibility is that the *DjPiwi-1*-positive cells play some role in body patterning, as these cells reside in a region for the planarian body, the dorsal midline, which is of crucial importance for patterning and specification of bilateral symmetry [32].

Sato and co-workers succeeded in identifying *D. japonica* germline stem cells that specifically express a *nanos*-related gene (*Djnos*) [17]. In asexual planarians *Djnos*-expressing cells are distributed in the presumptive ovary or testis-forming region (Fig. 4d, e). Specimens of the *S. mediterranea* asexual strain also express *nanos* transcripts in the presumptive ovary or testis-forming region [33, 34]. During sexualization, *Djnos*-expressing cells produce germ

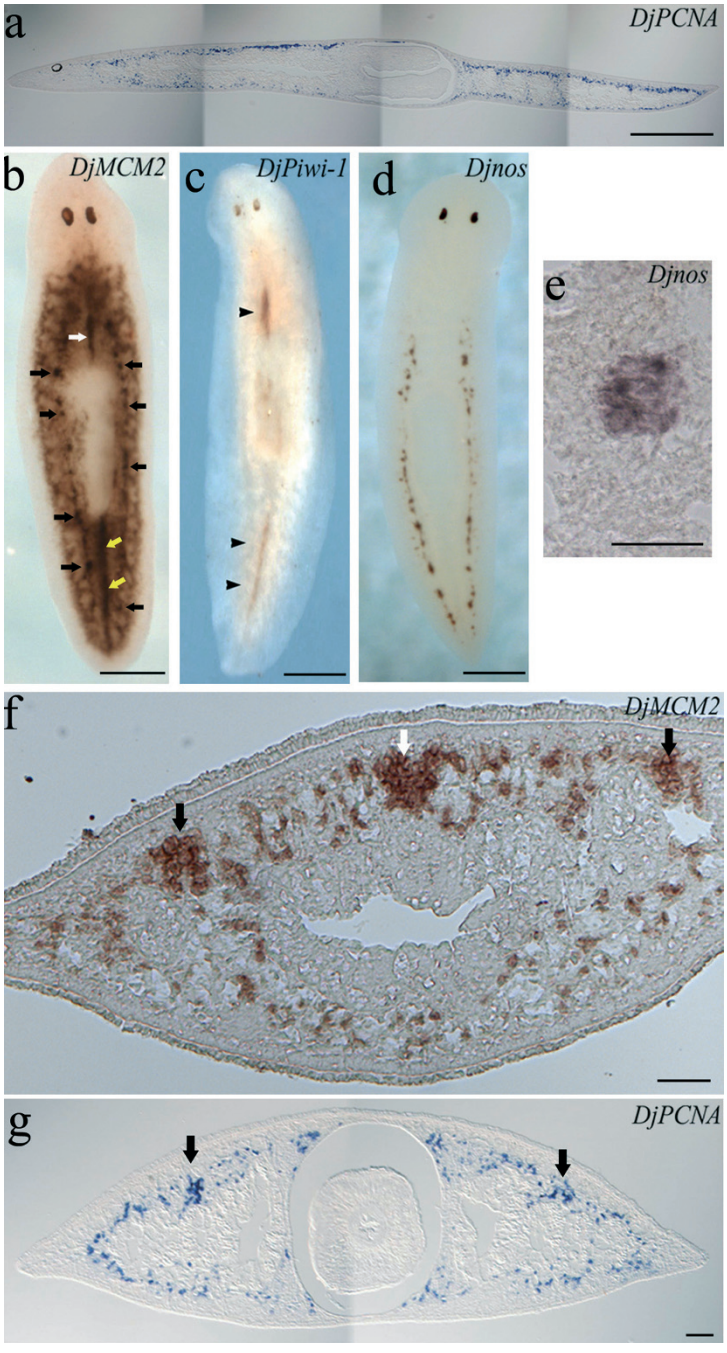

Figure 4. Neoblasts in *Dugesia japonica*. (*a*) *DjPCNA* expression in a longitudinal section of an intact planarian (courtesy of Dr H. Orii). Scale bar, 500 μm. (*b*) *DjMCM2* expression in an intact planarian (dorsal view) as visualized by whole mount *in situ* hybridization. *Black arrows* indicate the dorso-lateral patches; the *white arrow* indicates neoblasts clustered along the dorsal midline anterior to the pharynx; *yellow arrows* indicate the accumulation of neoblasts in the parenchyma between the two main gut branches posterior to the pharynx. Scale bar, 500 μm. (*c*) *DjPiwi-1* expression in an intact planarian (dorsal view) as visualized by whole mount *in situ* hybridization. *Arrowheads* indicate the expression signal accumulated along the dorsal midline. Scale bar, 500 μm. (*d*) *Djnos* expression in an intact planarian (dorsal view) as visualized by whole mount *in situ* hybridization. Scale bar 500 μm. (*e*) *Djnos* expression in a cross section of the pre-pharyngeal region. Scale bar, 35 μm. (*f*) *DjMCM2* expression in a cross-section of the pre-pharyngeal region of an intact planarian. Scale bar, 60 μm. (*g*) *DjPCNA* expression in a transversal section of the pharyngeal region of an intact planarian (a gift from Dr H. Orii). *Black arrows* indicate the dorso-lateral patches; the *white arrow* indicates the dorsal clustered neoblasts along the midline anterior to the pharynx. Scale bar, 60 μm.

cells, indicating that these cells are kept as germline stem cells even in the asexual state. Interestingly, the germline stem cells are morphologically indistinguishable from the neoblasts but do not seem to contribute to the regeneration process at all. *Djnos*-positive cells actually accumulate the S-phase marker *DjPCNA* but do not incorporate BrdU [17]. These results suggest that the cell cycle of *Djnos*-positive cells is arrested in asexual planarians.

As further support for this hypothesis, it has been found that *Djnos*- and *DjMCM2*-positive cells clustered in the dorso-lateral region survive longer in planarians exposed to either low- (5Gy [our unpublished results]) or high-doses of X-rays [30]. The low-dose X-ray treatment also sheds light on the presence of an additional population of cells localized in the ventral parenchyma. These cells tolerate 5Gy X-ray and allow the irradiated animal to survive as, after irradiation, they proliferate, migrate and repopulate the planarian body, rescuing other populations [A. Salvetti et al., unpublished].

In addition to molecular marker-driven identification of neoblast subpopulations, the recent application of FACS technology to the study of planarian neoblasts distinguished two different populations, named X1 and X2, of X-ray sensitive cells on the basis of nuclear DNA content and cytoplasm volume [22, 28]. Ultrastructural studies of X1 and X2 cell fractions revealed the presence of two neoblast subtypes (Type A and B) that differ in size, chromatin organization and number of chromatoid bodies [35]. The Authors suggest that the type B cells (smaller in size, with a higher nucleus/cytoplasm ratio, a large amount of chromatin and few chromatoid bodies) could represent a new class of stem cell, which is in the G0 state.

Thus, the apparent totipotency of neoblasts probably reflects the compound activities of multiple types of stem cells. Pioneering evidence of such plasticity was described by Gremigni and co-workers [36–38] who provided a strong argument for the transdetermination of young germ cells into somatic cells during planarian regeneration. They used a strain of *Dugesia lugubris s.l.* in which the somatic cells are triploid, the female germ cells hexaploid and the male germ cells diploid. This mixoploidy made it possible to follow the fate of the germ cells and to demonstrate that both very young male and female germ cells can contribute to the regeneration of somatic tissues.

Maintenance versus differentiation

Neoblasts can indefinitely renew themselves as well as differentiate into any specialized cell of the body, thus representing an exceptional model system to study molecular mechanisms that regulate stem cell behavior *in vivo*. The capability of stem cells to maintain their undifferentiated state is a critical parameter for the comprehension of several biological phenomena, such as embryonic development, tissue remodeling and neoplastic transformation. Regulatory molecules that operate as translational regulators act, in addition to the transcriptional operating system, in defining the intrinsic molecular program of a stem cell [39]. Up to now, two of these post-transcriptional regulators have been studied in planarians: *DjPum,* the planarian homologue of the *Drosophila Pumilio* gene, and *bruli,* a *Bruno-like* gene, identified in *D. japonica* [24] and *S. mediterranea* [19], respectively. They appear to act in a similar fashion, being both required for stem cell maintenance. *DjPum* and *bruli* are both expressed in the parenchyma, with a pattern that resembles that of other neoblast markers, and in the central nervous system. In regenerating animals, *DjPum* and *bruli* transcripts accumulate in the post-blastema area. After X- or gamma-radiation, transcripts of these genes are no longer detectable in the parenchyma, while their neuronal expression remains unaffected. Planarians amputated after *bruli* RNAi show regeneration defects and start to die 6 days after transection. However, *bruli* RNAi animals die slower than specimens treated with dsRNA designed to silence genes interfering with the cell-cycle progression. Interestingly, *bruli* RNAi animals initiate the regeneration process normally, giving rise to slightly smaller blastemas after the first 5 days of regeneration. However, 9 days after transection, regeneration stops and the blastema begins to regress, suggesting that bruli protein is not required for blastema formation or the neoblast response to the wound but rather for some other aspect of neoblast function [19]. Thanks to the use of several neoblast markers, these Authors demonstrated that along with the regeneration defects, *bruli* RNAi animals showed a severe reduction in the number of neoblasts, although the neoblast loss is slower than that observed after gamma-radiation. According to these findings the Authors concluded that in the absence of bruli protein, neoblasts can divide and differentiate, but lose the ability to self-renew, resulting in the gradual depletion of the neoblast population.

DjPum RNAi animals also show a significant inability to regenerate, although this result can be detected only during the second regeneration round. In fact, the majority of the animals transected after *DjPum* RNAi complete regeneration, but are totally unable to regenerate after a second amputation. The stem cells are dramatically reduced at the end of the first regeneration round and almost completely absent 5 days after the second transection, leading to animal death 20–30 days after the second transection [24]. Taking in mind the species-specific differences between *D. japonica* and *S. mediterranea,* the *bruli* and *DjPum* RNAi effects actually appear very similar since the silencing of both genes results in a severe reduction in the number of neoblasts: the remaining neoblasts are sufficient to produce a normal first-regeneration round in *DjPum* RNAi animals, while they only allow the formation of a small blastema in *bruli* RNAi animals. Thus, the proposed function in neoblast self-renewal for both of these genes appears the most plausible.

In contrast to *bruli* and *DjPum,* the *S. mediterranea Piwi* homologue (*smedwi-2*) is not primarily needed for neoblast maintenance but rather for the production of neoblast progeny capable of replacing either aged differentiated cells during homeostasis or missing tissues during regeneration. As a secondary effect *smedwi-2* RNAi animals also lose their neoblasts, probably because of their failure to quench an ever-increasing demand for differentiated cell replacement

[22]. Further detailed studies on neoblast regulatory genes are necessary to understand how these cells and the differentiated tissues communicate with each other to properly adapt their self-renewal/differentiation rate to the demands of the body. A first move toward the identification of candidate regulatory genes (see below) to be studied in depth has been taken via a large-scale RNAi screen [40] and a transcriptional profile analysis of neoblasts [41].

The genomic era

The introduction of a molecular resource database for studying stem cells and regeneration in planarians [42–44] paved the way to renewed strategies to decipher planarian regeneration and stem cell-related issues. Reddien and co-workers [40] launched a systematic gene perturbation system, combining non-redundant cDNAs from *S. mediterranea* and the RNAi by feeding methodology. They screened 1065 genes and found that RNAi of 240 of them generated abnormal phenotypes. To further classify among the 240 genes those with candidate function in regeneration, Reddien and co-workers focused their attention on phenotypes perturbing the events that follow the wounding and produce functional regenerated planarians. For example, to identify genes involved in the initiation of regeneration (i.e. genes identifying signaling mechanisms that specifically activate neoblasts following wounding), they sorted among all the genes causing regeneration defects those unable to perturb the normal tissue homeostasis of intact planarians. Among those the Authors identified SMAD4 as a gene necessary for any blastema formation but dispensable for neoblast function in homeostasis. Since SMAD mediates TGF (transforming growth factor)-beta signals [45], the Authors suggest that TGF-beta signaling may control regeneration initiation in planarians [40]. A more complicated issue was to distinguish between genes involved in neoblast maintenance or proliferation and those affecting neoblast progeny. Thus, the Authors counted the number of mitoses after RNAi, compared the effects induced by gamma-irradiation with the obtained phenotypes and sorted these data according to the blastema phenotypes observed after RNAi. In conclusion, they identified three classes of genes: I) genes putatively involved in neoblast maintenance or mitosis whose RNAi-mediated silencing reduces the mitoses number, inhibits blastema formation and produces a phenotype similar to that of irradiated planarians; II) genes influencing the progression of mitosis whose RNAi-mediated silencing increases the mitoses number and inhibits or reduces regeneration; III) genes involved in regeneration initiation or needed for the functioning of neoblast progeny whose RNAi silencing affects regeneration without significant changes in the number of mitoses. A different approach has recently been applied in our laboratory.

Taking advantage of the unique possibility to selectively eliminate neoblasts by X-ray treatment, we analyzed the molecular machinery that regulates neoblasts by comparing the transcriptional profile of planarians exposed to lethal doses of X-rays (30 Gy) with that of untreated wild-type worms (controls). To this end we designed and validated a brand new high-throughput oligonucleotide microarray platform containing 600 selected sequences enriched for genes putatively involved in stem-cell-related processes [41]. Class comparison analysis between the 30 Gy group and the untreated controls identified 60 differentially expressed genes. Most of these genes were selectively downregulated after treatment. Although the reduction of their expression could be taken as a specific consequence of the X-ray treatment, genes silenced when neoblasts are selectively destroyed have a high probability of being neoblast-specific, highlighting thus a possible neoblast signature. Among those, the genes known to be selectively expressed in stem cells, such as *DjPiwi-1, 2* and *3* [31], *DjMCM2* [23], *DjPCNA* [21], *DjVLGB* [46] and *DjHSP60* [unpublished] could be found. Most genes of the neoblast signature belong to RNA metabolism and chromatin modeling functional categories and appear interconnected in related pathways involved in post-transcriptional regulation and epigenetic modification. The neoblast signature genes, as well as the genes involved in neoblast regulation identified through RNAi, represent new tools to further investigate the various neoblast subpopulations and their respective roles in maintenance and/or differentiation processes.

Conclusions

The availability of a molecular resource database [42–44, 47], the sequencing of the genome of *S. mediterranea,* the optimization of genetic interference tools [48, 49], the successful isolation of a pure neoblast cell fraction [28] and the production of specific gene platforms for transcriptional profile analysis [41] allow planarians to be included among the elected model systems for studying regeneration in the genomic era. With this scenario and due to their strategic phylogenetic position, planarians offer the possibility to collect information about the genetic circuitry involved in stem cell biology. The trans-

lation of this information to higher organisms might allow a better understanding of the mechanisms that enable stem cells to self-renew or undergo differentiation.

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