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REVIEW

Regeneration of Planarians: Experimental Object

I. M. Sheiman† and N. D. Kreshchenko

Institute of Cell Biophysics, Russian Academy of Sciences, ul. Institutskaya 3, Pushchino, Moscow oblast, 142290 Russia
e-mail: nkreshch@rambler.ru

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Abstract—We discuss the expediency of using invertebrates, such as flatworms and planarians, as experimental objects. Free-living planarian flatworms (phylum Platyhelminthes, class Turbellaria) are invertebrate animals in which a bilateral symmetry appears for the first time in evolution and organs and tissues form. As the highest ecological link of the food chain—predators—these animals are characterized by a set of behavioral reactions controlled by a differentiated central nervous system. Planarians have unsurpassed ability to regenerate lost or damaged body parts. Owing to the ease of their cultivation and their convenience for manipulations, these animals are used to study the influence of chemical and physical factors on the processes of life, growth, and reproduction. Currently, planarians are recognized as a model for biological research in the field of regeneration, stem cell biology, study of their proliferation and differentiation, as well as the regulatory mechanisms of morphogenetic processes. The genome of the planarian *Schmidtea mediterranea* was fully sequenced, which opened up the opportunity to work with this object at the molecular biological level. Furthermore, planarians are used in neurobiological and toxicological studies, in studying the evolutionary aspects of centralization of the nervous system, mechanisms of muscle contraction, and in the development of new antiparasitic drugs. This review aims to demonstrate the relevance and diversity of research conducted on simple biological objects—planarians—to a wider audience to show the historical continuity of these studies and their wide geographical distribution and to focus on the studies carried out in Russia, which, as a rule, are not included in the foreign reviews on planarian regeneration.

Keywords: invertebrates, planarian, regeneration, morphogenesis, stem cells

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The structure of a scientific publication has traditionally included a description of the methodology with the representation of an object, when it comes to biological experimental work. Deviation from the usual and most common objects requires a detailed description of the characteristics of the new object and a rationale for its selection. Nature has endowed some animal species with such characteristics that it seems as if it has taken care of their use in the study of certain biological phenomena. Owing to the unique properties of some representatives of living matter, a number of breakthroughs in biology were achieved. Among invertebrates, true model objects can be listed that have led to scientific discoveries. Suffice it to mention the proverbial fly Drosophila with its polytene chromosomes and its relevance to genetics, the giant neurons of clams and stretch receptors of the crayfish for neurophysiology, and ants and bees for ethology. The purpose of this publication is to draw attention to an object that has a sizeable research experience.

One of the most important sections of developmental biology is regeneration. In the study of the principles, sources, and mechanisms of the regeneration process, it is difficult to compete in terms of research volume and contribution with such an object as a flat worm—planarian (Figs. 1a, 1b, 2). Planarians are endowed by nature with a number of remarkable properties that are attractive in many experimental tasks. The following is a brief description of the object.

Planarians are the lowest worms. They are widely distributed in nature, in fresh and salt waters and moist soils. The species with a body length of 1–3 cm are more common than others. Freshwater planarians from moderate latitudes inhabit the underside of aquatic plants or rocks, avoiding light. The flat body of a planarian can be transparent, brown, almost black, mottled, and covered with mucus. Planarians occupy a key position in the evolution of the animal world. They were the first to evolve a bilateral symmetry, to concentrate the neural elements into the central nervous system, and to evolve a pair head ganglion ("brain") in the front part of their body (Figs. 2c, 2d). Due to lack of a circulatory system in the planarians, most of their cells communicate with each other and the environment via signaling molecules or chemical messengers that are synthesized by neurons. In the nervous system of planarians, the following substances were detected: serotonin, acetylcholine, catecholamines, GABA, neuropeptides and growth factors (Welsh and Williams, 1970; Tiras et al., 1975; Kabotyanski et al., 1991; Maule et al., 1994; Johnston et al., 1995, 1996; Eriksson and Panula, 1994; Reuter et al.,

† Deceased.

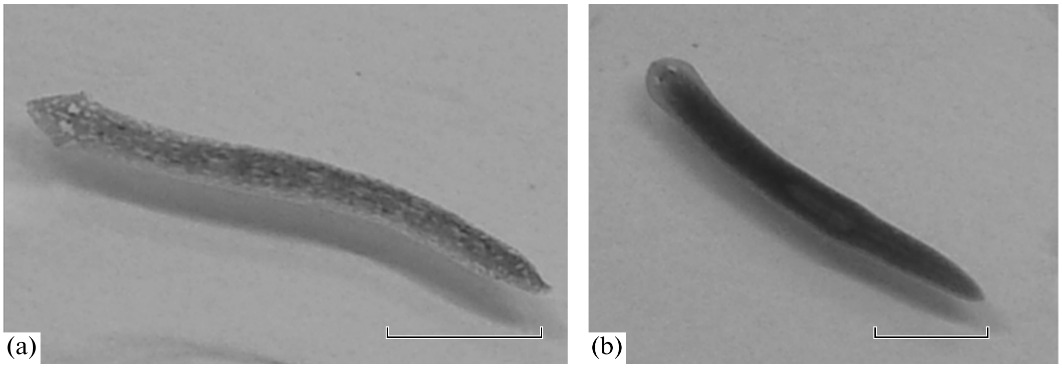

Fig. 1. Fresh-water planarians. (a) *Girardia tigrina* and (b) *Schmidtea mediterranea*. Scale: 1 mm.

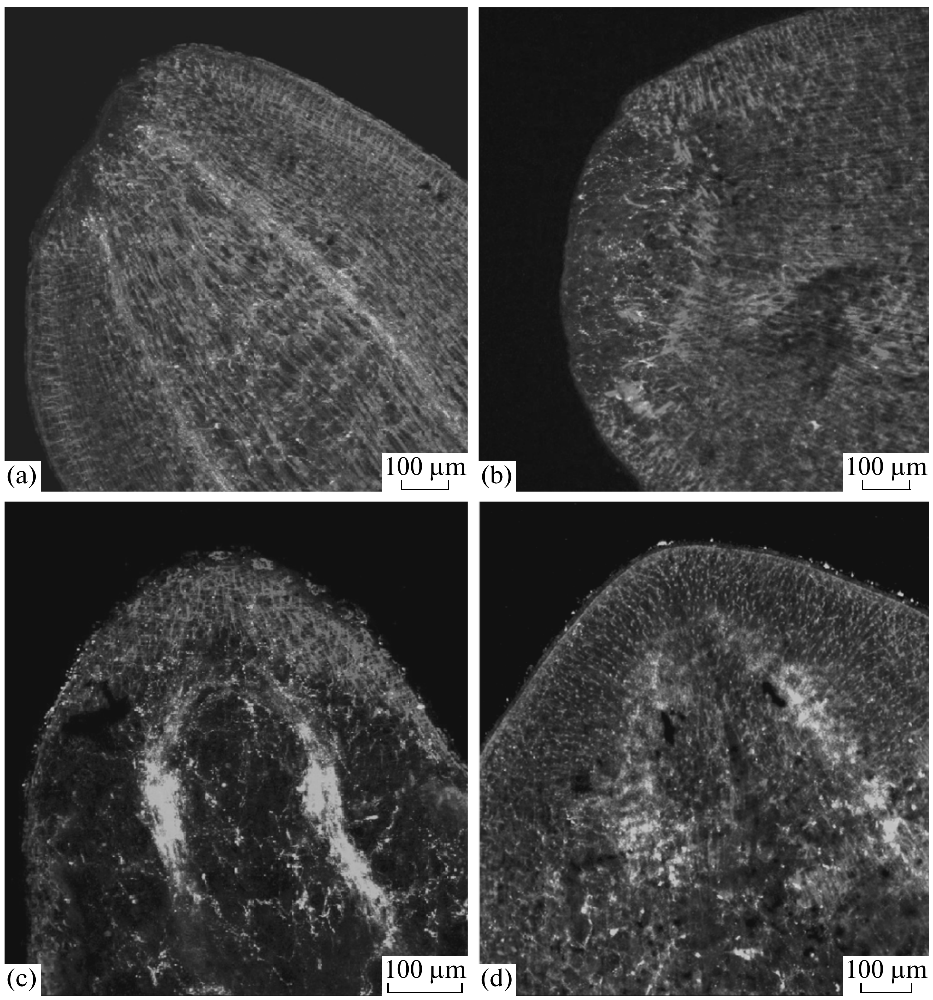

Fig. 2. Regeneration of the head part of the body of planarian *G. tigrina*. Immmunopositive coloring to neuropeptide NPF (white color) in the regenerating head nerve ganglion of the planarian, the grey color shows the muscles of the body; (a) 1 day, (b) 2 days, (c) 4 days, (d) 7 days of regeneration. Scale: 100 μm.

1995; Cebria et al., 2002; Nishimura et al., 2007; Cebria, 2008; Kreshchenko et al., 2008; Kreshchenko and Tolstenkov 2012).

Planarians belong to the family of triclads (characterized by triply branched intestine). Their intestine consists of a tube, the wall of which consists of a single layer of cells. When taking food, cell walls break down naturally, but the cells naturally recover at the end of digestion, showing a characteristic model of physiological regeneration of the planarian. This behavior of the intestine provides two functions: digestion and nutrient distribution (Sheiman and Sakharova, 1974; Sakharova and Sheiman, 1977).

Planarians are hermaphrodites and reproduce sexually. But there are species and races that reproduce asexually, by a simple separation of the tail fragment through a constriction of the body in the "zone of division." From the two halves of the planarian, two new full bodies are restored. This is another natural model of planarian regeneration. From these fragments, populations of pure lines can be obtained. Some species of planarians possess both modes of reproduction (Best et al., 1974, Kobayashi and Hoshi, 2011) Thus, planarians possess the capacity not only for reparative regeneration caused by damage to their body from outside but also physiological regeneration, due to the nature of their life. A continuous renewal of cells and structures as a result of the enormous morphogenetic plasticity of these animals provides cycles of digestion, asexual reproduction, and daily self-renewal of tissue.

Interest in planarian regeneration has its own long history. Darwin already tried to look into the remarkable property of planarians to recover from the dissection of their body into two parts (Darwin, 1884). A systematic study of this phenomenon dates back to the 20th century, when such classics of biology as Morgan (Morgan, 1901) and Child (Child, 1941) conducted experiments on planarians and described the main hallmarks of their regeneration. The next contribution to the study of planarian regeneration belongs to the French school of embryologists led by Wolff (Wolff, 1962) and Lender (Lender, 1965) and refers to the mid-20th century. They have added significantly to our understanding of the process, and set a time frame and the morphological characteristics of regeneration in planarians. In the 1960s and subsequent years, researchers from the Department of Embryology at Leningrad University worked in the same direction under the leadership of B.P. Tokin (Tokin, 1959). Brondsted collected and published the most complete for that time summary of publications on planarian regeneration (Brøndsted, 1969). During this period, cells were described in the body of planarians, called neoblasts, which possessed the ability to divide and differentiate into cells of different tissues. Neoblasts were identified histologically due to the intense basophilia of the cytoplasm and relatively poorly developed cytoplasmic organelles, a large nucleus and the presence of the nucleolus and rich RNA (Pedersen, 1959; Baguna, 1975a, 1975b; Romero and Baguna, 1981; Hori, 1992). Many researchers studying regeneration in planarians held the opinion that neoblasts are cellular sources of regeneration (Brønsted, 1969; Wolff, 1962; Lender, 1965).

A raise of interest in planarians occurred in the 1950s in connection with the work of the American zoopsychologist McConnell, who touched closely upon the problem of regeneration (McConnel, 1967). He studied simple reflexes in planarians: he worked out the reaction to the light signal supported by electrical stimulation. He cut the trained planarians, which responded to light as to the current, in halves and found that the planarians that regenerated from both halves retained the memory trace formed in the initial parent individual. Since this trace was preserved equally in the head and tail fragments devoid of the cerebral nerve ganglion, McConnell decided that the memory trace must persist in special cells, neoblasts, from which a new ganglion formed during the regeneration. He attributed this function to RNA molecules, which are very plentiful in neoblasts. At the time of universal interest in DNA in the middle of the last century, the possible association of RNA with the storage and "transfer" of memory sounded sensational. In many laboratories around the world, the experiences of McConnell were repeated and modified, transferred to other animals, and the results were developed or refuted. Numerous failures in maintaining of the memory trace in similar experiments led to their phasing out. However, in a modest laboratory of the Pushchino Biological Center, in the same period, there was an attempt to reproduce the experiments of McConnell and find an explanation through targeted experimental analysis. The experimental results obtained by McConnell proved indeed to be true, but the researchers managed to show that memory traces were not stored in neoblasts or in RNA molecules but were in some way related to the central nervous system of planarians (Sheiman, 1984). During the dissection of trained planarians, it was found that the memory trace in the regenerated individuals was retained only in those parts of the body in which a certain portion of the nervous system was preserved, and it was not maintained in the complete absence of it (Sheiman, 1984; Sheiman and Tiras, 1996). Recent studies have confirmed the findings that seemed unbelievable at the time of a long-term memory in planarians, which persists after the removal of the cerebral ganglion and its subsequent regeneration (Shomrat and Levin, 2013).

In recent decades, there was a change of course dictated by the time of research. New directions are associated with the emergence and implementation of modern methods into the experimental practice, such as immunocytochemistry, electron microscopy, and fluorescent and confocal laser scanning microscopy, which allowed us to invade the intimate cell physiology. The study of stem cells in higher organisms led to renewed interest in the stem cells of planarians. This, in turn, led to a new stream of research papers on planarian regeneration. Presently, modern works on planarians are being successfully developed in the laboratories of different countries: Finland (Reuter et al., 1995; Reuter and Kreshchenko, 2004), Spain (Salo and Baguna, 2002; Baguna, 2009), Italy (Salvetti et al., 2000, 2005; Isolani et al., 2012), Great Britain (Evans, 2011), Japan (Agata and Watanabe, 1999; Ishizuka et al., 2007), United States (Newmark and Alva-

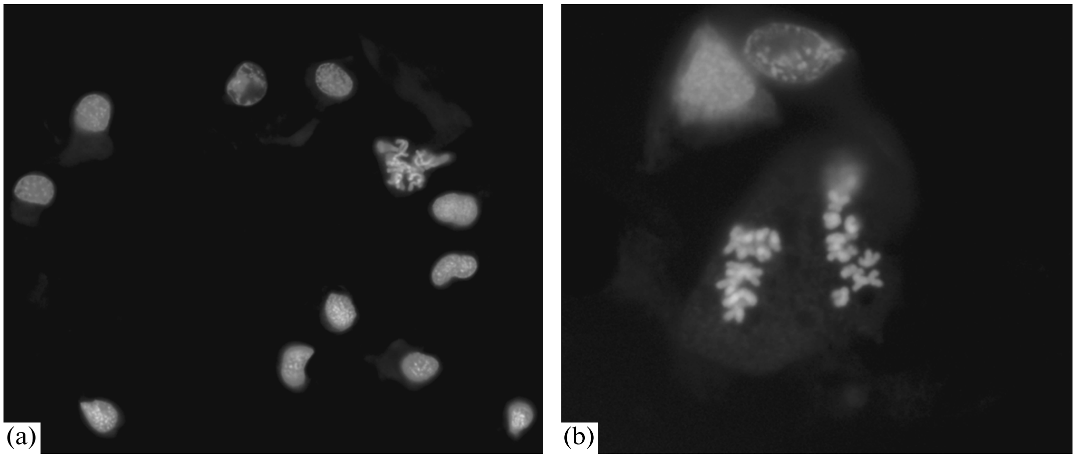

Fig. 3. Staining of nuclei in a cell suspension obtained from tissues of planarians (a) *G. tigrina* using a fluorescent dye Hoechst33342. (a) Eleven stained nuclei of cells in the interphase stage of the cell cycle can be seen; in the upper right corner is the nucleus of a dividing cell in the early stages of anaphase, i.e., early divergence of daughter chromatids to different poles of the nucleus (×20 objective). (b) In the center, the mitotic cell (nucleus) after exposure to colchicine is seen; it can be noticed that the chromosomes have doubled, but the chromatids did not diverge; two interphase nuclei are located above it (×63 objective).

rado, 2000; Oviedo and Levin, 2007; Lapan and Reddien, 2011; Sanchez Alwarado, 2012), Germany (Gentile et al., 2011; Sandmann et al., 2012), France (Galloni, 2012), China (Yuan et al., 2011), and India (Rangiah and Palakodeti, 2013). Cellular and molecular studies of regeneration are widely conducted. The hypothesis of axial polarity and dorso-ventral interaction in the process of morphogenesis in planarians is being investigated (Kato and Orii, 1999; Molina et al., 2011; Blassberg et al., 2013). Information regarding cellular sources of regeneration and genes involved in the morphogenetic process is being accumulated. It is shown that reparative regeneration in planarians at the initial stage is accomplished by epimorphic formation of a blastema, from which amputated tissues and organs are restored. Morphallaxis is involved in the structural and functional combination of old and new tissues. The regenerative capacity is provided by the presence of a pool of pluripotent stem cells, neoblasts, in planarians. Some features of the biology of neoblasts were revealed, their morphological traits were described, and the distribution of neoblasts in several species of planarians was studied (Baguna et al., 1975a, 1975b; Newmark and Sanchez Alvarado, 2000; Baguna, 2002; Orii et al., 2005). It was found that a population of neoblasts is maintained at a certain level due to their continuous proliferation (Newmark and Sanchez Alvarado, 2000; Kreshchenko, 2012; Ermakov et al., 2012). It was shown that it is the descendants of neoblasts in planarians that form the regeneration blastema after injury or experimental transection, where they subsequently differentiate and give rise to all cell types of the body: nervous, muscle, intestinal, parenchymal, and germ cells (Salo, 2006; Handelberg-Thorsager et al., 2008).

A comparative study of regeneration after the amputation of various organs and body parts (the anterior end of the body, ganglion, photoreceptors, and pharynx) in planarians has been conducted over the past decade at the Institute of Cell Biophysics (Pushchino, Moscow region). The regulatory factors of morphogenesis during the asexual reproduction of planarians are being investigated. These studies on planarians are unique in Russia and post-Soviet states. Due to these studies, the existing experimental models of regeneration in planarians can easily be described now using the available adequate quantitative and qualitative criteria of regeneration. The regeneration processes of the intestine, pharynx, eyes, or body fragments after transection at different levels of the body of planarians were found to have both common features and differences (Kreshchenko et al., 1999; Sheiman et al., 2004, 2006; Kreshchenko, 2009; Sheiman et al., 2010). The distribution of proliferating neoblasts in different regions of the body of both intact and regenerating planarians *Girardia tigrina* and *Schmidtea mediterranea* was described and the distribution of the dividing neoblasts over the phases of the mitotic cycle was studied. The dynamics of the proliferative activity of stem cells during the regeneration of the cerebral ganglion and after exposure to the blocking agents colchicine and melatonin was studied in a suspension of the cells obtained from the tissues of regenerating planarians (Fig. 3) (Kreshchenko et al., 2008; Kreschenko, 2012; Ermakov et al., 2012). The regulation of regeneration in planarians was shown to involve the natural neuropeptides NPF and FMRF (Kreshchenko, 2008; Kreshchenko and Maule, 2010). The physical factors, namely, weak electromagnetic radiation, were found to affect the morphogenetic processes in planarians (Sheiman et al., 2009; Novikov and Sheiman, 2012).

New cellular and molecular technologies have appeared over the last 20 years, including genetic and genomic analysis, in situ hybridization, and fluorescence-activated cell sorting (Hayashi et al., 2006; Romero et al., 2012). Chimeric analysis allowed researchers to examine the transplantation of tissue from one species of planarians to another and study its consequences (Kato et al., 1999); the introduction of bromodeoxyuridine made it possible to mark and identify the proliferating cells (Newmark and Sanchez Alvarado, 2000). Immunocytochemical markers (specific antibodies) are a tool to study cell differentiation during regeneration in planarians (Reuter et al., 1996; Bueno et al., 1997; Agata et al., 1998; Kreshchenko et al., 1999; Kreshchenko, 2008; Cebria, 2008; Fraguas et al., 2012). Recently, the fate of a single transplanted stem cell in the body of planarians was traced (Wagner et al., 2011). Thus, it was confirmed that neoblasts are somatic stem cells that retain the properties of pluripotency in adult organisms.

A number of genes were revealed in the genome of planarians *Schmidtea mediterranea* (Robb et al., 2008), including the genes *piwi*, *pumilio*, *bruno* and *musashi*, *vasa* and *nanos*, *argonaute-2*, *STAG*, and *wnt*, which take a definite part in the regulation of development processes (Shibata et al., 1999; Salvetti et al., 2002; Sato et al., 2006; Handberg-Thorsager and Salo, 2007; Petersen and Reddien, 2011; Yuan et al., 2011; Riddiford and Olson, 2011; Iglesias et al., 2011; Almuelo-Castilio et al., 2012; Roberts-Galbraith and Newmark, 2013; Blassberg et al., 2013). It was shown that some genes were expressed only in neoblasts, for example, *piwi* (Reddien et al., 2005), while others were represented in both neoblasts and differentiated cells (*pumilio*, *bruno*, and *musashi*) (Salvetti et al., 2005; Sato et al., 2006), and still others were expressed only in the germinal cells (homologs of the genes *vasa* and *nanos*) (Shibata et al., 1999, 2010; Sato et al., 2006; Wang et al., 2007). Researchers created the EST and the genomic and proteomic databases of several species of planarians (Mineta et al., 2003; Zayas et al., 2005; Ishizuka et al., 2007; Robb et al., 2008; Abril et al., 2010; Adamidi et al., 2010; Fernandez-Taboada et al., 2011; Fraguas et al., 2011; Nishimura et al., 2012; Labbe et al., 2013), which can now be used to determine the signaling pathways involved in regenerative processes.

The use of the modern RNA interference method for planarians made it possible to study the function of certain genes in the key processes of development and morphogenesis (Sanchez Alvarado and Newmark, 1999). Thus, it was found that the RNA-mediated knockout of the genes *pumilio*, *bruno*, and *piwi* in planarians led to the death of neoblasts and the loss of their ability to regenerate. Salvetti (Salvetti et al., 2005) found that silencing of the gene *bruno* violated the proliferation of neoblasts, whereas their differentiation proceeded properly. These animals formed a normal blastema but soon died because of the limited stock of neoblasts. Silencing of the gene *piwi* in planarians caused the opposite effect: the proliferation of neoblasts was normal, but the differentiation was defective (Reddien et al., 2005). The knockout of the expression of the gene *nanos* by RNA interference did not affect the regeneration in general but stopped the formation of gonads in planarians that reproduce sexually (Handberg-Thorsager and Salo, 2007). In the planarians that cannot regenerate or have a limited capacity for regeneration, the Wnt-signaling pathway is activated. In contrast, the knockout of the gene *beta-catenin-1* involved in the wnt-signaling pathway led to the recovery of the ability to regenerate in the tail part of the body of two species of planarians, *Procotyla fluviatis* and *Dendrocoelum lacteum*, which was lost during the evolution (Liu et al., 2013; Sikes and Newmark, 2013). Thus, studies on planarians led to a certain progress in the regeneration research.

The products of the above-mentioned genes are protein molecules: transcription factors, enzymes, hormones, and other molecules that play an important role in the regulation of morphogenetic processes (Cebria et al., 2002; Fraguas et al., 2011; Ermakova et al., 2009). Separate studies revealed that proliferation and differentiation of stem cells in planarians can be activated or suppressed by a number of hormones, neuropeptides, growth factors, and neurotransmitters (Franquinet and Martelly, 1981; Baguna et al., 1989; Sheiman et al., 1985, 1989; Villar and Schaeffer, 1993; Kreshchenko and Sheiman, 1994; Hori, 1997; Hori and Kishida, 2003; Reuter and Kreshchenko, 2004; Kreshchenko, 2008; Ermakova et al., 2009; Fraguas et al., 2011). Later, several researchers found that the genes and proteins that play the key role in the regulation of tissue regeneration, proliferation, and differentiation of stem cells and morphogenesis have a high degree of homology with the same genes and proteins in higher animals (Oviedo and Levin, 2007; Onal et al., 2012; Labbe et al., 2013).

Despite the efforts of scientists, studies of regeneration in planarians still have many challenges. For example, the efforts on the *in vivo* creation of a long-lived culture of stem cells of planarians were not acheived. The mechanisms of functioning of the system of stem cells in planarians are being investigated, but they are not clearly defined. The trigger mechanisms of regeneration remain unknown. There is no general theory that would explain the regeneration phenomenon. Currently, the scientific community has to analyze and rationalize the results and create a unified theory that would summarize the accumulated data and explain the mechanisms of regeneration and its triggering in planarians, including the complex set of events from expression of the proper genes to proliferation of stem cells and differentiation of their descendants, and formation of a fully functioning body. In our opinion, investigation of the fundamental regenerative capacity and the mechanisms of life span, functioning, and regulation of stem cells in planarians is an essential condition for the successful use of the obtained information and controlled and directed differentiation of embryonic stem cells in higher animals and humans and their safe practical applications in biology and medicine. Furthermore, the use of invertebrates as experimental objects has a number of advantages. Their collection and maintenance are inexpensive, and multiple, statistically significant experiments are possible. Finally, vivisection can be excluded to comply with the ethical factor. The words of Watson, one of the fathers of molecular biology, should be remembered: "What holds for *Esherichia coli* also holds for the elephant."

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