The planarian flatworm: an in vivo model for stem cell biology and nervous system regeneration

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Planarian flatworms are an exception among bilaterians in that they possess a large pool of adult stem cells that enables them to promptly regenerate any part of their body, including the brain. Although known for two centuries for their remarkable regenerative capabilities, planarians have only recently emerged as an attractive model for studying regeneration and stem cell biology. This revival is due in part to the availability of a sequenced genome and the development of new technologies, such as RNA interference and next-generation sequencing, which facilitate studies of planarian regeneration at the molecular level. Here, we highlight why planarians are an exciting tool in the study of regeneration and its underlying stem cell biology in vivo, and discuss the potential promises and current limitations of this model organism for stem cell research and regenerative medicine.

Introduction

Neurodegenerative and cardiovascular diseases, as well as stroke, infection and injury, require therapies that aim to replace lost, damaged or inoperative tissues. Regenerative medicine is therefore a major focus of medical research. Whereas regeneration in humans is limited, several vertebrates, such as salamanders and fish, can regenerate amputated body parts with high efficiency (reviewed in Stoick-Cooper et al., 2007). The master of regeneration is, however, the planarian flatworm.

Planarians are free-living Platyhelminthes that can regenerate any part of the body, including the central nervous system (CNS). In addition to Dugesia japonica and Girardia tigrina, Schmidtea mediterranea is one of the most commonly used species in planarian research. This freshwater planarian is small in size (0.1-2 cm), has a diploid genome of about 800 Mb distributed on four chromosomes, which accounts for about 30,000 predicted genes (Cantarel et al., 2008), and can reproduce sexually as well as asexually by fission. The regenerative abilities of planarians depend on a large population of somatic stem cells (reviewed in Handberg-Thorsager et al., 2008). This feature, which, among bilaterians, is unique to planarian flatworms, means that planarians can serve as an in vivo Petri dish for the study and manipulation of stem cells in their natural environment.

In recent years, the unique properties of planarians, combined with the development of new technologies and the genome sequencing of S. mediterranea (http://genome.wustl.edu/genomes), have sparked planarian research. The application of RNA interference (RNAi) for gene-specific knockdown in planarians (Sanchez Alvarado and Newmark, 1999; Newmark et al., 2003) allowed identification of several genes and signal transduction pathways that regulate different aspects of regeneration, such as polarity and patterning, and stem cell proliferation, maintenance and differentiation (Guo et al., 2006; Oviedo et al., 2008; Adell et al., 2009; Rink et al., 2009; Felix and Aboobaker, 2010; Fernandez-Taboada et al., 2010; Scimone et al., 2010). The amenability to efficient RNAi treatments, rapid development of clear phenotypes and established cell biological readouts, combined with new post-genomic technologies, make planarians an outstanding tool for gene discovery and can reveal unidentified functions of known and unknown genes involved in human regeneration, development and disease. Table 1 summarises several planarian genomic regions that have significant similarity to human disease-related genes.

In this Primer article, we review the state-of-the-art of planarian research, focusing on stem cells, neural regeneration and re-establishment of polarity, and discuss how the knowledge gained from planarian research might be translated to higher organisms. We aim to bring the attention of the broader scientific community to these amazingly plastic animals as a promising model organism for the rapidly progressing fields of regenerative medicine and bioengineering.

Studying planarian regeneration: insights into how polarity is re-established

Freshwater planarians can perform all manner of amazing tricks when it comes to regeneration. Thomas Hunt Morgan was one of the first people to systematically study planarian regeneration in the late 19th century. Inspired by the observations of Harriet Randolph, he defined the minimal size of a fragment capable of regeneration as 1/279th of the intact animal’s volume (Morgan, 1901). Morgan and others were well aware of the ‘problem’ of polarity during animal regeneration: if an animal capable of regenerating is transversely amputated, a
new head or anterior region develops from the anterior-facing wound, whereas a new tail or posterior region regenerates from the posterior-facing wound. As cited by Morgan (Morgan, 1901), Allman was the first to give the name of ‘polarity’ to this phenomenon (Allman, 1864). It was known that polarity reversal (two-headed or Janus head) in planarians could occur following amputation unless the polarity of the piece is sufficiently strong to overcome this tendency, and cause a tail to regenerate” (Morgan, 1904).

Planarians also possess dorsoventral (DV) polarity. Recently, the bone morphogenetic protein (BMP) pathway was shown to be essential for the re-establishment of the DV axis. Inactivation of this pathway leads to a dorsal-to-ventral conversion, accompanied by the duplication of the CNS (Molina et al., 2007; Orii and Watanabe, 2007; Reddien et al., 2007; Orii and Watanabe, 2007). In some cases, a second planarian forms out of the dorsal side of the treated animals, similar to a Siamese twin (Molina et al., 2007; Orii and Watanabe, 2007; Reddien et al., 2007).
et al., 2007). Thus, similarly to other invertebrates, the BMP pathway specifies the dorsal identity in planarians.

Recent research is helping to answer long-standing questions regarding planarian polarity (Meinhardt, 2009; De Robertis, 2010). For example, additional elements related to the Wnt/β-catenin and Hh pathways involved in AP polarity have been characterized (Adell et al., 2009; Petersen and Reddien, 2009; Rink et al., 2009; Yazawa et al., 2009; Gurley et al., 2010). Moreover, the block of gap-junction-mediated communications, coupled or uncoupled with the disruption of the nervous system continuity, results in the alteration of the AP polarity of regenerating planarian fragments (Nogi and Levin, 2005; Oviedo and Levin, 2007; Oviedo et al., 2010). Thus, one of the next challenges in the field is to integrate the functions of the signalling pathways with long-range neural cues and gap-junction-mediated patterning signals in order to understand how the AP and DV axes are determined during regeneration (Forsthoefel and Newmark, 2009; Adell et al., 2010). A second important issue to be addressed is to determine how stem cells respond to the activation of the different signalling pathways and how they interpret them to generate their progeny in the correct spatial and temporal manner.

The functional conservation of signal transduction pathways – exemplified by the roles of Wnt/β-catenin and BMP pathways in axis polarity in both planarians and vertebrates – offers the opportunity to study mammalian development and disease using planarian regeneration as a model.

**Planarians: performing artists of nervous system regeneration**

Owing to the presence of chemo-, mechano- and light-sensory neurons that are assembled with others into a CNS, planarians react rapidly to environmental conditions, contact and light (reviewed in Cebríà, 2007; Agata and Umesono, 2008). Two cephalic ganglia constitute a bi-lobed brain, which is connected to a pair of nerve cords that extends ventrally from the head to the tip of the tail and that sends projections to nearly all regions of the body (Fig. 1A). Although relatively simple at the morphological level, the planarian CNS displays a much higher complexity when its molecular compartmentalization (Umesono et al., 1999; Cebríà et al., 2002b) and the degree of conservation with vertebrate CNS genes is considered (Mineta et al., 2003). Amazingly, whereas the mammalian CNS is highly limited in its regenerative abilities, planarians structurally and functionally recover damages to their nervous system in only a short time. Even more spectacularly, they can regenerate a whole brain de novo within a few days.

Planarian brain regeneration can be divided into three major processes (Cebríà et al., 2002c). Inside the newly formed blastema, a brain rudiment can be detected within 1 day after head amputation (step 1; Fig. 1B). This rudiment grows and develops into the properly patterned brain (step 2). In the third step, old and new nervous tissues connect, and function is recovered after 4-7 days (step 3). As regeneration proceeds, different sets of genes are expressed that promote proper re-growth, patterning and functional recovery of the planarian brain. One of the early proteins that is activated during brain regeneration is the conserved fibroblast growth factor receptor (FGFR)-related protein Noudarake (Ndk). Ndk is expressed in the head region of the animal and is pivotal for positional identity of the brain. RNAi against Ndk leads to FGFR-dependent ectopic brain formation throughout the animal, suggesting that Ndk determines the location of brain tissue development through capturing a putative FGF-like molecule, thereby restricting brain formation to the head region (Cebríà et al., 2002a). However, whether an FGF-like molecule is required for brain formation remains to be determined.

Another conserved family of proteins involved in brain regeneration is the netrin family. These proteins act as chemoattractants or chemorepellents and are essential for neuronal network formation. RNAi against planarian netrins and netrin receptors results in a disorganized meshwork of axonal projections and interrupted growth of visual axons, suggesting that these proteins act as axon guidance signals during regeneration (Cebríà and Newmark, 2005). Slit, a member of another conserved family of axon guidance cues, is also crucial for axon guidance in the planarian brain.
planarians (Cebrià et al., 2007), as previously shown in other organisms (Long et al., 2004; Dickson and Gilestro, 2006).

Recent studies have also uncovered the role of *Smed-Wnt5* in planarian neural regeneration. Released from cells along the CNS and body edge, SMED-WNT5 protein seems to restrict axonal growth and assist integration of new and existing neuronal networks, because RNAi against *Smed-Wnt5* leads to the expansion of the brain and disconnections along the ventral nerve cords (Adell et al., 2009; Gurley et al., 2010). How the different axon guidance cues are regulated, and how they act together, possibly in combination with uncharacterized proteins and microRNAs (Friedlander et al., 2009; Lu et al., 2009), is currently unclear.

The reasons why planarians can regenerate an entire brain whereas humans struggle to repair a single axon is still obscure. Data on intrinsic and extrinsic inhibitors of axonal regeneration in humans is still minimal (for a review, see Sun and He, 2010), and just as little is known about the factors that facilitate this process in planarians. However, owing to the relatively high degree of conservation between neural proteins in planarians and vertebrates (Mineta et al., 2003), planarian research should help to better understand the regulatory programmes that control axon regeneration and circuitry re-formation, and might lead to the ability to activate the competence of mammalian neuronal networks to regenerate after damage- or disease-caused neurodegeneration.

**Pluripotent stem cells: planarians as a Petri dish**

The classical definition of a stem cell – a cell that can indefinitely self-renew and maintain...
the ability to differentiate into a diverse range of specialized cell types – encompasses the important concept of ‘potency’, which describes the capacity of a stem cell to differentiate into specialized progeny (Schiöler, 2007). Foetal and adult (or somatic) stem cells are usually multipotent (can give rise to a limited number of cell types), whereas inner blastomeres of blastocysts and their in vitro counterparts [cultured embryonic stem (ES) cells] are pluripotent and can differentiate into all three germ layers (Tiedemann et al., 2001). Pluripotency is maintained in mammalian ES cells by what is referred to as the ‘pluripotency network’, a transcriptional regulatory circuitry, integrated by external signalling pathways, that specifies an ES-cell-specific gene expression (Boyer et al., 2005; Mathur et al., 2008).

Owing to their ability to differentiate into various cell types, stem cells hold great potential for regenerative medicine. Although somatic stem cells are widely used in medical therapies (Stocum, 2001), the powerful pluripotent ES cells have not been of clinical use thus far owing to the associated high risks of teratoma formation, as well as legal and ethical concerns. The recently developed induced pluripotent stem (iPS) cells, which are derived from terminally differentiated cells by overexpressing a specific combination of transcription factors (Takahashi and Yamanaka, 2006), seem to bypass these controversies. These cells can be patient-specific, derived without the need for viral integration (Si-Tayeb et al., 2010) and are claimed to have the same potential as ES cells to treat a wide variety of diseases. iPS cells, however, also share the same drawbacks as ES cells: the differentiation process must take place in vitro (with several limitations, above all chromosomal and epigenetic instability) (Humphreys et al., 2001) and the transplanted cells must be devoid of pluripotent cells to prevent the formation of teratomas. A possible alternative to overcome these problems is to therapeutically stimulate resident progenitor cells to promote and enhance the endogenous regenerative process, as in the case of the TWEAK-dependent activation of oval cells in the liver (Jakubowksi et al., 2005) or the Tβ4-induced restoration of multipotency in adult epicardial cells (Smart et al., 2007). In order to develop this strategy to its full potential for future clinical applications, it is pivotal to obtain a clearer understanding of the molecular mechanisms that underlie every aspect of stem cell biology in vivo. Because these studies are relatively limited in mammalian models, planarians and their pluripotent stem cells represent a suitable complementary system for dissecting relevant molecular details.

Planarian adult stem cells (pASCs), historically referred to as neoblasts, are small (5–10 μm diameter) undifferentiated cells that localize in the parenchyma, a loose tissue that lies beneath the muscle layers and surrounds the organs. Although definitive proof of their pluripotency has not yet been provided, planarian neoblasts are regarded as adult pluripotent stem cells (reviewed in Shibata et al., 2010) and are responsible for the remarkable regeneration ability shown by these animals (Saló and Baguñà, 1984; Rossi et al., 2008) (Fig. 2A).

As is the case for other metazoans, a typical pluripotency network has not been characterized for planarian stem cells. However, pASCs possess their own molecular signature, a set of irradiation-sensitive genes (Table 2) that have different roles in the regeneration process (Fig. 2B). Most of these genes are RNA-binding proteins (Rouhana et al., 2010); at least three of them [D. japonica Cbc-1 (DjCbc-1), Schmidtea polychroa Tud-1 (Splotud-1) and Smed-SmB] localize to a ribonucleoprotein granule called a chromatoid body that is typically found in stem cells, germ cells and neurons (Coward, 1974; Yoshida-Kawashita et al., 2007). Remarkably, transcription factors that are specific to pASCs have not yet been identified, although genes involved in other aspects of neoblast biology were described, such as the chaperone Djmot and the chromatin-remodelling factors Smed-CHD4 and DjRbpA48, which are needed for planarian stem cell viability and differentiation, respectively (Conte et al., 2009; Bonuccelli et al., 2010; Scimone et al., 2010).

There is also evidence to suggest the existence of different stem cell subpopulations in planarians. Although germine stem cells are morphologically indistinguishable from the neoblasts (Coward, 1974), they express the germ-cell marker nanos, which localizes to the gonads, or to the gonadal primordia in asexual strains (Sato et al., 2006; Handberg-Thorsager and Salo, 2007; Wang et al., 2007). Moreover, although previous reports indicated that all pASCs cycle regularly (Newmark and Sanchez Alvarado, 2000), it has recently been suggested that there is a subpopulation of G0-arrested stem cells in the planarian species D. japonica (Higuchi et al., 2007). In the same species, the argonaute family gene DjPiwi-1 was found to be expressed by a subpopulation of neoblast-like cells that are preferentially located at the anterior dorsal midline (Rossi et al., 2006). Together with the different levels of radiotolerance displayed by pASCs exposed to low-dose irradiation (Salvetti et al., 2009), this suggests the existence of distinct stem cell subpopulations in D. japonica.

Although they differ in several respects from mammalian ES cells, pASCs offer a unique opportunity to study pluripotent stem cells and their contribution to the regenerative process in vivo in an intact adult animal.

**Planarian research community: hopes and efforts**

Although planarians are a promising model system for in vivo stem cell biology and nervous system regeneration, we are still in the fledgling stage in understanding the molecular principles governing the associated regulatory mechanisms. This is largely owing to the absence of important techniques, such as the generation of transgenic animals and maintenance of planarian stem cells in culture, but also to the lack of manpower. Until 5 years ago, few laboratories worldwide worked on planarian regeneration research. However, the exploitation of RNAi and sequencing technologies have now attracted many new researchers from different areas of biology, and the number of planarian laboratories has exploded within a short time. Thus, to generate a collaborative research community to drive planarian research forward, EuroPlanNet (www.europlannet.org) was launched during the 1st International Meeting on Planarian Biology (IMPB), held in May, 2010 in Münster, Germany. As with other scientific communities, the joint efforts of the network are likely to succeed in tackling current problems and overcoming existing technical limitations. Immediate goals of the network are to develop missing techniques and to create an open-access database to accommodate integrated genomic and transcriptomic datasets. The network will also provide the planarian community with common infrastructures and a student exchange programme. These innovative actions will be essential for
Table 2. Planarian genes involved in stem cell biology

<table>
<thead>
<tr>
<th>Planarian gene</th>
<th>Homologous human protein (accession no.)</th>
<th>Expression pattern</th>
<th>RNAi phenotype (regeneration)</th>
<th>Proposed function in planarian stem cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Djp1i-1</td>
<td>HIWI (Q96J94)</td>
<td>Dorsal midline</td>
<td>N.D.</td>
<td>Unknown</td>
<td>Rossi et al., 2006</td>
</tr>
<tr>
<td>Smediwi1</td>
<td>HIWI (Q96J94)</td>
<td>Parenchyma</td>
<td>No phenotype</td>
<td>Unknown</td>
<td>Reddien et al., 2005</td>
</tr>
<tr>
<td>Smediwi2</td>
<td>HIWI (Q96J94)</td>
<td>Parenchyma</td>
<td>Blastema formation, then regression; death in 2-3 weeks</td>
<td>piRNA expression; stem cell differentiation</td>
<td>Reddien et al., 2005; Palakodeti et al., 2008</td>
</tr>
<tr>
<td>Smediwi3</td>
<td>HILI (Q87C59)</td>
<td>Parenchyma</td>
<td>Reduced blastema formation, then regression; death in 3 weeks (after three rounds of RNAi and amputation)</td>
<td>piRNA expression; stem cell differentiation</td>
<td>Palakodeti et al., 2008</td>
</tr>
</tbody>
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Other RNA-binding proteins

| DJ/1gA         | DDX3X (Q000571)                          | Germ cells, parenchyma, blastema | N.D.                        | Post-transcriptional regulation | Shibata et al., 1999 |
| Djpum          | PUM2 (Q8TB72)                            | Parenchyma (strongly expressed in the posterior dorsal stripe), brain | Reduced blastema formation; death in 3-4 weeks (after two rounds of RNAi and amputation) | Post-transcriptional regulation | Salvetti et al., 2005 |
| Smed-Bruli     | TNRC4 (Q98TF3)                           | Parenchyma, brain | Reduced blastema formation, then regression; death in 3 weeks | Stem cell self-renewal | Guo et al., 2006 |
| Smednos/ Djos  | NANOS1 (Q8W4Y1)                          | Primordial germ cells, spermatagonia, oogonia, eye precursor cells | No regeneration of gonads and copulatory apparatus | Zinc-finger-containing RNA-binding protein; germline specification | Sato et al., 2006; Handberg-Thorsager and Salo, 2007; Wang et al., 2007 |
| Spoltud-1      | TDRD gene family                         | Ovaries, testes, CNS, parenchyma | Regeneration defects; loss of stem cells (after 4 rounds of RNAi and amputation) | Chromatoid body component; stem cell long-term self-renewal | Solana et al., 2009 |
| Djbc-1         | RCK (D17532)                             | Parenchyma, brain, germline stem cells | No phenotype | Chromatoid body component | Yoshida-Kashikawa et al., 2007 |
| Smed-SnB       | SMB (P14678)                             | Parenchyma, brain | No blastema formation; loss of stem cells; death in 2 weeks | Chromatoid body component; stem cell self-renewal | Fernandez-Taboada et al., 2010 |

DNA replication

| Djmc2          | MCM2 (P49736)                            | Parenchyma (strongly expressed in the dorsal midline) | N.D.                        | DNA replication | Salvetti et al., 2000 |
| DjPCNA         | DDBJ (M15796)                            | Parenchyma (strongly expressed in the dorsal midline and in the bilateral lines) | N.D.                        | DNA replication | Orii et al., 2005 |

Chromatin remodelling

| Smed-Chd4      | CHD4 (Q14839)                            | Parenchyma, CNS | Reduced proliferation response; decreased number of neoblast progeny cells; death in 3-4 weeks | ATP-dependent helicase; stem cell differentiation | Scimone et al., 2010 |
| DjRbp48        | RBP4 (Q09028)                            | Parenchyma, blastema | Reduced blastema formation; ventral curling; reduced motility; death in 6 weeks | Histone-binding protein; nucleosome remodelling | Bonuccelli et al, 2010 |

Other functions

| Smedinx-11     | Not present                             | Parenchyma, pharynx, region surrounding the brain and region anterior to photoreceptors | No blastema formation; reversal of the neoblast gradient; death in 5 weeks | Gap junction protein (innexin) | Oviedo and Levin, 2007 |

| Category 2 and Category 3 genes | Various Early blastema, outer parenchyma of intact animals | N.D.                        | Early stem cell progeny differentiation | Eisenhoffer et al., 2008 |
| Smed-p33       | TPS3 (P04637)                            | Parenchyma, stem cell progeny | Decreased number of neoblast progeny cells | Early stem cell progeny differentiation | Pearson and Sanchez Alvarado, 2010 |
| Djmot          | HSPA9 (Q8N1C8)                           | Parenchyma (strongly expressed in the dorsal midline), blastema | Reduced blastema of neoblast reduced regeneration; death in 5-7 weeks | Chaperone, sequestration of p53 | Conte et al., 2009 |

N.D., not determined; piRNA, Piwi-interacting RNA.
promoting the use of the planarian model in the field of regenerative medicine, to which the planaria community aims to contribute by providing novel insights into the molecular machineries controlling stem cell behaviour and CNS regeneration.

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COMPETING INTERESTS
The authors declare no competing financial interests.

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