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The planarian neoblast: the rambling history of its origin and some current black boxes

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ABSTRACT First described by Randolph in 1897, the nature and main features of planarian neoblasts have a long rambling history. While their morphologically undifferentiated features have long been recognized, their origin and actual role during regeneration have been highly debated. Here I summarize the main stages of this rambling history: 1) undifferentiated, wandering cells of uncertain origin with a main, albeit undefined, role in regeneration (1890-1940s); 2) quiescent, undifferentiated cells whose main function is to build the blastema during regeneration, an idea which culminated in the 'neoblast theory' of the French School (1940-1960); 3) neoblasts as temporal, undifferentiated cells arising by dedifferentiation from differentiated cells (the 'cell dedifferentiation theory'; 1960-1980s); 4) a new paradigm, starting in the late 1970s-early 1980s, that brought together the role of neoblasts as the main cell for regeneration, with its more important role as somatic stem cells for the daily wear and tear of tissues and as the source of germ cells; and 5) more recent developments that culminate in the report of rescuing lethally irradiated planarians by injection of single neoblasts, which makes of neoblasts an unrivaled toti-, pluripotent somatic stem cell system in the Animal Kingdom. I finally discuss some "black boxes" regarding neoblasts which still baffle us, namely their phylogenetic and ontogenetic origins, their role in body size control, how their pool is regulated during growth and degrowth, the logic of their proliferative control, and some 'old' long-sought missing tools.

KEY WORDS: *planarian, neoblast, totipotency, dedifferentiation, stem cell*

A general overview of cell potency during development

The developmental potential of cells within embryos, and by extension in adult organisms has been one of the biggest riddles in Developmental Biology. A large amount of observations and experiments soon made clear that egg cells and early blastomeres in most phyla are totipotent cells (cells able to give rise to all cell types including germ cells). It also became evident that as development goes on, such potentialities, however ample, become restricted. After the morula stage in mammals, cells of the inner cell mass and from the outer trophoblasts are no longer totipotent but pluripotent (able to give rise to cell types of all three germ layers or to the placenta, respectively). Later, embryonic germ layers segregate. Embryonic outer cells (ectoderm), give rise to several epidermal derivatives together with central nervous system and neural crest cell types but not to any embryonic inner cells, or endoderm derivatives, and vice versa. Such cells are considered multipotent (able to give rise to several cell types). The final stage, the adult organism, is formed of thousands, millions, billions, or trillions of cells of different types (over 200-300 in complex vertebrates) patterned, from sponges to humans, into an almost endless array of sizes and shapes.

For a long time, differentiated cell types in the adult organism were considered irreversibly differentiated; in other words, its developmental potential was nil. The only cells bearing an unlimited potential were germ cells (sperms and unfertlized eggs) provided they fuse to form a fertilized egg from which a new organism arises. By the mid 20th century however two exceptions to this rigid panorama sprung. First, some tissues, namely epithelial (e.g. the skin and the gut), the hematopoietic system, and the germinal tissues (namely in males), made by a limited repertoire of differentiated cell types that carry out the main function of each tissue and organ, do not proliferate, have a limited lifespan, and are continuously replaced

Abbreviations used in this paper: EGFP, enhanced green flourescent protein; FACS, fluorescent activated cell sorting; LTC, long-term cultures.

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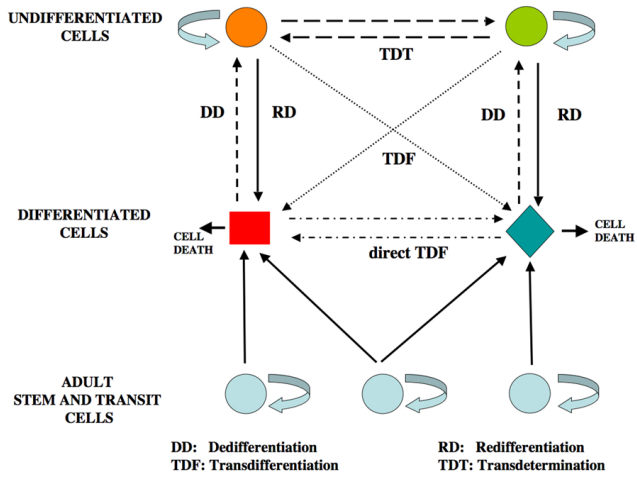
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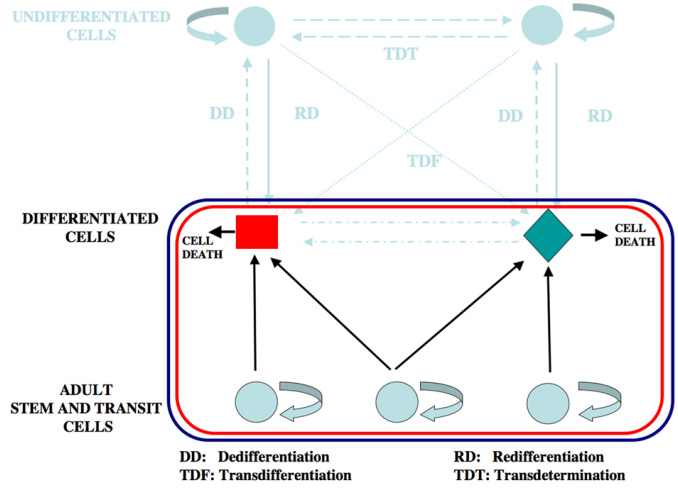
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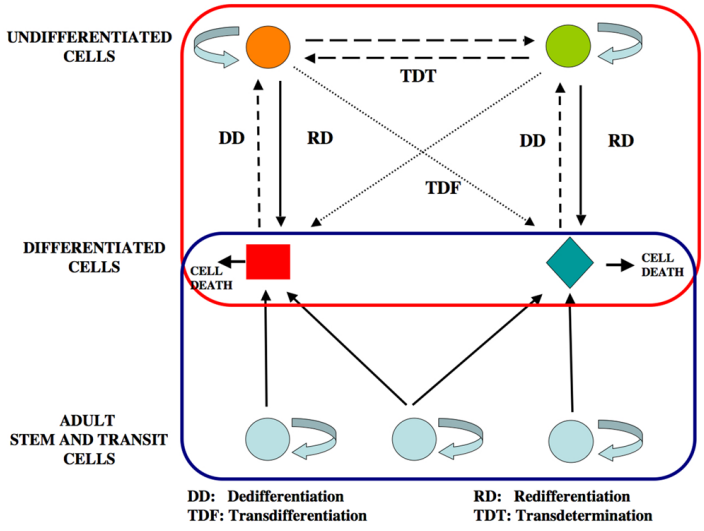
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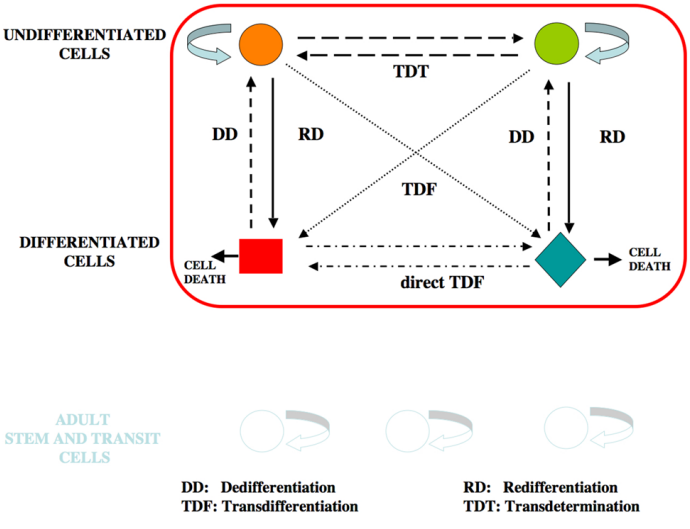
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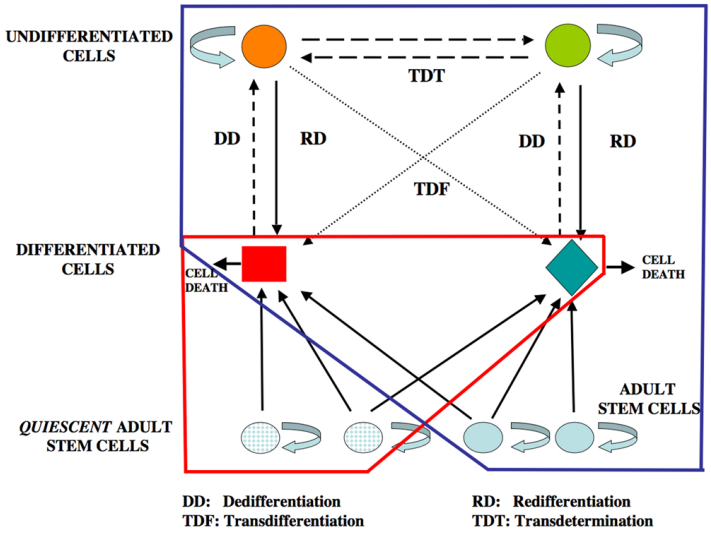
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by new cells differentiating from a restricted pool of multipotent and proliferating undifferentiated cells located at specific places (crypts, inner layers, etc,). These adult stem cells have two main properties: the capacity to *self-renew* and the *potency* to give rise to one or more than one differentiated cell type. The presence of stem cells in adult organisms did not question the irreversibility of the differentiated state; only posed the problem of the embryonic origin and the maintenance by self-renewal of the stem cells.

The second exception had far more reaching consequences. Several experiments and observations in plant embryonic development and tissue culture and some reports, going back to the late 18th century, on the abilities of different groups of organisms to regenerate lost parts (whole bodies, arms, legs, tails, and even some internal organs), indicated that cells (differentiated or not) at, or far from, the cut surface (the wound) were able to restore complex organs and tissues and even most of a lost body. Of particular importance were experiments in plants showing that small bits and even single cells from differentiated tissues were, under different culture conditions, able to regenerate whole plants (Steward *et al.,* 1958; Vasil and Hildebrandt, 1965). Similar experiments performed with cells from stems, leaves, flowers, and even pollen grains, gave similar results. In other words, most differentiated cells from plants, under proper conditions, do have the potential to give rise to all differentiated cell types, germ cells included. In addition, adult plants are endowed with permanent multipotent undifferentiated cells located at specific places (meristems and quiescent centres; Weigel and Jürgens, 2002) that give rise to new stems, roots, leaves, and flowers having key roles in plant growth, morphological adaptation, cell turnover, and homeostasis. In contrast to plants, no whole adult animals have so far been produced from single differentiated or undifferentiated cells. However, after removing large or very large parts of their bodies, sponges, hydras, flatworms (namely freshwater planarians), some annelids and nemerteans, and some ascidians, are able to regenerate whole again (reviewed in Ferretti and Geraudie, 1998).

Fig. 1. **Reported transformations among cell states in adult organisms and its role during regeneration**. *For the sake of clarity only undifferentiated cells, differentiated cells, and adult stem (and transit) cells are represented. The upper panel depicts reported (proven and very likely) cell-cell transformations during tissue homeostasis and regeneration. In the middle tier, two differentiated cell types (red square and blue lozenge) represent most of the body mass which is kept in a steady-state balancing the cell losses due to cell death with the entrance of new cells differentiating from adult stem and transit cells (solid light blue circles) which maintain themselves by cell proliferation (curved light blue arrows). The upper tier depicts reported transformations usually occurring during regeneration and in stressful conditions: dedifferentiation (DD; dashed black arrows) to undifferentiated cells (orange and green circles), followed by proliferation (light blue curved arrows), and redifferentiation (RD; solid black arrows) to the same cell type. Occasionally, after dedifferentiation and proliferation, cells might change their determination state (TDT: transdetermination) and redifferentiate to different cell types (TDF: transdifferentiation; thin black arrows). Only rarely, differentiated cells transdifferentiate directly (without dedifferentiation and proliferation) into other cell types (direct TDF; dash and point black arrows) or engage in cell proliferation (here not represented).* **(A)** *A common pool of pluri- or multipotent adult stem and transit cells serves daily cell renewal (blue rounded rectangle) as well as most regenerative phenomena (red rounded rectangle); dedifferentiation and transdifferentiation have no reported roles. This is the situation in planarians and, likely, in sponges, and to some extent in some cell lineages of cnidarians.* **(B)** *Dedifferentiation of differentiated cells into undifferentiated cells, followed by proliferation and redifferentiation into identical, similar, or different cell types (through TDT and TDF) is the basis for regeneration (red rounded rectangle). Direct TDF is also contemplated. This is the classical cell dedifferentiation scenario of vertebrate and several invertebrate regenerative models. Adult stem (and transit) cells have no role in them. Tissue homeostasis by daily cell renewal is not represented because it usually was not analyzed.* **(C)** *Separate multi- or unipotent undifferentiated adult stem (and transit) cells serve the daily wear and tear of renewing tissues during growth and homeostasis (blue rounded rectangle), while dedifferentiation of differentiated cells is also called upon when whole or partial regeneration is needed (red rounded rectangle). This is the modern scenario for classical regeneration models in complex organisms that take into account both the presence and role of stem cells and cell dedifferentiation, and even transdifferentiation.* **(D)** *The old (some even recent) concept of 'reserve cells' for regeneration. Toti- or pluripotent stem cells exist as a set aside population of quiescent cells (the planarian 'reserve cells' of Dubois, 1949) ready to be activated when regeneration is needed (red polygonal form). In turn, cell renewal and growth relies on different populations of undifferentiated adult stem cells either permanent ('beta-cells' in planarians, Hay and Coward, 1975) and/or produced transiently by cell dedifferentiation (blue polygonal form).*

The cellular basis of tissue growth, homeostasis and regeneration

Question arises as to whether regeneration and tissue growth and homeostasis have a common cellular basis or whether different cells and mechanisms serve separately each of these processes. From the known cell-cell transformations in adult organisms (top panel in Fig 1) several models, summarized in Fig 1A-D, could be contemplated. First, a common pool of undifferentiated, likely pluri- or multipotent adult stem cells exist able to proliferate and, to different extents, differentiate on demand into cell lineages to maintain tissue/organ integrity during growth and homeostasis and to restore the pattern during regeneration (Fig 1A). Second, undifferentiated adult stem cells, either toti-, pluri- and multipotent, are mental constructions, while cell dedifferentiation into undifferentiated cells, followed by proliferation and redifferentiation into identical, close, or different cell types is the sole mechanism to account for partial or whole regeneration, growth, and even tissue renewal (Fig 1B). Third, pluri- or multipotent undifferentiated adult stem cells namely serves the daily wear and tear of renewing tissues during growth and homeostasis, while dedifferentiation of differentiated cells is called upon when whole or partial regeneration is needed (Fig 1C). Finally, undifferentiated cells, likely toti- or pluripotent, could exist as a set aside population of quiescent cells (the so-called, 'reserve cells', Dubois, 1949) ready to be activated when regeneration is needed, while daily cell renewal and growth relies on different populations of undifferentiated adult stem-cells either permanent (the so-called 'beta-cells', Hay and Coward, 1975) and/or produced transiently by cell dedifferentiation (Fig 1D).

The planarians and the neoblasts: a case study of cell plasticity

Planarians in brief

"Planarian" is the generic name applied to species of the Order Tricladida of the Phylum Platyhelminthes (the flatworms) (Sluys *et al.,* 2009). Planarians are bilaterally symmetric, triploblastic, unsegmented, acoelomate, with a clear anteroposterior polarity and

usually dorsoventrally flattened. They lack circulatory, respiratory or skeletal structures. Adult planarians range in size from 4-5 mm in length and up to 1 meter of some land planarians. They are hermaphrodites with cross-fertilization and have sexual, asexual and mixed (sexual/asexual, usually seasonal) modalities of reproduction. Between the monolayered epidermis and the gut, the space is filled by a solid mass of rather unstructured tissues and cells, called parenchyma or mesenchyma, made by different cell types (neoblasts among them), within which organs such as the brain and nerve cords, ovaries, testes, excretory system, and the copulatory complex are embedded. In cellular terms, planarians are made by two compartments: 1) a proliferative one formed by a single, morphologically identifiable population of toti-, pluri- and multipotent stem-cells, or neoblasts (approx. 20-35% of the total number of cells; see below), which, by differentiation, give rise to all differentiated cell types, while maintains its own density by cell proliferation (Baguñà, 1981; reviewed in Baguñà *et al.,* 1990), and 2) a functional compartment, made by 20-25 non proliferating differentiated cell types (approx. 65-80% of total cells) that are continuously replaced during the life-time of the individual (Baguñà and Romero, 1981; Romero and Baguñà, 1991).

When a planarian is cut, the epithelium around the wound closes up and after one hour a thin film of epidermal cells from the stretched old epidermis covers it. Below the wound epithelium groups of undifferentiated cells aggregate to form the regenerative blastema. The blastema, whose cells do not proliferate, increase in size by the continuous entrance at its base of new undifferentiated cells, alike to neoblasts, produced by active cell proliferation in the old stump (postblastema: Baguñà, 1976b; Saló and Baguñà, 1984). Indeed, soon after cutting an still unidentified wound-specific signal induces between 2-6 hrs a first mitotic peak by shortening the G2 phase of neoblasts close to the wound (Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). Of note, in the species *Schmidtea mediterranea* this first mitotic peak occurs allover the organism (Wenemoser and Reddien, 2010). After the first mitotic peak a relative minimum follows at 1 day of regeneration and a second, higher and temporally more sustained, peak ensues betwen 2-4 days. Beggining at 3-4 days of regeneration new structures (e.g. brain primordia, eye spots, and pharynx) are determined within the blastema and postblastema areas following a disto-proximal sequence as showed using several grafting techniques (reviewed in Baguñà *et al.,* 1990, 1994) and more recently using molecular markers. The lost structural pattern is thus restored and normal body proportions finally attained (by remodelling or morphallaxis) after 3-4 weeks of regeneration.

Besides their extraordinary powers to regenerate, planarians also show another unsual feature: a much greater plasticity both in the growth of an individual and on its final size, and an enduring capacity to degrow when starved. In common with other phyla (cnidarians, nemertines, and some annelids, molluscs and ascidians; Calow, 1978) most planarians can stand long periods of starvation, and during this time may shrink from an adult size to, and sometimes beyond, the initial size at hatching. When feeding is resumed, they grow again to adult size. This process, back and forth, may go alternatively for long periods without apparent impairment to the individual nor to its future maturing and breeding capacities.

Neoblasts in brief

The origin of the term 'neoblast'

Although most reviews and textbooks state that Harriet Randolph coined the term neoblast to refer to *small*, undifferentiated, embryonic-like cells found in some species of earthworms (Randolph, 1892), this is incorrect. Actually, she first introduced the term neoblast in a short preliminary paper (Randolph, 1891), and a year after in her 1892 paper, to refer to *large* cells with large nuclei and nucleoli placed near the peritoneum in the oligochaete *Lumbriculus*. She regarded these cells, formerly known as chorda cells, 'as specialized embryonic cells set apart for the formation of new mesodermic tissue immediately after the fission of the worm' (Randolph, 1892). Accordingly, she considered that each germ layer gave rise to the corresponding tissues in the regenerated part. Therefore, neoblasts in oligochaetes gave only mesodermal derivatives. When she extended the term neoblasts to similarly staining, albeit smaller, cells in planarians (Randolph, 1897) she made no explicit reference to its germ layer derivations.

Neoblasts after the introduction of Electron Microscopy

Until the arrival of electron microscopy and better fixation techniques (Pedersen, 1959), and because planarian parenchyma cells (fixed and secretory; see Baguñà and Romero, 1981, for the first and thorough description of planarian cell types) have numerous intermingled processes without clear limits at the optical microscope, the parenchyma was considered to form a syncytial mass within which small wandering cells reside. As already mentioned, these cells were named neoblasts after Randolph (1897). The typical planarian neoblast is a small (7-12 μm) cell, rounded or more or less spindle shaped, with a large ovoid nucleus bearing two or three nucleoli, a scant rim of cytoplasm, a high nucleocytoplasmic (N/C) ratio (Fig. 2) and often bearing a single filopodia.

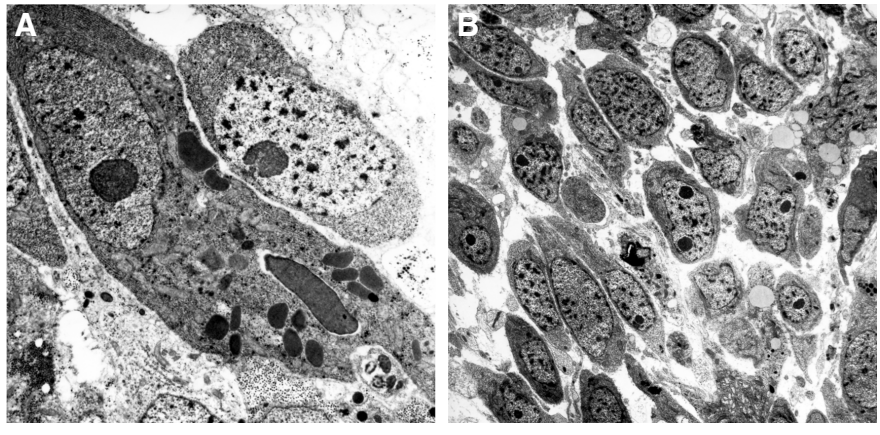

Fig. 2. **Electron micrographs of planarian neoblasts**. **(A)** *A single neoblast (upper right) alongside a differentiated cell (a rhabdite cell; middle) within the parenchyma of* Schmidtea mediterranea. *Note the scant cytoplasm of the neoblast bearing ribosomes and a few mitochondria in contrast with the larger and more complex cytoplasm of the rhabdite cell with several rhabditic inclusions, endoplasmic reticle, and different vacuoles. x 9,000.* **(B)** *Groups of neoblasts at the postblastema area of a 3-day regenerating* Schmidtea mediterranea. *Note the loose intercellular spaces that ease neoblast movements. Bits of cytoplasm from differentiated cells are also seen. x 3,200.*

Their cytoplasm is strongly basophilic due to the presence of high amounts of ribosomal RNA and easily stains with most basic dyes (e.g. methylgreen pyronin, Azur A- eosin B, chromotrope 2R, etc,.). They are distributed all over the parenchyma (nowadays known to be entirely cellular) except in the head region above the eyes and in the pharynx (Baguñà, 1976a). Neoblasts are the only cell type in planarians able to proliferate, the stem cell of all differentiated cell types, germ cells included, and the source of all blastema cells during regeneration (see below).

The rambling history of neoblasts as stem cells and regenerative cells

To our knowledge, a full historical account on the wanderings and ramblings of neoblast nature until they were accepted as a multipurpose toti-/pluripotent stem-cell for the daily tissue and organ renewal, germ cell formation, and regeneration, has yet to be produced. To fill this gap here I give a brief account of the main stages. For a more detailed account of particular stages and subjects, see Bronsted (1955, 1969), Gremigni (1988), Baguñà (1998), Baguñà *et al.,* (1994), Saló (2006), and Gurley and Sanchez Alvarado (2008).

Because of its key importance, I start the story from the end.

The end:* Science*, May 13, 2011

In the 13th of May issue of the journal Science, Wagner *et al.,*2011) from Peter Reddien's lab, reported the final answer to the long-standing question of whether neoblasts in planarians contain within them stem cells that are pluripotent (and even totipotent) at the single cell level. They used transplantation of single neoblasts, isolated by flow cytometry from different body regions of one strain of the planarian *Schmidtea mediterranea,* into lethally irradiated hosts of a different strain of the same species. Injection of single neoblasts resulted in the formation of large descendant-cell colonies *in vivo* that later gave rise to several differentiated cell types restoring both body homeostasis and the ability to regenerate. This study is the first clear evidence that neoblasts are fully pluripotent (and even totipotent, as they also give rise to the germ cells), and closes the debate between the so-called neoblast theory and the dedifferentiation theory (Dubois, 1949; Gremigni and Miceli, 1980; Slack, 1980; Baguñà, 1981; Baguñà *et al.,* 1989; Kobayashi *et al.,* 2008), whose main stages are now considered.

The early stage (1890-1940): neoblasts as undifferentiated wandering cells of uncertain origin but with a role, albeit undefined, in regeneration

At the end of 19th century, ideas on cell dedifferentiation and transdifferentiation and stemness as regards cells participating in regenerative processes were too vague and undefined. Most authors thought planarian regeneration to be accomplished by totipotent cells of embryonic character to which they gave different names ('Bildungszellen', 'Wanderzellen', 'Stoffträger', or 'Stammzellen'; see Bronsted, 1955) until H. Randolph christened them as neoblasts in 1897. Another common feature assigned to these cells was its power to migrate to the wound or to other places of the body where tissue/organ formation occurred, as reported by Randolph (1897) for the spindle-shaped strongly basophilic cells that she followed during regeneration up to the wound to form the blastema. It remained unclear, however, whether cells other than neoblasts retained some proliferative capacity and were able to produce some cellular material to build the regenerate. The last view was hold, among others, by Randolph herself, Flexner, Stevens, Schultz, Bardeen, and Bandier (references in Bronsted, 1969). Another view was that 'Wanderzellen' or neoblasts were formed partly by transformation from other cell types (today's dedifferentiation), a view namely espoused by big names such as Lang and Steinmann and lesser ones as Bartsch and Weigand (references in Bronsted, 1969). Interestingly, the view of neoblasts as non-permanent temporally dedifferentiated cells was called upon again in the 1960s-70s (see below).

Meanwhile, the two big names of planarian regeneration in the first third of the 20th century, Thomas H. Morgan and Charles M. Child, remained undefined or uninterested as regards neoblasts. Morgan acknowledged the role of neoblasts in epimorphic regeneration but was unsure whether morphallaxis was also due to transformations from other cell types, was it direct (today's direct transdifferentiation) or indirect (by dedifferentiation through neoblasts). His main interest was to explain regeneration, and namely axial polarity, as related to some 'structure or composition' in the regenerating piece. He strove at it doing all sorts of cutting experiments trying to deduce some general rules. Despite his work is nowadays considered the first 'classic' of planarian regeneration, he actually failed to answer the main questions posed regarding axial poplarity, as we still fail today to understand it completely despite the enormous progress reported in the last 5 years (Reddien *et al.,* 2007; Gurley *et al.,* 2007; Iglesias *et al.,* 2008; Oviedo *et al.,* 2010; Molina *et al.,* 2011). Morgan's main conclusion was that "something" (meaning some factor or factors) in the structure or composition of the old tissue determined totipotent cells to regenerate the missing parts, with an antero-posterior and/or posterior-anterior qualitative gradation of such "factors" determining axial polarity (Morgan, 1905). The main stumbling blocks he faced were the very primitive, for today's standards, fixation and histological staining methods, and the evident difficulties to explain his results at the cellular level. As it is well know, finding himself unable to solve these mysteries in his lifetime, he wisely left the field for genetics.

Charles M. Child took a physiological stance to regeneration and held it for life. His hypothesis, in sheer contrast to Morgan's, was that morphological and functional differences within and between planarians (e.g. head versus tail, head dominance, frequency of head regeneration; in other words, axial polarity) relied in quantitative differences in metabolism, an idea that culminated in the notion of axial gradients (namely from head to tail). According to Child, axial gradients were reflected on gradations of oxygen consumption, sensitivity to toxins, and differences in electrical potential, which were highest in the head and decreased posteriorly (Child, 1941). Polarity was therefore interpreted as an expression of dominance due to a quantitative and not to a qualitative gradation as in Morgan's scheme. Moreover, with the exception of a very early paper (Child, 1903) acknowledging the role of neoblasts in brain regeneration of *Stenostomum* (a catenulid plathelminth), he paid little attention to cells; and hence, to neoblasts. Although highly influential in his time, the axial gradient theory of Child and others is today only marginally present in modern textbooks on embryology and development. As regards planarian regeneration, and to neoblast in particular, Child's approach to analyze it on a level other than the cell did not provide solid grounds to deduce general rules.

All in all, as regards planarian regeneration in general and to neoblasts in particular, the first third of the 20th century ended like the economy: in a great depression. Morgan's and Child's postulated gradients of substances or metabolic activities could not be substantiated and lead nowhere. In that, it anticipated the rather bleak period of Embryology from the 1930s to 1950s: accumulation of data without a clear conceptual framework using coarse histological, cytological and biochemical techniques unable to solve the problems posed. All of it concurred to make progress extremely slow. The best example was the fruitless efforts to isolate any substance having the properties and cellular potentialities of Spemann and Mangold's amphibian organizer. These efforts were only fulfilled when Biochemistry and Genetics merged into Molecular Biology well into 1960s.

1940s-1960s: neoblasts as quiescent undifferentiated cells ready to migrate and proliferate to build the blastema: the 'neoblast theory'

To analyze how a system behaves, one of the most powerful methods is to eliminate single components, one by one, and test the system's behavior in their absence. This is the basis of classical genetic analysis using mutants. Loss-of-function mutations, epistatic effects aside, allow us to make educated guesses as to the likely function of any particular gene. In cellular terms, this would imply to eliminate or dispose of specific cell types and test how the system functions or develops in its absence.

Although they were unaware of, the first experiments to test the role, by absence, of planarian neoblasts in regeneration were performed by X-irradiation (Bardeen and Baetjir, 1904; Curtis and Hickman, 1925). After X-irradiation planarians were unable to regenerate and died in 3-5 weeks. Whether this was due to lack of proliferation of neoblasts (difficult to spot and count) or to lack of proliferation of other cell types (e.g. epidermis, gut,) was not (and actually could not be) addressed. At that time, and for almost 20-30 years, contradictory reports were published on the extent and cell types able to proliferate in intact planarians as well as on the presence and rate of mitoses in the blastema area.

The first strong evidence in favor of planarian neoblasts as the true, and likely sole, regenerative cells came from a series of experiments of the Strasbourg school (later on best known as the French School) in the late 40s- early 50s, best exemplified by Dubois (1949) classic paper. She first confirmed that total X-irradiation killed planarians in 3-5 weeks along which neoblasts were progressively lost. Second, she introduced a simple and ingenious technique: shielding with lead an area of an intact planarian during irradiation and cutting the day after the irradiated part at different axial levels. Results were striking. While in the irradiated areas of some planarians necrotic spots appeared scattered here and there in the dorsal and ventral epidermis, these spots began to fade after three weeks in parallel to the formation, in a high percentage of cases, of a small blastema at the wound area. Histological analyses showed the blastema to be made by groups of basophilic (RNA rich) cells akin to neoblasts of the intact worm. This suggested that blastema was formed, after a delay proportional to the distance between the wound and the healthy unirradiated tissue, by undifferentiated cells (neoblasts) migrating from the unirradiated area (Wolff and Dubois, 1948; Dubois, 1949). These results were confirmed using unirradiated grafts into irradiated hosts followed, or not, by cutting. In intact irradiated hosts, neoblasts from the graft repopulated host tissues, whereas in regenerating irradiated hosts, neoblasts from the graft made a new blastema and also repopulated the rest of the body. Importantly, it was claimed that migration of neoblast was only stimulated by a cut or a wound, was preferentially directed towards it, and during migration neoblasts did not proliferate. A final test on the role of neoblasts used grafts labeled with tritiated uridine into non labeled hosts. Because labeled cells were found in the host blastema after cutting it was concluded that labelled neoblasts from the graft migrated to build it (Lender and Gabriel, 1965).

To summarize, the experiments carried out by the French School from 1947 to the late 1960s provided the first evidence backing the presence in planarians of a population of undifferentiated cells scattered within the parenchyma, bearing extensive migratory powers, and able to build the regeneration blastema and to restore within the blastema and the stump all damaged or lost tissues, organs and cell types, germ cells included (Fig 1A). Importantly, most data also indicated that neoblasts were the only proliferative cell type and, hence, the most likely candidate to be the stem-cell of all differentiated cell types in the daily wear and tear in intact organisms. However, the regeneration feats of planarian neoblasts shadowed its much more important physiological role as stem cell. Indeed, ever since the work of the French School went into textbooks, neoblasts were unfortunately referred as 'reserve cells for regeneration', 'quiescent cells' and the like and not, as it should be, as 'adult stem cells' leaving open the possibility that cell and tissue renewal could not be based on a neoblast-like system (Fig. 1D).

The concept of 'reserve cells for regeneration', or 'neoblast' in short, squeezed for a while into other regenerating organisms, such as polychaete and oligochaetae annelids, nemertines, colonial ascidians and even urodele amphibians (Wolff, 1961). In the upcoming years this gave rise to heated controversies between this view and those claiming cell dedifferentiation as the main way to produce blastema cells (see below).

1960s-1980s: neoblasts are not quiescent reserve cells but temporal undifferentiated cells arising by cell dedifferentiation. The 'cell dedifferentiation theory'.

The view of dedifferentiation of differentiated cells as the leading mechanism in planarian regeneration, advanced by Lang, Bartsch, and Steinmann in the first third of the 20th century (see references in Bronsted, 1969), was resuscitated in the early 1960s. Using vital dyes, enzyme staining, and electron microscopy, several authors (see main references in Coward, 1969; and Hay and Coward, 1975; and Gremigni, 1988 for a thorough review) described in intact and regenerating planarians the 'transformation' of intestinal, goblet, and secretory cells, as well as cells from the ovaries, testes, and copulatory organs, into undifferentiated neoblasts that, later on, made the blastema. Therefore, neoblasts could not be considered a permanent population of undifferentiated cells but transient undifferentiated cells produced by cell dedifferentiation (Fig 1B). An alternative scenario proposed that the so-called neoblasts (there renamed beta-cells) did actually exist but were specialized cells set aside for physiological regeneration, while cell dedifferentiation provided most of the regenerative cells for the blastema (Fig 1D) (Hay and Coward, 1975). In retrospect, as it was the case for the early authors, the methods they used were insuficient and the evidence produced too weak to make such a conclusion. Indeed, parallel electron microscopy studies clearly showed the transformation of neoblasts into different cell types (rhabdite, nerve, muscle,

flame, and gut cells among others) in both intact and regenerating worms, and failed to find clear signs of dedifferentiation within the blastema and in areas behind it.

The 'dedifferentiation theory' of planarian regeneration was indirectly supported by data gathered from other regenerating systems. In the 1960s and 70s considerable progress was made in amphibian leg regeneration by the combined use X-ray irradiation, thymidine labelling, ploidy markers, and grafting techniques. In amphibian leg regeneration, cell dedifferentiation was found to be the rule and 'reserve cells' could not be substantiated (for a general review, see Wallace, 1981). Similar arguments were held when annelid regeneration was again analyzed (Hill, 1970), and from dedifferentiation and transdifferentiation phenomena in Hydra regeneration. All of it reinforced dedifferentiation as the main, if not the sole, mechanism to build a blastema and cast serious doubts on the concept of neoblasts as cells set aside or reserved for regeneration (Slack, 1980).

What seemed to be the final blow against the 'neoblast theory' came from a set of simple and beautiful experiments in planarians from Vittorio Gremigni's group in Pisa (Gremigni and Miceli, 1980; Gremigni *et al.,* 1980; reviewed in Gremigni, 1988) using chromosomal markers. Using a strain of the species *Dugesia (S) polychroa* (now *Schmidtea polychroa*) that are naturally occurring mosaics: the somatic cells triploid, the male germ cells diploid, and the female germ cells hexaploid; they showed that regeneration from a cut surface through the gonadal region gave rise to blastemas and regenerates that contained mainly triploid cells but also diploid (from male germ cells) and/or hexaploid cells (from female germ cells) from which somatic cells (e.g. pharyngeal muscle cells) originated. This suggested that dedifferentiation and transdifferentiation (and hence, metaplasia), however limited, occurred during planarian regeneration. These results, held as conclusive evidence for dedifferentiation in planarians and, hence, for similar mechanisms of blastema formation in most animal groups (Slack, 1980), were criticized on the grounds that they did not demonstrate the occurrence of dedifferentiation and metaplasia but, at the most, suggested the existence of dedetermination (or transdetermination) (Baguñà, 1981). This is because the loss of an haploid complement during spermiogenesis and its doubling during oogenesis, though one of the first steps from neoblasts to germ cells, is only a small step in cell determination and occurs in undifferentiated cells of the germinative epithelium, which are undistinguishable from somatic neoblasts. Moreover, it is known that differentiating and differentiated germ cells like spermatocytes, spermatids and spermatozoa, and its counterparts in the female germ line, degenerate and lyse after transection and, therefore, cannot dedifferentiate to give blastema cells. In a later overview of planarian regeneration, Gremigni (1988) took an intermediate view asuming that both neoblasts and dedifferentiated cells take part in blastema formation.

Overall, 1970s and early 1980s were rough times for undifferentiated stem cells as the basis for regeneration (Slack, 1980). While the function of undifferentiated stem-cells for the daily wear and tear in renewing organs and tissues was, soon after the introduction of radioactive labelling (Leblond *et al.,* 1959) and cell transplantation techniques (Till and McCulloch, 1961) at the dawn of 1960s, widely accepted (see Potten, 1983), most researchers concurred they had no role in traumatic regeneration. The baseline argument was dual. First, in most regenerating systems the experimental evidence was for dedifferentiation and against specific stem-cells. Second, whereas the presence of some multipotent stem cells in vertebrates (haematopoietic system, epidermis, gut,.) was clearly accepted, the existence of permanent toti- or pluripotent undifferentiated cell types (e.g. neoblast-like) in complex adult organisms, was difficult to accept. This is because it implied, by necessity, an excesive number of binary decissions to produce so many different cell lineages stemming from a single pool of pluripotent undifferentiated cells. This appeared unreasonable to occur within an adult complex organism because of the burdens and complexities needed to control it. Furthermore, such view equalled the potential of neoblast-like cells to those of egg cells and early blastomeres. And last but not least, the mere thinking on specific stem-cells for organs or tissues as complex and sophisticated as the heart, the central nervous system, the striated muscles, the retina, the liver and pancreas, with complex ontogenetic histories, was considered odd and unrealistic.

The new paradigm: neoblasts as permanent stem cells for physiological regeneration (wear and tear) and for occasional traumatic regeneration. Adult stem cells begin to explode.

Because neoblasts were known to be the only mitotic cell type, efforts in the 1960s concentrated to analyze their distribution in the intact worm, their mitotic rate, and the changes occurring during regeneration. Lender and Gabriel (1960), Bronsted (see references in his 1969 book) and, most notably, Lange (1967) published the first relevant work on the number and distribution of neoblasts in several species of planarians of different sizes and ploidies. The last author found neoblasts to be distributed within the parenchyma with two relative maxima, one anterior behind the eyes and one posterior to the intercalary minimum associated with the position of the pharynx. Further, he estimated the absolute number of neoblasts and correlated them with animal length and volume. His main finding was that larger animals had a lower density of neoblasts, although more of them; in other words, during growth neoblast density decreases while it increases during degrowth. The decrease in neoblast density with size could explain the decreased regenerative abilities of large versus small animals reported by many authors in several species as well as the species-specific maximum size reported by Abeloos (1930). In addition, it also had some bearing, albeit speculative, in the problem of senescence in planarians (Lange, 1968).

Because feeding leads to body growth, likely by cell proliferation, the next step was to quantitate it. The old Giemsa technique proved excellent to spot every mitotic cell in whole mounts (Baguñà, 1974), showing the existence of size- and temperature-dependent mitotic rates. Cells in mitosis were small cells, alike to neoblasts, as they were the proliferating cells seen in the first succesful short-term *in vitro* culture of planarian cells (Betchaku, 1967). After feeding, mitosis increase three to fourfold compared to basal rates and level off at 4-5 days (Baguñà, 1976a). During degrowth by starvation, size- and temperature-dependent basal mitotic rates were found, which indicates that neoblasts are always cycling, a common feature of all stem cells. And during regeneration, mitoses increased three-fourfold in two separate temporal maxima in areas below the wound (the postblastema) while blastema formed and grow, with no proliferation within it, by the steady accumulation of neoblast at its base (Baguñà, 1976b; Saló and Baguñà, 1984).

A step further in the analysis of the intact, growing or degrow-

ing, and regenerating planarians came from the introduction of a maceration technique to dissociate them into single cells. This allowed to estimate the total number of cells and to characterize up to 14 different cell types and their percentages during growth, degrowth and regeneration, in organisms of different sizes kept at different temperatures (Baguñà and Romero, 1981). As expected, the total number of cells increased with increasing body size (area or volume) during growth and decreased during degrowth, changes in cell volume only accounting a mere 10% of total body volume changes. Interestingly, neoblasts were found to account between 20% of total cells in large animals (≥11mm in length) to 35% in small ones (≤3mm in length), validating the changes in neoblast density with size reported by Lange (1967). In addition, other cell types increased or decreased reproducibly in percentage with length, in agreement with some previous results from Abeloos (1930), being the base of organ, tissue and region alometries. Finally, during regeneration, an increase in neoblast density in the blastema (despite the lack of mitotic figures; Baguñà, 1976b) followed later on by increases in nerve cells were the more significant changes detected.

The high percentage of neoblasts found in intact worms for every length studied (20 to 35% of total cells), was very surprising even considering them as the stem cell of all differentiated cell types. Hence, it was postulated that planarian neoblasts should comprise three populations: a small one representing uncommited, proliferating, toti- or pluripotent cells, a bigger one of still undifferentiated and proliferating cells, but commited at different extents to different lineages and differentiated cell types, and a likely third population totalling up to 50% of all neoblasts of fully commited non proliferating neoblasts (Baguñà *et al.,* 1990). The existence of these populations was confirmed 20 years later by Florescent activated Cell Sorting (FACS) analyses: The first two will roughly correspond to the X-ray sensitive X1 and X2 populations, first described in Hayashi *et al.* (2006), whereas the last one (made by some X2 cells and some X-ray insensitive XIS populations) still awaits confirmation.

All in all, most data on the total number and percentages of neoblasts in intact, growing and degrowing, and regenerating worms, favored the role of neoblasts as stem cells and regenerative cells and strongly argued against the ill-defined and not well-founded claims of cell dedifferentiation. Nevertheless, two basic tennets of the 'neoblast theory', that is, its *long migratory capacities* and that migration was *stimulated* by the wound and *directed* towards it, were still under suspition. Indeed, in his then highly influential book 'Cells into Organs', Trinkaus (1984) wrotte: "these cells, called *neoblasts,* have been assigned full pluripotency and legendary migratory capacities. They have even been thought to move from one end of a creature to another,... there is certainly no evidence that such cells engage in their postulated peregrinations', (p. 38, *op. cit.)*.

To answer the criticisms against its migratory capacities, permanent cell markers able to distinguish graft from host cells were needed. Hence, grafts were made between the asexual race of *Dugesia* (now *Schmidtea) mediterranea* bearing a chromosomal marker (a heteromorphosis) into hosts of the sexual race of the same species lacking it, or between diploid and tetraploid biotypes of *Dugesia (*now *Schmidtea) polychroa* with clear differences in nuclear size. Moreover, to track the movements, if any, of differentiated cells, fluorescent latex beads, taken up by specific differentiated cell types were used. Results were straightforward (Saló and Baguñà, 1985a, 1989): 1) graft cells moved evenly and at low rates (40μm/day) into host tissues in intact organisms, and doubled its rate in irradiated hosts; 2) regeneration did not speed up the rate of movement nor drove cells preferentially to the wound, the sole exception being within local regions close to the wound where cells moved up to 90-140μm/day and aligned preferentially its mitotic axes in parallel to the AP body axis; 3) differentiated cells moved at rates low enough (10-15μm/day) to be compatible with random movements; and 4) higher rates of movement were correlated with higher mitotic rates. Hence, the so-called 'migration' of neoblasts was not a true cell migration but a slow, even, and progressive spreading of neoblasts mainly caused by random movements linked to cell proliferation (Solé *et al.,* 1988). In addition, mitotic activity together with local cell movements and preferentially aligned mitotic axes within a 200-300μm area (the so-called postblastema) below the blastema/stump boundary was sufficient to explain the kinetics

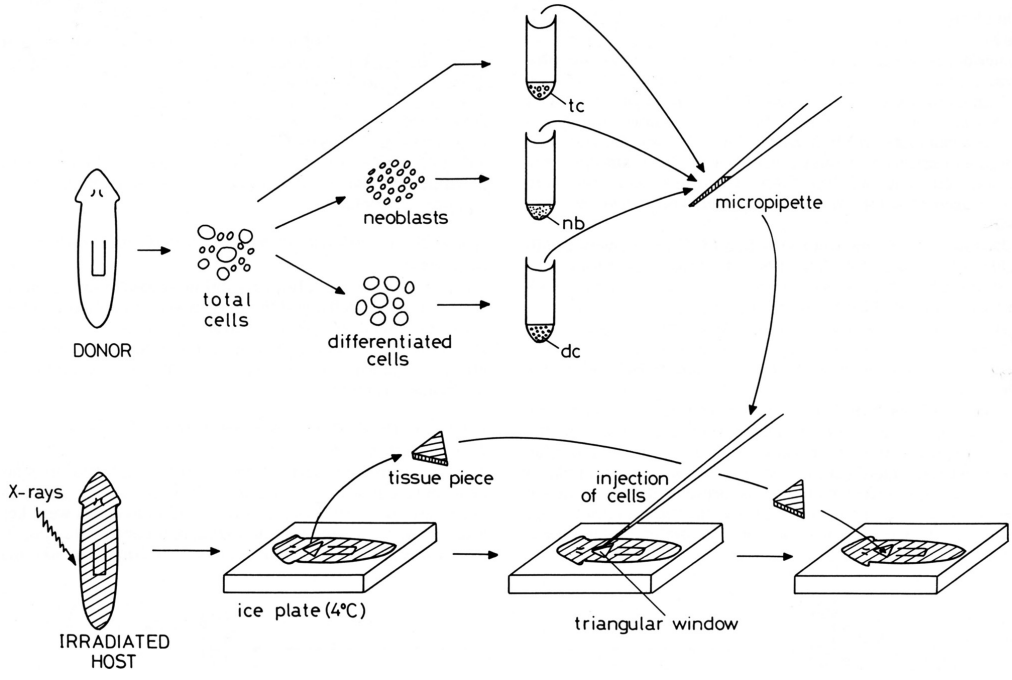

Fig. 3. **Rescue of irradiated planarians by injection of enriched neoblast fractions**. *Procedure employed to isolate and inject total cells (tc), enriched neoblast fractions (nb) or enriched differentiated cells (dc) from nonirradiated donors into the parenchyma of irradiated hosts in the planarians* Girardia tigrina *and* Schmidtea mediterranea *(from Baguñà* et al., Development *107, 77-86 (1989)).*

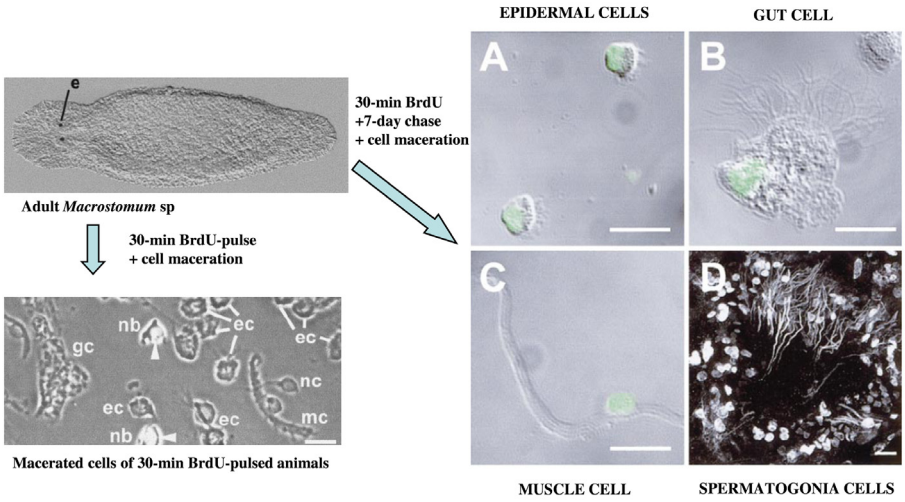

Fig. 4. **BrdU-pulse and chase labelling in planarians**. *Labelling S-phase cells in* Macrostomum *sp with a 30-min bromodeoxyuridine (BrdU) pulse and 7 days chase (no BrdU) to show labelling of somatic and germ cells. On the left, after a 30-min BrdU pulse, animals are macerated into single cells and labelling observed by phase-contrast and fluorescence microscopy. Neoblasts (nb, arrowheads) are the only labelled cells while no differentiated cells contain labelled nuclei. In the right, after a 30-min BrdU pulse, organisms are chased (no BrdU) for 7 days after which they are macerated into single cells. All somatic cells (here only represented by epidermal, gut and muscle cells) and germ cell (spermatogonia) are labelled (A, B, C: nuclei stained in green [FITC-conjugated secondary antibody]; D: round bright nuclei of stained spermatogonia at the periphery of the testes). Ec, epidermal cells; gc, gastrodermal cells; mc, muscle cells; nc, nerve cells. Scale bars: 10μm. Taken, with some modifications, from Ladurner* et al., Dev Biol *226, 231-241 (2000).*

of blastema growth (Saló and Baguñà, 1989).

The last and most enduring criticism against the neoblast theory was that, after all, neoblasts could be a temporal population of undifferentiated cells continuously produced by cell dedifferentiation (Fig 1 B,D). This was the main tennet of the 'cell dedifferentiation' theory. Although to produce such a large population of undifferentiated cells (20-35% of total cells) by cell dedifferentiation was thought to be very unlikely, it should nonetheless be tested and either proved or disproved. Due to the lack of methods to label planarian cells by radioactive substances (e.g. tritiated thymidine), chromosomal markers together with injection of purified neoblasts provided the key. The same nuclear and cytoplasmic markers from the asexual and sexual races of *S. mediterranea* employed to test their migratory capacities were used to test the regenerative and stem cell capabilities of partially purified neoblasts and differentiated cells (80 to 90% enrichment) when introduced, separately, into the parenchyma of irradiated hosts which have neither functional neoblasts nor mitotic activity (Fig. 3). The results obtained showed that when purified neoblasts are introduced into the host, mitotic activity resumes leading, in a fair percentage of hosts, to long survival and blastema formation (Baguñà *et al.,* 1989). On the contrary, injection of differentiated cells never gave mitotic recovery nor blastema formation. From these results it was concluded that the ease of recovery and regeneration of irradiated host was proportional to the number of neoblasts introduced and that, at least under the experimental conditions employed, differentiated cells were not able to rescue the host nor make it regenerate. A similar set of experiments and results were recently reported using enriched neoblast fractions from the OH strain of the planarian *Dugesia ryukyuensis* injected into irradiated hosts of the *menashi* (eye-deficient) mutant strain of the same species (Kobayashi *et al.,* 2008). Injection of OH neoblasts rescued the irradiated hosts and made them form normal eyes. Altogether, these results turned cell dedifferentiation, namely at the scale needed to produce a transient but large population of neoblasts, an unnecessary, unlikely and unproved mechanism, suggesting instead that neoblasts (or at least a subpopulation of them) in the intact organism were totipotent (or pluripotent) stem cells for the daily wear and tear and the main source of blastema cells in regenerating organisms (Fig. 1A).

The totipotent nature of planarian neoblasts was reinforced by the fact that injection of neoblasts from the asexual race of *S.mediterranea* to irradiated hosts of the sexual race while rescuing the host also transformed it into an asexual individual able to reproduce by fission but unable to reproduce sexually. Conversely, the introduction of neoblasts from the sexual race into irradiated asexual hosts transformed the latter into individuals unable to reproduce asexually and capable, after developing germ cells and the copulatory complex, to mate and lay cocoons (Baguñà *et al.,* 1989).

Coda

When these results were published in 1989 the bets for stem cells having a leading role in regeneration were at its lowest. Ironically, this was also when, in the wake of Till and McCulloch (1961) pioneering studies on the bone marrow and of Altman and Das (1967) demonstration of adult neurogenesis in the brain of guinea-pigs (contradicting Cajal's 'no new neurons' dogma), the field of stem cells began to explode onto the scene of biological research. From the 1990s and namely from the 2000s the number of publications on stem cells increased by an order of magnitude. As of today, no organs and tissues are known without proven or putative adult stem cells. As regards regeneration, new analyses of classical and new models have shown the increasing roles of adult stem cells together with the once-thought unique processes of cell dedifferentiation and transdifferentiation (Fig. 1C).

Last developments and the end of the story

The rescue of body integrity and homeostasis and the restoring of regenerative capacities of lethally irradiated hosts after the injection of purified neoblasts (Baguñà *et al.,* 1989) represented a very strong argument for the stem nature of neoblasts in both intact and regenerating planarians. However, it was also self-evident that a further proof of the exclusive (or even a main) role of neoblasts asked to label them permanently and follow the lineage of descendant differentiated cells. Finally, a ultimate proof contemplated the introduction of single neoblasts into irradiated hosts and testing whether all differentiated cell types were formed and body homeostasis and the ability to regenerate restored.

Whereas to label proliferative cells in non-triclad plathelminthes (germinative cells in cestodes; Bolla and Roberts, 1971; neoblasts in microstomids; Palmberg, 1986) was routine from the 1970s, the long-sought attempts to label planarian neoblasts had to wait the year

2000 when Newmark and Sanchez-Alvarado labeled proliferating neoblasts in *S.mediterranea* with the analog bromodeoxyuridine (BrdU)(Newmark and Sanchez-Alvarado, 2000). Short BrdU pulses followed by different chase times showed neoblasts to be the only cells to take the label and to form the blastema in regenerating organisms. Further, 1-2 days after chasing labeled neoblasts start to differentiate into several somatic cell types. Very similar experiments, with the added bonus of differentiation of labelled neoblasts into all somatic cell types, germ cells included, were reported for the plathelminth macrostomid *Macrostomum* sp (Ladurner *et al.,* 2000) (Fig. 4). A further improvement was the development of a fluorescent activated cell sorting (FACS) method for cells of *Dugesia japonica*, based on Hoechst staining for DNA content and Calcein-AM staining for cell size (Hayashi *et al.,* 2006). Comparing the cell sorting profiles of intact nonirradiated and X-ray irradiated organisms revealed the presence of two different X-ray sensitive cell fractions: X1 and X2. The first corresponded to S and M/G2-phase neoblasts; the second to an overlapping mixture of G1/G0 X-ray-sensitive cells (G1/G0 neoblasts, making 30-40% of X2) and X-ray insensitive cells (G0/G1 differentiating and differentiated cells; 60-70% of X2). A large third fraction made by pure X-ray-insensitive cells (XIS; G0/G1 differentiated cells) was also detected. The relative proportions of the three cell populations X1:X2:XIS were about 1:2:6, with X1+X2 close to the expected percentage (25-30% of total cells) of neoblasts for 6-8mm length intact worms found in cell maceration studies (Baguñà and Romero, 1981).

In parallel, a flood of molecular markers, labelling either all or most neoblasts or specific sets of them, were reported. Prominent among them were the homologues of the genes *piwi*, *vasa*, *nanos*, *pumilio*, *tudor*, *bruno*, *MCM2*, *pcna*, *cyclin B*, *cbc*, *innexin*, and many other found in microarrays studies and transcriptome and proteomic analyses (for further details see Reddien *et al.,* 2005; Rossi *et al.,* 2007; Oviedo and Levin, 2007; Eisenhoffer *et al.,* 2008; Fernández-Taboada *et al.,* 2011). These markers were used in the first single-cell simultaneous analysis of gene expression and cell cycle state of neoblasts using FACS and real-time PCR (Hayashi *et al.,* 2010). Most neoblasts analyzed expressed the *piwi* gene (now considered a general marker of neoblasts) together with different sets of genes in patterns related to the phases of the cell cycle (X1 at the S/G2-phase, and X2 at the G1-phase). This was the first demonstration that planarian neoblasts are, as expected, a heterogeneous set of cells made by different subpopulations co-expressing different sets of genes.

The final blow against cell dedifferentiation as a source of transient or permanent neoblasts in planarians came from experiments using sublethally irradiated planarians. In *Dugesia japonica*, sublethal X-rays doses (1-5 Grays) produce a dramatic reduction of neoblasts (mostly from the X1 fraction) 1-3 days after irradiation, followed by a *de novo* proliferation of radioresistant neoblasts (likely commited non proliferating neoblasts from the X2 fraction) situated at the ventral side close to the nervous system (Salvetti *et al.,* 2009). Proliferating neoblasts migrate and repopulate the dorsal parenchyma so that number of neoblasts is re-established 2 weeks after irradiation. These results were interpreted in terms of neoblast population plasticity: commited, radioresistant, non-proliferating neoblasts, re-acquire stem cell capacities and restore the complex neoblast system; in other words, like in Gremigni's experiments (Gremigni, 1988), determined neoblasts could step back and become non determined stem cells. More precise and definitive results came from experiments using sublethally (1750 rads) irradiated *S.mediterranea* (Wagner *et al.,* 2011). In some of these individuals, a few isolated *smedwi+* cells (neoblasts expressing the general marker *piwi*) survived and after exponential growth yielded, 2-3 weeks after irradiation, clusters of hundreds of *smedwi+* cells that restored body homeostasis and the ability to regenerate. Crutially, when a pulse of BrdU was given before sublethal irradiation, isolated and clustered *smedwi+* cells were also $BrdU^+$ cells, suggesting that cluster expansion results from division, by clonal growth, of existing *smedwi+* cells. If a process such as cell dedifferentiation from any differentiated (and non proliferative) cell had been the source of neoblasts it should have produced clusters of *smedwi+* $BrdU^-$ cells. Such cells or clusters were never observed. These observations, together with the rescue of lethally irradiated planarians by injection of single neoblasts reported by the same authors, represents the last nail in the coffin of cell dedifferentiation as the primary source of neoblasts in planarians.

Conclusion

The 114 years long rambling road from Randolph's 1897 description of planarian neoblasts to Wagner *et al.,* 2011 report showing the clonal potentialities of single neoblasts is an enlightened résumé of the difficulties (conceptual, technical, and sometimes ideological) found by generations of embryologists and developmental biologists to sort out the nature of cells building the lost parts during regeneration in a rather simple organism. Leaving aside technical advances, always playing to the advantage of last generations, the difficulties at the level of concepts and ideas were threefold. First, most researchers ignored the many ways evolution has had to attain or maintain a simple goal (to regenerate) in different organisms. Linked to it, the second important stumbling block was the naïve persistence of some (or most) researchers searching for a unique valid mechanism of regeneration from sponges to humans. Finally, the most important hindrance was to consider separately the rare process of regeneration (most organisms, even planarians, never or very rarely go into it, and just a few use it regularly) from the evergoing universal process of tissue and cell renewal.

Some current black boxes on planarian neoblasts to think about

In what follows I briefly summarize some important unknowns (black boxes) on planarian neoblasts. Being a personal recolection it might, inevitably, include some personal biases for which I am completely responsible. Needless to say other important black boxes have been left out for editorial reasons despite some of them (e.g. Somatic versus germline tradeoffs: the bearing of neoblasts on germ line stem cell production in semelparous and iteroparous reproduction and on the senescence/longevity/inmortality problem; Do dedifferentiation, transdetermination and transdifferentiation occur in planarian homeostasis and regeneration?; How many and where are located the true toti-, pluripotent neoblasts?; The hierarchycal cell lineage of toti-, pluripotent neoblasts: topographical, typological, or stocastic?; and how neoblasts acquire axial (AP, DV and bilateral) positional information from differentiated tissues/cells?), are equally or even more important that the items dealt with here.

The phylogenetic origin of neoblasts: did they result from a progenetic event?

Because they lack circulatory, respiratory or skeletal structures and because they bear a blind gut lacking an anus, Platyhelminthes were considered early derived bilaterians (Hyman, 1951); hence, of

considerable interest for the evolution of body plans, developmental processes, and regeneration. However, recent phylogenetic and phylogenomic analysis have shown the phylum Platyhelminthes to be polyphyletic with the Orders Acoela and Nemertodermatida (the Acoelomorpha) to be early divergent bilaterians (Ruiz-Trillo *et al.,* 1999) or derived deuterostomates (Philippe *et al.,* 2011), shifting the rest of plathelminthes (including the Order Tricladida) to a more derived phylogenetic position within the superphylum Lophotrochozoa of protostomian bilaterians (Baguñà and Riutort, 2004).

Shifting the bulk of plathelminthes to a derived position questions their primitiveness and simplicity and asks whether they are instead a simplified phylum derived from more complex ancestors. Indeed, although Platyhelminthes share the quartet-spiral type cleavage with complex lophotrochozoan phyla such as Annelida, Mollusca and Nemertea, they lack coelom and anus. These absences, for long considered indicative of primitiveness, could instead be interpreted as losses produced by a heterochronic process of progenesis (for a thorough revision of heterochrony, see Gould 1977). Under progenesis, development and growth stop in parallel to the early development of gametes and sexual reproduction. As a consequence, mature adults are miniatures of ancestors and keep their embryonic or larval features. Under this framework, the undifferentiated embryonic-like features and the high potency and plasticity of planarian neoblasts might be features of embryonic cells now within an adult body that was frozen out in a 'embryonic' state in the past by progenesis when compared with the ancestor they might have come from. However speculative, this proposal merits to be tested analyzing the expression of hindgut and coelomic markers in the embryonic development of the less derived (modified) plathelminthes groups (e.g. polyclad and catenulid flatworms).

The ontogenetic origin of neoblasts: where do they come from?

The embryonic development of most triclads, and more so in freshwater triclads, is extremely modified and difficult to study (for a classic description see Le Moigne, 1969; for a recent update see Cardona *et al.,* 2005). Despite the hurdles, when, where, and how neoblasts or neoblast-like cells originate during planarian development was studied in depth in the 1960s namely by Le Moigne (Le Moigne 1966, 1968, 1969), and in the last 10 years in a handful of papers. Even so, its current understanding, namely at the molecular level, is rather poor. Using histological staining techniques, electron microcscopy, X-ray irradiation, and assesing the regenerative power of embryos at different developmental stages, Le Moigne found that cells morphologically similar to neoblasts at the EM and highly rich in RNA, began to be found at the end of stage 4B, being numerous at stage 5 when a burst of mitotic activity occurs in blastomere-like cells which soon differentiate into the different tissues and organs of the late embryo (see also Cardona *et al.,* 2005). Importantly, from stage 4B on, X-irradiation of embryos do not prevent differentiation and hatching, but prevent regeneration after cutting. The logical outcome of these results is that from stage 4B on, undifferentiated neoblasts, more sensitive to irradiation, are killed, while those already determined to form the anlage of the main organs and tissues are spared, differentiate, and give a normal, albeit smaller, organism than control non irradiated embryos.

Although scores of neoblast-specific markers are on the shelves ready for use, very few of them have been employed to detect them backwardly in embryos (from hatchlings to late stage 8 and to earlier ones) during development. The markers used so far are the planarian homologues of germ cell genes: vasa-like (*vlg*), tudor-like (*tud*), and nanos-like (*nos*) genes from *Dugesia japonica* (Shibata *et al.,* 1999) and *Schmidtea polychroa* (Solana and Romero, 2009; Solana *et al.,* 2009). *Vlg*-like and *tud*-like genes are expressed in several blastomeres from stages 1-4, in most parenchyma cells (neoblast-like) from stages 5-7, and only in neoblasts from the stage 8. After hatchling, they are expressed in a restricted set of them; when ovaries and testes develop both genes are up-regulated, namely in spermatogonia and oogonia (*tud*-like) and in spermagonia, oogonia and their immediate products, spermatids and primary oocytes (*vlg*-like). In contrast, *nos*-like genes are not expressed during embryogenesis except in few neoblasts at the stage 8 which later on become the progenitors of adult germ cells. Depletion of *nos* by RNA interference (or RNAi; introduced into planarian research by Sanchez Alvarado and Newmark, 1999) eliminate testes, ovaries and germ cell precursors, but not neoblasts, suggesting a clear role for *nos* to determine the germ cell program and to repress the somatic one (see main references in Shibata *et al.,* 2010). Importantly, *vasa* and *nanos* are also expressed in the undifferentiated toti- or pluripotent cells in embryos and adults of sponges and cnidarians (Funayama, 2010; Extavour *et al.,* 2005).

Despite other markers, such as *piwi*-like, *PCNA*-like, *pumilio* (*pum*)-like, *Bruno*-like (*bruli*), etc,. are expressed in all or in discrete sets of neoblasts, their embryonic expression have unfortunately not been analyzed. Of those, *Piwi*-like genes are of particular interest. They are expressed in all cycling (neoblast) cells, being thus considered a general marker of planarian neoblasts (Reddien *et al.,* 2005). Moreover, they are essential for germline development and renewal, epigenetic regulation, suppression of phenotypic variation, and repression of transposable elements. They are also found and expressed in undifferentiated pluripotent cells in sponges (Funayama *et al.,* 2010) and some cnidarians (Denker *et al.,* 2008) that can give rise to gametes. Therefore, to study the expression of *Piwi* genes in planarian embryos seems a good approach to spot at which stage and from which cells and/or cell layer/s this key cell type arise during embryogenesis. Moreover, finding *Piwi*-like genes in planarian embryos will further support the proposal that during animal evolution the germline molecular program originated first from toti-, pluripotent cells in adult organisms (sponges, cnidarians, flatworms) able to give rise both to somatic and germ cells. This program was subsequently co-opted by more specialized germ cells either during embryogenesis (fly, frog, mouse, etc,..) or in larval and juvenile stages (sea urchin, snail, annelids) (Juliano and Wessel, 2010).

How is the size of a planarian controlled? The roles of cell birth and cell loss.

The sheer differences in size among species of freshwater planarians (from <5mm up to 50cm in length) make obvious that size is encoded in the genes, is species-specific, and depends on cell number and cell size. Because changes in cell size/volume barely represents around 10% of total body volume (Romero and Baguñà, 1991; Romero *et al.,* 1991), the actual size of a planarian could be expressed, after cell maceration, into total number of cells, in relation to body length, area, or volume. Therefore, changes in body size with time, under controlled conditions of temperature

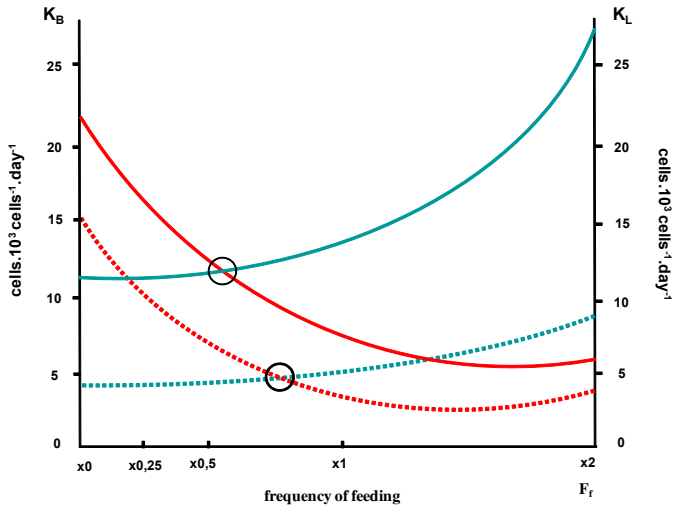

Fig. 5. Growth and degrowth in planarians related to length, temperature, and feeding conditions. *Comparative curves of cell birth (K_B, or cells born per 1,000 cells per day; green lines) and cell loss (K_L, or cells lost per 1,000 cells per day; red lines) for 3mm (solid lines) and 11mm (broken lines) long organisms of the planarian* Girardia tigrina *kept at 12ºC along one year at different feeding regimes (F_f: x0, no feeding; x0,25, once a month; x0,5, once every two weeks; x1, once a week; x2, twice a week). Large circles indicate, for each body length, the cross-points between K_B and K_L values where 'steady-state' body size holds if the frequence of feeding (F_f) is maintained. To the left of each cross-point, degrowth occurs; to the right, growth holds. Note that for 3mm long organisms the area where growth occurs is much larger and starts at lower feeding intakes (Ff) than that for 11mm long organisms; hence, the higher rates of body growth for 3mm long organisms. Instead, degrowth areas are slightly larger for 11 than for 3mm long organisms and start at higher feeding intakes (Ff); hence the higher rates of degrowth for 11mm long organisms. Importantly, during starvation (F_f = 0) K_B (cell birth or cell proliferation) is maintained at a basal level in both groups and, similarly, a basal level of K_L (cell loss or cell death) occurs even at optimal feeding regimes (F_f = x1 and x2). Based, with modifications, from Romero (1987) and Baguñà* et al., *1990).*

and feeding, could in cellular terms be expressed as the difference between new cells born by proliferation per 1000 cells per time and cells lost by death and apoptosis per 1000 cells per time (Baguñà *et al.,* 1990; Romero and Baguñà, 1988; Romero *et al.,* 1991). The peculiar neoblast system in planarians with a single proliferative type and 25-30 differentiated cell types turning over (by apoptosis and cell death) is, in kinetic terms, equivalent to the crypt-villae system of mammalian gut which bear a single stem cell and 4 different cell types turning over continuosly at different rates.

Two decades ago, a kinetic analysis was undertook measuring at weekly intervals along a year the plan area (in mm^2) of growing and degrowing organisms to calculate the changes in total number of cells per time unit (growth or degrowth rates). Moreover, a stathmokinetic method that blocks metaphase with colchicine, was used to calculate the number of cells born daily (cell birth rates or K_B; see Fig. 5). From the later and the rates of growth/degrowth the rates of daily cell death (cell loss rate, or K_L; see Fig. 5) were estimated. When applied to growth and degrowth of 5 species of planarians of different lengths grown at different temperatures and feeding conditions, the results were extremely informative (Baguñà and Romero, 1981; Romero, 1987; Baguñà *et al.,* 1990). Briefly: 1) as expected, cell birth rate is directly proportional to temperature and frequence of feeding, though the specific cell birth rate decrease with increasing body length; 2) contrary to expectations, neoblast proliferation (cell birth rate) is maintained at a basal, but sustained, rate during degrowth by starvation (e.g. 1500 neoblasts born per day in starving 3mm long individuals kept at 22ºC); 3) also as expected, the rate of cell loss (by cell death or autophagy) is directly proportional to temperature and inversely proportional to the frequence of feeding, and follows an exponential negative curve with body size; 4) again, as expected, a basal rate of cell loss is maintained even at optimal conditions of temperature and food availability (e.g. 1000 cells (likely differentiated) lost per day in well fed 3mm long individuals kept at 22ºC); and 5) importantly, the rates of body growth and degrowth and cell birth and cell loss, for a given length, temperature, and frequence of feeding, set the conditions when growth or degrowth occurred (Fig. 5) and the minimal and maximal sizes attainable. Finally, the maximal and minimal rates of cell birth and cell loss set the species-specific limits of cell proliferation and cell loss. These limits were both found to be higher for asexual and sexual populations of perennial (iteroparous) species than for sexual annual (semelparous) species and had a clear bearing on these reproductive strategies and regenerative abilities (Romero and Baguñà, 1988, Romero *et al.,* 1991; and see below).

Despite this kinetic simplicity and the availability of BrdU, PCNA, histone H3P and other markers of the cell cycle as well as the TUNNEL assay and other markers of apoptosis and autophagy (reviewed in González-Estévez and Saló, 2010), as well as scores of cell lineage-specific markers, no attempts have so far been produced to analyze in cellular terms (cell birth and loss) the kinetics of body size changes (growth and degrowth); only data on the role of cell death in regeneration, with short detours to apoptosis/autophagy during starvation, have been reported (Hwang *et al.,* 2004; González-Estévez *et al.,* 2007; Pellettieri *et al.,* 2010).

How is the neoblast pool size controlled during growth/degrowth? Its bearing on organ, tissue and cell type alometries as related to body size.

A main unsolved question in planarian growth and degrowth is how the size of the neoblast pool is controlled. The total number of neoblasts between 11mm and 3mm long *S.mediterranea* differs by an order of magnitude (from 400.000 to 40.000 neoblasts). How the increase in total number of neoblasts during growth and its decrease during degrowth is implemented? Conventional wisdom suggests that during starvation neoblast loss should be due either to neoblast cell death or more likely to direct differentiation of both of its daughter cells (symmetric differentiation) to replace the increasing number of differentiated cells turning over by apoptosis, leading to partial neoblast extintion. On the contrary, upon refeeding and growing after starvation, the increase in neoblast numbers could occur by two mechanisms: symmetric division of remaining neoblasts to give more of them (symmetric renewal) or, less likely though possible, by dedetermination (or transdetermination) of determined neoblasts back to stem neoblasts (e.g. Gremigni, 1988; Salvetti *et al.,* 2009). After degrowth, degrown organisms are very similar in terms of body length and volume, percentage of neoblasts, mitotic indices, and rates of regeneration to newborn individuals; in other words, they seem to be 'rejuvenated'. Whatever that means, this poses the intriguing possibility that toti-, pluripotent stem neoblasts are differentially selected (or kept) during degrowth. This could now be assessed checking a likely increase in the number of piwi$^+$ cells

or in telomere's lengths (Montgomery *et al.,* 2011).

Because feeding have such an important role for growth and degrowth in body size in planarians, the Insulin/Insulin Receptor (InR) and Target of Rapamycin (TOR) pathways are key pathways to be analyzed. The InR/TOR system coordinates cell growth and proliferation with the animal's nutritional status. If, as in other systems (Kaczmarczyk and Kopp, 2010), insulin peptides and receptors are key agents in stem cell maintenance and proliferation, it could be anticipated that loss of function mutants or RNAi against homologues to planarian genes for insulin, insulin receptors and transducers, or a reduced TOR signalling would lead, even in well fed animals, to a decrease in neoblast and germ stem cell (GSC) numbers and to enhance late reproduction and longevity. Conversely, constitutive or sustained activation of components of these pathways would lead to increasing numbers of neoblasts and GSC even during periods of severe starvation and to early reproduction and short lifespans. Indeed, RNA interference against *Smed-PTEN-1* and *Smed-PTEN-2*, the planarian gene homologues coding for a phosphatase that inhibits the activity of the insulin transducer PI3K (phosphatidylinositol-3 kinase), produce a hyperproliferation (x 2-3 of control levels) of neoblasts and the accumulation of postmitotic cells with impaired differentiation (Oviedo *et al.,* 2008). Rapamycin, an inhibitor or negative modulator of TOR activity, partially reverses this effect. This confirms that TOR and InR signalling work together to enhance neoblast proliferation above normal control levels in response to feeding. More work is however needed to detect all the planarian homologous genes from both pathways, to define its role and interactions in neoblast proliferation, and finally to link them to the cell kinetics of growth/degrowth in normal worms, to its reproductive strategies (iteroparity *versus* semelparity; Romero and Baguñà, 1988, Romero *et al.,* 1991), and to senescence, longevity and, however impossible to test and most likely incorrect, to the claimed immortality of asexual strains of planarians (Calow, 1978).

Moreover, the InR/TOR pathways are known to interact, likely indirectly, with morphogen gradients patterning the AP and DV axis of the whole body or of specific organs and tissues, which also control cell proliferation (Parker, 2011). Coordinating cell proliferation according to morphogen and nutrient levels should lead organs and tissues to scale with body size. It may be anticipated that neoblasts, either the stem cell and/or transit-amplifying populations, should integrate both types of signals (morphogens and nutritional state) to produce different outputs according to region, organ, and tissue features. In addition, differential sensitivities and gene variation to InR/TOR and to axial morphogens may hold the key to understand the organ and tissue alometries in planarians first described in detail by Marcel Abeloos (1930) in his excellent monograph on growth, degrowth and regeneration in *Planaria (*now *Dugesia) gonocephala*, and later on at the level of cell types in several planarian species (Baguñà and Romero, 1981; Romero and Baguñà, 1991).

The logic of neoblast lineage and its proliferative control. Which ones and where are the stem cell inhibitors?

Despite the decade elapsed since proliferating neoblasts were first labelled with BrdU (Newmark and Sanchez Alvarado, 2000; Ladurner *et al.,* 2000), the logic underlying the control of its proliferation is very poorly known. In other words, knowledge on the percentage and sites of residence of the true stem neoblasts, on how many transit-amplifying stages occur and where they are, and namely which is the structure and main features of the hierarchy leading from the true stem neoblast to the 25-30 differentiated cell types is almost non existent. And more specifically, are transit amplifying stages related to topography (e.g. anterior vs posterior; dorsal vs ventral; inner vs outer, etc,.) or to typology (ectoderm vs endomesoderm; endoderm (gut) vs mesoderm (parenchyma); epidermis vs nervous system; and so on) or to mixtures and combinations of them? Finally, whichever the actual lineage turns out to be, how is it regulated? Thus, besides activators of neoblast proliferation, are there inhibitors produced either by stem neoblasts, by their transit-amplifying populations, by differentiated cell types or by altogether to control and fine tune the increase and decrease of the whole pool or of particular lineages during regeneration and growth/degrowth? A set of related questions has recently been raised and partially answered by Gurley and Sanchez Alvarado (2008).

A tentative answer to this set of questions was recently advanced by Eisenhoffer *et al.,*2008). Examining the expression patterns of genes downregulated differentially in time after irradiation, they identified three main categories of genes: a) those downregulated very soon after irradiation and detected in small discrete cells throughout the animal but absent anterior from the photoreceptors and the pharynx (Category 1), very likely corrresponding to cycling neoblasts; b) those downregulated 24h postirradiation and expressed in cells slightly anterior to the photoreceptors (Category 2), likely being postmitotic neoblasts; and c) those downregulated by day 7 after irradiation and expressed in small cells closer to the organism margin than those of Category 2 (Category 3), and being either postmitotic neoblasts or cells in differentiation. Category 1 genes were found after FACS to be highly expressed in the X1 fraction (G2/M cycling neoblasts) and less so in some X2 cells (G0/G1 non cycling neoblasts). Instead, Categories 2 and 3 were expressed in some cells of fraction X2 and not in those of the X1 fraction. From these results they proposed a model (Fig 6 in Eisenhoffer *et al.,* 2008) in which centrally located Category 1 cycling neoblasts gives rise to more peripheral Categories 2 and 3 of nondividing neoblasts accompanied by corresponding changes in gene expression. In other words, the lineage from stem proliferating neoblasts to more determined non proliferating ones occurs in parallel to its progressive displacement from central to peripheral regions. This model, first applied to the anterior head border of the intact worm, was extended to the whole body where stem neoblasts located in the inner parenchyma close to the gut give rise by division to postmitotic neoblasts that migrate to the outer parenchyma and later on to subepidermal areas where they differentiate. A similar scheme was suggested to hold in anterior regeneration. Surprisingly the cell lineage model proposed contemplated a mere one-stage lineage, in which cycling stem cells produced non-cycling (non transit) population of neoblasts which, after migration, differentiated into several cell types.

However interesting, this scenario is likely an oversimplification. First of all, it only applies to the anterior head border but not to posterior and lateral borders where mitotic neoblasts occur well into subepidermal areas. Second, neoblast determination and differentiation also takes place in the inner regions (e.g. the gut) and at the base of the pharynx; therefore one should expect these three categories to occur well within the organism. Indeed, proliferating cells (neoblasts) have recently been described at the base of the gut lumen (Forsthoefel *et al.,* 2011), some of them co-expressing neoblast-specific and gut-specific markers. Third, and most importantly, the lineage suggested does not contemplate transit-amplifying stages. This is rather odd for a system based in a

putative single stem cell population from which 25-30 different cell types emerge. Therefore, it seems sounder to think of Category 1 neoblasts as a mixture of true stem cells together with several transit amplifying (hence proliferative) populations at different stages of their topological, typological or mixed determination. Identical arguments apply to Category 2; in that case, broadly-determined postmitotic cells could be sorted out to different fates by differential inhibition of complementary sets of active genes likely triggered by external cues. In any case, a final answer to this conundrum need deeper analyses combining BrdU labelling, the large set of markers so far identified in *S.mediterranea* using double and triple FISH, together with polyclonal and monoclonal antibodies (Bueno *et al.,* 1997; Robb and Sanchez-Alvarado, 2002), and RNAi of selected genes to spot and define the true stem cells, the transit amplifying cells and their subpopulations, and the final differentiated cell types.

Central to the logic of proliferative control is the replication probability, or p_o. If a stem cell undergoes obligatory asymmetric divisions (p_o= 0,5), one daughter enters differentiation and one remains a stem cell. In the alternative model, often called 'stochastic', stem cells have a mixture of asymmetric and symmetric divisions, the later producing either two stem cells (symmetric renewal) or two differentiating cells (symmetric differentiation). Although the all-asymmetric division model is seen as simpler and more perfect, it does not fit systems like planarians, in which the stem cell pool and transit amplifying populations expand and contract. Computer simulations of stochastic models (Lander *et al.,* 2009) point out the need for a tight control to obtain homeostasis and lineage control. This is best attained by negative feedbacks from differentiated cells modulating the replication probabilities (p_o) and the division rates (v_o) of transit-amplifying and stem cell populations, and from transit-amplifying on the p_o and v_o of stem cell populations, and even from stem cells onto themselves. In other words it is external, non autonomous, inhibitors on stem cell properties which are at a stake.

The search of inhibitory substances for planarian growth and degrowth, and by extension for regeneration, has received scarce attention in the last 30 years. The idea of negative feedback and inhibitory substances to regulate tissue size and enhance regeneration was introduced by Bullough in 1965, to refer to secreted factors that inhibit the growth of the tissues that produce and secrete them. Together with secretion of stimulatory factors, partial removal of a tissue or a body part reduces the levels of inhibitors resulting in upregulation of cells. Several factors, many of them belonging to the TGFβ superfamily, that exert negative feedback on the growth of skin, bone, brain, blood cells, retina, and hair (for references, see Lander *et al.,* 2009) have recently been uncovered.

Several approaches to uncover stem cell inhibitors could be envisaged. The first, and more obvious, is the gene candidate approach searching for homologues of members of the TGFβ superfamily in planarians, analyzing its expression in intact and regenerating worms and, eventually, the effects of its lack of function by RNA interference. A second, more focused approximation is looking at phenotypes of neoblast hyperproliferation produced by RNA interference (RNAi) of specific genes. In addition to those mentioned produced by RNAi to the members of the InR pathway *PTEN1, 2* of *S.mediterranea*, we could mention those produced by *Smed-p53* inactivation (Pearson and Sanchez Alvarado, 2010). *Smed-p53* is expressed in the stem cell progeny and not in stem cells proper. Its inactivation blocks the production of progeny and enhances the number and proliferative rates of stem cells; later on, stem cells are depleted. Although the precise mechanism of action of p53 in planarians is uncertain, it seems to act cell autonomously as it is for PTEN1,2, not being therefore good examples of extrinsic inhibitory factors. Interestingly, RNAi against the *Smed-egfr-1*, a gene coding for one of the receptors for Epidermal Growth Factor (EGF) in planarians, that seems expressed in gut cells, also causes a hyperproliferative phenotype (Fraguas *et al.,* 2011). *Smed-egfr-1 (RNAi)* upregulates *Smed-p53* and increase the numbers of *Smed-wi-1*$^+$ cells, a marker of stem neoblasts. These results suggest the presence of a negative regulator released from a differentiated cell type that control neoblast replication probability (p_0). Its absence due to inhibition by RNAi would increase the number of stem cells preferentially producing stem cells daughters (symmetric renewal) that could explain the increase in *Smedwi-1*$^+$ cells.

A third, more physiological approach, is to look for systemic signals that mediate short-range and long-range cues involved to control from axial polarity to neoblast homeostasis. The recent finding of gap junction proteins (innexins) specific for neoblasts and for nerve cells (Oviedo and Levin, 2007), together with the importance of nervous system integrity to transmit inhibitory signals from anterior to posterior regions to avoid duplication of existing structures, highlights the importance of those signals, whatever they turn out to be, to regulate stem cell behaviour in adult organisms (Oviedo *et al.,* 2010). Last but not least, the known effects of several ions, the role of ion channel proteins (Nogi *et al.,* 2009), the plethora of neuropeptides and their agonists and antagonists on neoblast proliferation (Saló and Baguñà, 1986; reviewed in Baguñà *et al.,* 1990), and of several signalling pathways (Wnt, BMP, Notch,..) acting on neoblast determination to specify axial polarity and regional identities (Gurley and Sanchez Alvarado, 2008; Reddien, 2011), also deserve further analyses.

Besides the proliferation probability, p_i, another key parameter involved in the expansion/contraction of stem cell and transit amplifying populations is the proliferative rate (v_i) of each lineage stage. While it is safe to asume that the later might be namely controlled by the levels of activators and inhibitors available and by the kinetics of cell cycle proteins, I would like to consider the likely influence from inhibitors produced by stem cells themselves. Two old observations, by Dubois (1949) and by Saló and Baguñà (1985b), are here instructive. In non-irradiated regenerating planarians, Dubois (1949) reported in regions near the wound during the first days of regeneration a 3 to 4-fold increase in neoblast proliferation when compared to non regenerating controls. However, in irradiated regenerating animals bearing an unirradiated graft, the number of mitoses of graft neoblasts in areas close to the wound escalated up to 10-12 fold compared to non regenerating controls. In other words, proliferative rates hinged somehow upon the number of neoblasts present.

Similar results were reported for mitotic rates in intercalary regenerates (blastemas) produced by non-irradiated head/non-irradiated tail combinations when compared to those produced by non-irradiated head /irradiated tail combinations (Saló and Baguñà, 1985b). While intercalary blastemas formed in both cases, the number of mitoses within the blastema and, namely, within the posterior (tail) pieces of non-irradiated head/irradiated tail combinations (tails have no neoblasts due to irradiation), was 6-8 fold compared to the 3-fold increase in blastemas in non-irradiated head/non-irradiated tail combinations (tails having a normal percentage of neoblasts). As in Dubois' observations, the most likely explanation is that lack of neoblasts in the irradiated posterior part allows there higher rates of proliferation when neoblasts from the

non irradiated head spreads into it. A molecular mechanism at the base of these results is only a matter of speculation: either more activators (from differentiated cells; nerve cells?) are available per neoblast, or inhibitors released by differentiated cells or by neoblasts themselves operate in a density-dependent way allowing more proliferation at lower neoblast densities and less proliferation at higher densities. Needless to say, a combination of both mechanisms may, in the end, be the most reasonable outcome.

The 'old' long-sought missing tools: in vitro long-term cultures of neoblasts and transgenics

Looking at the increasing number of groups involved in it (namely in the USA and Japan), the quantity and quality of new techniques introduced, and the scores of results produced, one has to conclude that the first decade of the 21st century represents the first golden period of planarian regeneration. A brief list of the molecular tools available attest this situation: BrdU and other cell labeling techniques, RNA interference, a plethora of regeneration stage-, area/region-, tissue, and cell-specific molecular markers used for expression analyses and RNAi perturbation, FACS technologies to isolate specific cells (namely neoblasts), transcriptional (RNA Seq) and proteomic profile analyses, sequencing the genomes of model species such as *S.mediterranea* and *D.japonica*, etc.

Even so, other key techniques have so far proved refractory to be introduced in planarian research. I briefly discuss two of them.

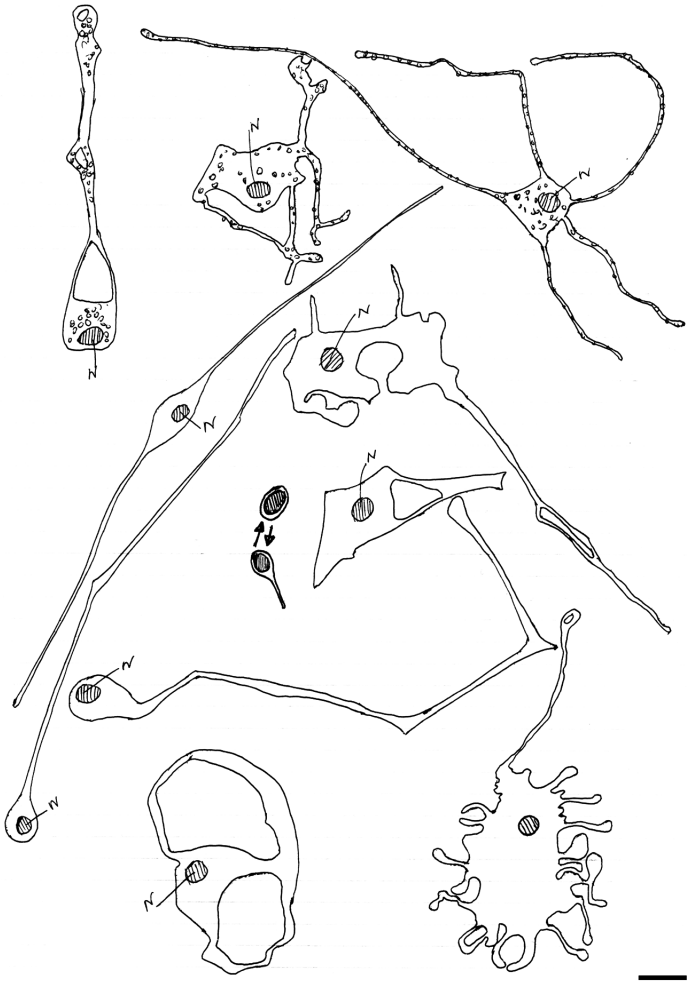

Fig. 6. **Planarian cells in culture.** *Hand-drawings of different parenchyma cell types of the planaria* Polycelis nigra *from a 4-day culture in DMEM-HAM F12 media supplemented with calf serum, grown at 20ºC between a coverslip and the bottom of a petri dish. In the center, two neoblasts (one bearing a filopodia, the other without) were drawn for comparison. Of note, most cells, some secretory (see inner granules) others not, have extensive, elaborate, and very dinamic cytoplasmic processes, and large vacuoles or even 'holes' within the cytoplasm. N (hatched), nuclei. Scale bars, 10μm (from J. Baguñà's neoblast culture notebook, Edinburgh 1973).*

In vitro long-term cultures (LTC) of neoblasts

For a stem cell system so powerful and versatile as planarian neoblasts, its culture *in vitro* is considered a must. A mere glimpse at the bewildering array of cell types and forms of planarian cells in culture (Fig. 6) support this need. No wonder several attempts, all failed because no permanent cultures have yet been achieved, have been published since the pioneering study of Murray (1927). She observed for a few days in culture different types of cells (gastrodermal, parenchymal, neoblasts, etc) and noted the prevalence of parenchymal cells and some changes in shape that she attributed to cell differentiation. Later attempts by Sengel (1960) and Ansevin and Buchsbaum (1961) culturing regenerative blastemas and isolated cells added not much, until Betchaku (1967) reported the first selective culture of neoblasts, now considered the first modern paper in the field. Disintegrating tiny fragments of *Dugesia* (now *Girardia*) *dorotocephala* by hypotonic treatment he noted that neoblasts, which are tough, less sensitive, cells to culture conditions, attached preferentially to the bottom of culture dishes while other cells, more sensible, remained less firmly attached or floated in the culture medium. After several rounds to discard and refill the media, the attached cells, mostly neoblasts, formed a sparse monolayer amenable to be studied and tracked using time-lapse recordings.

Betchaku produced two important observations. First, neoblasts in culture developed two cytoplasmic processes or filopodia in opposite directions, the longer one attaching preferentially to nearby neoblasts. Through repeated rounds of filopodia extension, attachment and contraction, neoblasts piled up to form small aggregates. These *in vitro* aggregates coalesced to form tridimensional large aggregates which at the slightest vibration or movement of the media detached from the substrate and floated in the medium. Interestingly, neoblasts *in vivo* are similarly distributed in clusters of different sizes, indicating its preferential attachment to each other, although they also occur single or in pairs. Second, the mechanics of neoblast movement and self-aggregation in culture were at odds with the extensive migrations postulated from graft-host experiments by the French School (Dubois, 1949) to explain blastema formation. According to Betchaku, neoblast movements towards the wound in regeneration organisms were not produced by active migration but by the cytolysis of parenchyma and gut cells near the wound and from neoblasts being carried to it by fixed parenchyma cells. While cytolisis of large cells near the wound is pretty obvious, the last assertion remains to be tested.

The four decades elapsed after Betchaku's seminal paper were spent to devise and optimize methods to isolate and culture neoblasts and other planarian cells (for short reference lists, see Schürman and Peter, 1988, and Asami *et al.,* 2002). Progress was, however, very scarce. Thus, despite several claims (most untested)

of weeks-long or months-long cell survival in culture, no primary cultures have so far been reported, neoblast proliferation has not been upheld beyond the first week in culture, and no clear cell differentiations nor cell lineages have been demonstrated. The only real advance reported was the enhancement of both the viability and the production of neurites from FACS isolated brain cells of *D. japonica* (Asami *et al.,* 2002) using different culture substrates (fibronectin, laminin, and poly-L-lysine).

Repeated failure to obtain neoblast LTCs likely hinge on the lack of suitable media (with specific growth factors included) and, namely, on finding proper substrate conditions. This is borne out by the easiness of neoblast proliferation when introduced, after purification (90% purity; Baguñà *et al.,* 1989) into non-irradiated or irradiated organisms, and namely by the recent report of repopulation of irradiated organisms by introducing a single neoblast (Wagner *et al.,* 2011). In both cases, neoblasts proliferate vigorously forming large colonies. In a very trivial sense, this points out that the proper combination of growth factors and substrates are right there.

A first avenue of research aimed at neoblast LTC is to use feeder layers onto which seed neoblast fractions or single neoblasts. One of the best, or likely the best, feeder layer available would be planarian differentiated cells isolated from X-ray irradiated organisms, a week after irradiation. The rationale is straighforward: this is, the substrate onto which injected neoblasts or single neoblasts best survive and proliferate (Baguñà *et al.,* 1989; Wagner *et al.,* 2011). A second and most sophisticated approach is to look at recent advances on *in vitro* regeneration of complex organs (e.g. mammary glands, gut crypt-villus, etc) from single stem cells. After a decade of failed attempts using conventional methods, mouse gut crypt-villus were recently succesfully grown *in vitro* (Sato *et al.,* 2009) using: 1) a growth media made by a mixture of DMEM and F12 media containing a coctel of growth factors based on previously defined insights in the growth requirements of intestinal epithelium (Wnt or Wnt agonists, epidermal growth factor (EGF), noggin, a Rho-kinase inhibitor, and Notch-agonist-peptides); and 2) as a substrate, either in plastic petri dishes or in microwells, the laminin-rich Matrigel (BD-Biociences) that supports three dimensional epithelial growth, previously used succesfully for the growth of mammarian epithelium from single stem cells (Stingl *et al.,* 2006). From single crypt-villus stem cells (isolated by FACS), organoids consisting of up to 40 crypt units, bearing all four differentiated cell types in position and percentages similar to *in vivo* crypts, were maintained for more than 8 months in culture.

The easiness to isolate single neoblasts by FACS, the increasing knowledge on factors stimulating neoblast proliferation, and the previous report by Asami *et al.,*2002) on the benefits of several EM substances on planarian neuron maintenance and viability, represent strong arguments to undertake similar experiments to obtain neoblast LTC.

Transgenics

In classic genetic analysis, the complementary technique to loss-of-function (in short, lof) mutants is the induction and detection of gain-of-function (in short, gof; dominant) mutants. This type of mutants produce overexpression of an otherwise normal gene in the same tissue or cells, or the expression of normal genes in different organs, tissues or cells (ectopic or heterotopy), or at different times (heterochrony) compared to WT organisms. Gof studies, albeit temporal, are also performed by injection of synthetic mRNAs in eggs and embryos. Lof mutants give valuable information as regards the normal function of genes and the phenotypic effects of their absence; gof mutants and functional gof studies perturb the system and produce phenotypes useful to understand normal mechanisms of cell differentiation, pattern formation and, in the long run, evolution.

Classical genetic analysis is so far not amenable in planarians, and in any platyhelminth. This hindrance was partially relieved when RNA interference (RNAi) was first succesfully applied to produce temporal lof mutants (Sanchez Alvarado and Newmark, 1999), to analyze stem cell biology and planarian regeneration. From it, scores of reports and a wealth of very useful information were produced in the last decade on stem cell biology, cell differentiation, and pattern formation. As of today, however, gof studies by injection of RNAs or proteins are not amenable in planarians owing to its large and non permeable eggs, to its syncitial early embryonic stages, and to the short lifetimes of most mRNAs and proteins. Only the injection of planarian mRNAs in surrogate amphibian (usually *Xenopus*) embryos has allowed to produce functional analysis of several planarian genes (e.g. Molina *et al.,* 2011). On the other hand, planarian lines bearing permanent reporter genes (GFP, LacZ) or planarian genes under the control of universal or cell- or tissue-specific promoters to monitor cell lineage, cell movements, cell differentiation, and pattern formation, as well as to produce gof mutant lines; in other words, transgenics, are not yet available.

Efforts to introduce transgenes into planarian species were initiated in the late 1990s. Ultimately, permanent expression of a universal enhanced green fluorescent protein (EGFP) with several *Pax6* dimeric binding sites (3xP3) was achieved using the transposons *Hermes* and *piggyBac* by microinjection into the parenchyma of the sexual race of *Girardia tigrina* and subsequent electroporation. After repeated rounds of cutting and regeneration, transformed neoblasts gave rise to differentiated photoreceptor cells expressing EGFP (González-Estévez *et al.,* 2003). Transformed neoblasts also gave rise to germ cells from which pure transgenic lines were obtained and kept for one year when, for unknown reasons, EGFP expression was silenced. Another set of transgenic lines were established using the *Hermes* transposon and the autophagy gene *Gtdap-1* under the control of 3xP3. Such lines produced a gof phenotype of recurrent autolysis in the cephalic region (eyes included) (González-Estévez *et al.,* 2007). In the long run, though, such lines could not be kept. Meanwhile, all attempts to produce transgenic lines in *S. mediterranea* have failed, this species apparently being more sensitive to electroporation protocols than *G. tigrina* (E. Saló, personal communication).

Similar efforts in cnidarians, which bear a stem-cell type functionally similar to neoblasts (the interstitial cells), did recently succeed, after 20 years of failed attempts, by microinjection of plasmid constructs into *Hydra vulgaris* embryos at 2- or 8-cell stage (Wittlieb *et al.,* 2006) and to fertilized eggs in *Nematostella vectensis* (Renfer *et al.,* 2010). In both cases, plasmids instead of transposons were used, mosaic animals obtained, and pure lines developed after 3-6 months. In *H. vulgaris*, an actin promoter-EGFP construct was used. After clonal propagation of the polyps, it was found to label all endodermal stem cells. This allowed to study the movement, migration, and proliferation of this cell compartment during regeneration. In *N.vectensis*, a *Myosin Heavy Chain type II* gene (*MyHC1*) promoter was linked to the fluorescent reporter *mCherry*, and faithfully reproduced the expression pattern of the

MyCH1 gene in the retractor and tentacle muscles. These lines were used to monitor muscle differentiation and reorganization during head regeneration. Current transgenic work in cnidarians focuses on the isolation of other gene-specific promoters and their functional dissection, on the development of ubiquitously expressed or inducible promoters to target gene functions late in development, and to identify new enhancers by random insertion into the genome.

The non amenability of planarian eggs and its extremely derived embryonic development to microinjection makes compulsory the use of neoblasts as cell vectors to obtain transgenic lines. A likely protocol, suggested more than 20 years ago (Fig. 4 in Baguñà *et al.,* 1990) could now be closer to reality thanks to the reported re-population of irradiated body fragments by the injection of a single neoblast (Wagner *et al.,* 2011). Briefly, plasmid or transposons bearing reporter or specific genes driven by specific promoters could be electroporated into single neoblasts in microwells. After selection and, hopefully, some rounds of cell division in culture, clonal (or just single) neoblasts could be injected into small prepharyngeal pieces of irradiated hosts. Then, neoblasts proliferate and repopulate the irradiated hosts and makes them regenerate. After regeneration, remodelling, and growth, the expression of reporter or specific genes could be assessed by the wealth of techniques to identify gene expression, and stable transgenic lines identified. A similar protocol could be used to identify, as already suggested (Baguñà *et al.,* 1990), new enhancers by random insertion of gene constructs into the genome of neoblasts.

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