Planarian Stem Cells: Pluripotency Maintenance and Fate Determination

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Abstract: Basic molecular mechanisms that orchestrate stem cell maintenance and fate are widely conserved across kingdoms, allowing for cross-species studies from simple model systems to mammals. In this context, planarians offer extraordinary possibilities containing a reservoir of experimentally accessible adult pluripotent stem cells, "the neoblasts". Indeed, in vivo reverse genetic manipulation of crucial neoblast regulators allows a fine study of adult stem cell fate in their natural environment. Recent extensive transcriptomics analysis revealed that planarian neoblasts are a widely heterogeneous population including clonogenic and lineage-committed stem cells, constituting a dynamic compartment that talks with differentiated tissue for proper physiological homeostasis and tissue regeneration. In this chapter, we review, in a chronological perspective, the most recent findings in the comprehension of neoblast biology, including their embryonic origin, and compare the most accredited models of pluripotency maintenance and fate determination.

1. Introduction

You can hurt them, cut them, or even decapitate them, they will rapidly heal and regrow. This is not a mythological tale, nor is it a sentence of a fantasy book; this is the truth for regenerating organisms, especially planarians, flatworms of the phylum Platyhelminthes (Box 1). The ability to reconstitute missing body parts through the formation of a transient mass of undifferentiated cells, i.e., the epimorphic regeneration, relies on the coexistence of three fundamental factors: (i) a pluripotent reservoir of stem cells that will produce the "bricks" to form the blastema mass; (ii) a sophisticated molecular machinery to address undifferentiated cell fate and de novo tissue morphogenesis; (iii) a permissive inflammatory status that favors regeneration versus scarring. All these features enable planarians to rebuild an entire organism with perfect novel organs from almost any tiny piece of their body. The presence of a pluripotent reservoir of stem cells and active body patterning cues allows for continuous turn-over of specialized cells and tissue homeostasis also in intact organisms, thus making planarians virtually immortal.

Box 1. Planarian—an overview.

"Planarian" is the generic name applied to free-living members of the order Tricladida of the phylum Platyhelminthes (the flatworms) (Sluys et al. 2009). A new higher classification of planarian flatworms (Platyhelminthes, Tricladida). Planarians are unsegmented acoelomates included in the Lophotrochozoan clade with bilateral symmetry and possess all three germ layers. They have a clear anteroposterior polarity with a head and a tail and are usually dorso-ventrally flattened. A mesenchyma intercalates among the various organs. The nervous system is composed of two cephalic ganglia connected to various sensory structures of the anterior part of the head and to two ventral longitudinal nerve cords, linked by commissural neurons and connected to a submuscular plexus that runs beneath the body wall musculature. Among sensory structures, the planarian eye is composed of two cell types, pigment cells, and photoreceptors. Pigment cells organize to form a cup-shaped structure, photoreceptors located at the opening of the pigment cup project their dendrites into the cup and their axons to the cephalic ganglia. Photoreceptor dendrites terminate with multiple microvilli-like structures called rhabdomeres and contain the photoreceptive molecule opsin.

The muscular system is organized into longitudinal, diagonal, and circular muscle fibers. In the midline of the animal, there is a muscular extensible organ, called the pharynx, connected to the digestive system, composed of three gut branches—one directed in the anterior part of the animal and two toward the tail region. The excretory system includes flame cells that remove unwanted liquids from the body by passing them through ducts, which lead to excretory pores on the dorsal surface of the body. The main nitrogenous waste product is soluble ammonia; thus, they are referred to as ammoniotelic. They lack circulatory, respiratory, and skeletal structures.

Freshwater planarians reproduce either asexually by transverse fission, generating two identical organisms (clones) or sexually as cross-fertilizing hermaphrodites.

If a planaria is cut, shortly after the amputation an unpigmented outgrowth, named the regenerative blastema, is observed near the site of injury, and cells within this structure will differentiate and spatially reorganize to restore the preexisting missing body part. Normal body proportions are attained after 3–4 weeks of regeneration. Freshwater planarians are easy and cheap to maintain in the laboratory and several species are used as model systems for cellular and molecular biology studies, in particular *Schmidtea Mediterranea* and *Dugesia japonica* species, belonging to the sister genus *Schmidtea and Dugesia*, respectively. Both species have excellent regenerative abilities, and clonal strains originating from single animals are used. Results from studies using either *S. mediterranea* or *D. japonica* are assumed comparable also in light of the preliminary *D. japonica* cell type atlas, which demonstrates that the two species share similar cell types in relatively comparable abundances (García Castro et al. 2021). Gene names in the planarian literature carry a prefix designating the species (i.e., Smed for *S. mediterranea* and Dj for *D. japonica*). Additional species might offer further features useful to understand complex patterning phenomena such as the *Dendrocoelum lacteum*, which is a regeneration-deficient planarian species in that its tailpieces are unable to regenerate a head and ultimately die. Indeed, downregulation of *Dlac-β-catenin-1*, the Wnt signal transducer, enables tailpieces to fully regenerate functional heads, rescuing *D. lacteum's* regeneration defect (Liu et al. 2013). An integrated web resource of data and tools to mine Planarian biology, PlanMine database: http://planmine.mpi-cbg.de/ (accessed on 18 July 2021)) has been created collecting all transcriptomics and genomic data and allowing for comparative analysis of flatworm biology (Rozanski et al. 2019).

From a research point of view, planarians represent a *"laboratory platform"* in which the most complicated cellular and developmental phenomena are continuously recapitulated in an in vivo context, thus offering the possibility to gain information about molecular regulatory mechanisms, cell-to-cell cross-talk, epigenetic phenomenon, ECM–cell interactions, and morphogenesis of tissues and organs (Figure 1).

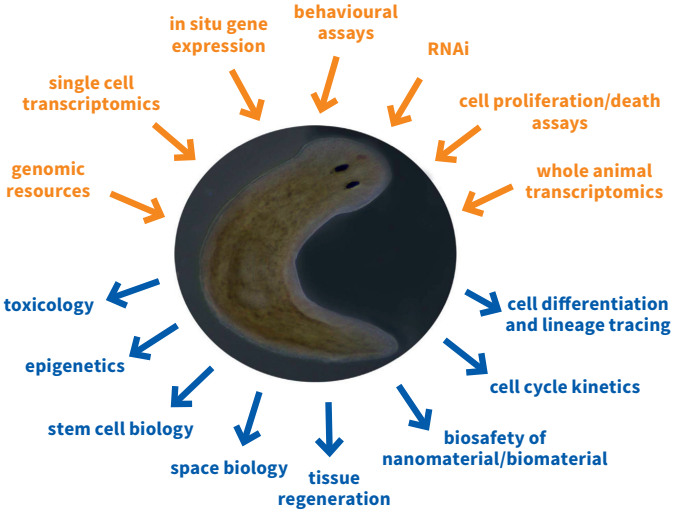

Figure 1. Scheme depicting the potential uses of the planarian model system. Orange arrows indicate methodological tools, while green arrows indicate some research fields. Source: Graphic by authors.

For this reason, this model system accompanies scientists since early 1900 up to nowadays despite, as perfectly described by Jaume Baguñà (2019) in his personal commentary, with "evident stumbling blocks due to hidden complexity and technical unfriendliness of planarians which explain why this model lagged, and still lags, behind other regeneration models and why and how they baffled and still baffle us" (ibid., p. 9). Today, most of the technical challenges have been overcome: interactive genomic/ transcriptomics databases are available (PlanMine database: http://planmine.mpi-cbg.de/ (accessed on 18 July 2021)) (Rozanski et al. 2019), even in the form of a single-cell atlas (Available online: https://digiworm.wi.mit.edu/ (accessed on 18 July 2021)) (Fincher et al. 2018; Zeng et al. 2018); RNAi is a widely used and validated technique (Sánchez and Newmark 1999); molecular markers for most of the differentiated tissues have been identified; protocols for several cellular assays have been successfully developed. Thus, in the last decade, molecular research in the planarian field jumped forward, revealing an extraordinary articulated cellular system in which multiple different specialized cell types, several postmitotic progenitors, and

a complex population of stem cells, generally referred to as "the neoblasts", interact to orchestrate perfect physiological homeostasis and tissue regeneration program. Here, we review, in a chronological perspective, the most significant findings in the comprehension of neoblast biology, including their embryonic origin, and compare the most accredited models of pluripotency maintenance and fate determination.

2. The Clonogenic Neoblasts

All the neoblasts share a similar morphology and show the presence in their scanty undifferentiated cytoplasm (Figure 2A) of the so-called chromatoid bodies, electron-dense non-membrane-bound aggregates rich in RNA (Coward 1974). Requirements used nowadays to define a cell as a neoblast are widely described in Alessandra and Rossi (2019). Among them, the expression of PIWI-encoding genes (*smedwi-1* for *Schmidtea mediterranea* and *DjPiwiA* for *Dugesia japonica*) (Figure 2B), X-ray sensitivity (Figure 2C), and their proliferating activity (Figure 2D).

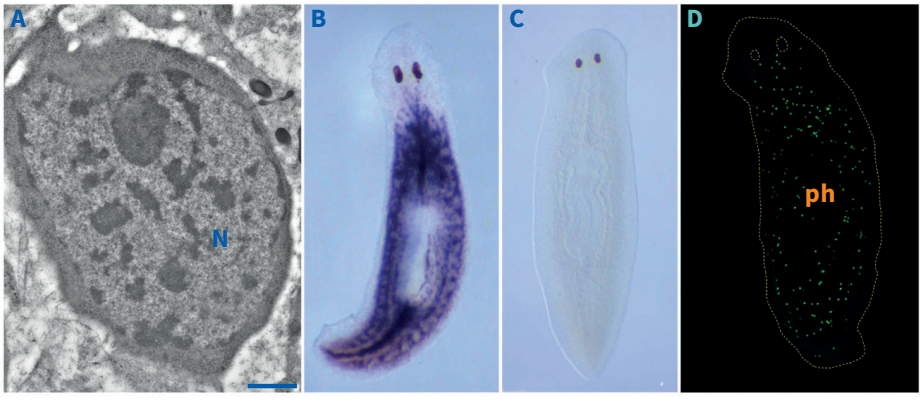

Figure 2. Neoblast features: (**A**) electron micrograph of a neoblast, mitochondria are highlighted in yellow. N—nucleus; (**B**,**C**) distribution of *DjPiwiA*-positive cells visualized by whole-mount in situ hybridization in wild-type (**B**) and in lethally irradiated (30 Gy) animals, 3 days after treatment; (**D**) phospho-H3 immunolabelling shows that proliferating cells are distributed throughout the entire planarian body with the exception of the pharynx and the anterior part of the head, especially behind the eyes. ph, pharynx. Scale bar corresponds to 800 nm in A and to 500 μm in (**B**–**D**). Source: Graphic by authors.

Despite these shared features, the neoblast population appears transcriptionally heterogeneous, as widely discussed in (Alessandra and Rossi 2019), and the discovery at the beginning of this century of the existence of some neoblasts able to resist low-dose X-ray treatment and repopulate the entire organism (Salvetti et al. 2009) opened the path toward the development of sophisticated assays

owing to which some secrets of these extraordinary cells have been unveiled. A question that remained unsolved for several years was whether regeneration or tissue homeostasis was accomplished by pluripotent cells or by the cooperative activity of multiple lineage-committed cell types. In 2011, a breakthrough was achieved by an elegant paper of the Reddien's group (Wagner et al. 2011) in which by coupling ionizing radiation and single-cell transplantation, they demonstrated the existence of neoblasts that could give rise to progenies covering different germ layers and restore regeneration in lethally irradiated hosts. These pluripotent cells were defined as clonogenic neoblasts (the cNeoblasts).

2.1. From σ Neoblasts to Deep Clustering by Single-Cell Transcriptional Profiling

cNeoblasts, initially simply defined as a subpopulation of the *smedwi-1*$^+$ cells, became the object of intense studies to try to characterize their molecular signature. Accordingly, the Reddien's group in 2014 identified, by a single-cell qRT-PCR assay, three prominent types of neoblasts—the ζ (zeta), the γ (gamma), and the σ (sigma) neoblast classes. σ-neoblasts proliferate in response to injury, possess broad lineage capacity, and can give rise to ζ-neoblasts, thus suggesting them as ideal candidates to include the cNeoblasts (van Wolfswinkel et al. 2014). The same authors also provide the observation that the conversion of the transcriptional profile from σ- to ζ-neoblasts begins directly upon entry into the S-phase. Indeed, the transcriptional profile of early S-phase ζ-neoblasts was more similar to that of G1-phase σ-neoblasts than to that of the G1-phase ζ-neoblasts, and that the ζ-neoblast identity became more resolved during progression through S-phase stages. Once produced, the majority of recently divided ζ-neoblasts are thought to exit the cell cycle permanently (van Wolfswinkel et al. 2014) and are not able of subsequent series of cell division and self-renewal (Lai et al. 2018).

Further evidence supported that σ-neoblasts might be the only neoblasts able to indefinitely proliferate (Lai et al. 2018) and, as a matter of fact, *Smed-soxP-1*, one of their molecular markers, is involved in stem cell self-renewal and is required in the rescue process after low-dose X-ray treatment for colony expansion (i.e., the ability of *smedwi-1*$^+$ cell colonies, formed by radioresistant neoblasts after low-dose X-ray, to grow in size), strengthen the idea that σ-class neoblasts include cNeoblasts (Wagner et al. 2012). The discovery of σ-neoblasts has led scientists to imagine a well-defined population of stem cells with its own molecular signature endowed with pluripotency. However, later, the expression of some σ-neoblasts molecular markers was found to be dispersed across all the neoblast classes identified by van van Wolfswinkel et al. (2014), unlike the ζ marker *zfp-1* and γ marker *hnf4*, which are largely specific to their respective classes (Molinaro and Pearson 2016). For this and further additional reasons, Molinaro and Pearson (2016) wondered whether σ-neoblasts were a truly distinct neoblast class or simply a collection of non-ζ and

non-γ cells. Accordingly, in a few years, advances in single-cell transcriptomic rapidly brought to light that σ-neoblasts are a heterogenous population themselves, not a single well-defined neoblast class. Indeed, both Fincher et al. (2018) and Zeng et al. (2018), focusing on the idea that *smedwi-1* differential expression levels might represent a discriminatory parameter for subclassifying neoblasts, identified several neoblast subclasses. Making the assumption that cNeoblasts might be included in the stem cell fraction with the highest level of *smedwi-1* transcript and its coded PIWI-1 protein, Zeng et al. (2018) identified a cluster (Nb2) that satisfies a series of selection criteria (expression of σ-neoblast markers and self-renewal regulators; negativity for fate specific transcription factors (FSTFs); increased expression of cluster markers within hours after amputation; decline in expression of cluster markers up to 6 days after sublethal irradiation with a markedly increased and sustained expression from 6 days after irradiation onward) was proposed to include the cNeoblasts. Nb2 cells show an enriched expression of *tspan-1* coding for the cell surface protein tetraspanin 1 (TSPAN-1).

Some concerns can be raised on the assumptions the author made delineating their strategy. First, the reason for which cells with the highest expression of *smedwi-1* should be considered as those that might contain cNeoblasts is a limiting assumption. For example, it is clearly a not sound strategy for *D. japonica* (the other principal planarian model system) in which both *DjpiwiA* and its coded protein show a very high expression in a dorsal midline population of neoblast-like cells that do not satisfy the previous selection criteria. Second, the "a priori" exclusion of clusters expressing FSTFs bias the analysis pre-assuming that a cell at the beginning of its commitments cannot revert its fate.

A common feature of adult pluripotent stem cells is that their self-renewal potential is proportional to their state of quiescence or deep dormancy (Post and Clevers 2019). This makes sense from an evolutionary point of view, as cell cycling exposes cells to propagate accidental DNA damage to daughter cells and future generations. Quiescent cells maintain DNA integrity and reenter the cell cycle only under appropriate stimuli.

A long debate on the existence of neoblasts with different cycling rates or on the length of the different cycle phases has characterized the 20th century. This question appeared definitely closed with the finding that up to 99% of neoblasts are labeled by BrdU in 3 days after treatment (Newmark and Sánchez 2000). However, very recently, the presence of a slow-cycling population of neoblasts, with low transcriptional activity (RNA^{low}neoblasts), has been identified and proposed as a regeneration-reserved neoblast population (Molinaro et al. 2021). RNA^{low}neoblasts show many characteristics reminiscent of quiescent stem cells, including very small size, slow division rate, and similarities in gene expression profile. RNA^{low}neoblasts undergo morphological changes after injury or low-dose X-ray and enter the cell

cycle during regeneration by a TORC1-dependent mechanism (Molinaro et al. 2021). A small fraction of RNA^{low}neoblasts expresses the tspan-1 marker, suggesting that some of them are part of the N2b cluster. Diverging lineage markers were often detected within individual RNA^{low} neoblasts, suggesting that some of these cells may not be specified to any one lineage (Molinaro et al. 2021). Further studies are necessary to characterize this novel subpopulation and its relationship with cNeoblasts and/or other neoblast subpopulations.

2.2. The Neoblast Fate Restriction Model

The last 15 years of scientific research in the planarian stem cell field was dominated by the line of reasoning that a clear hierarchical organization exists between neoblast subpopulations, with pluripotent stem cells (the cNeoblasts) giving rise to neoblasts with a restricted potency, the so-called lineage-committed neoblasts (Figure 3, left side). The first evidence of the existence of lineage-committed neoblasts was provided in 2006 owing to the work of Sato et al. (2006) that identified, in asexual *D. japonica*, germline stem cells that specifically express a *nanos*-related gene (*Djnos*), localized in the presumptive ovary or testis-forming regions, and morphologically indistinguishable from neoblasts. Although these $Djnos^{+}$ cells highly express the PCNA protein, they are blocked in the cell cycle and incapable to incorporate BrdU. Following the discovery of epidermal-committed ζ-neoblast and gut-committed γ-neoblasts (van Wolfswinkel et al. 2014), intense research was focused on identifying FSTFs that were also expressed in $smedwi\text{-}1^{+}$ cells and, thus, probably involved in neoblast commitment versus a specific lineage. In this way, putative neoblast precursors for cells of the eye, protonephridia, nervous system, pharynx, anterior pole, and gut were identified (Scimone et al. 2011, 2014a, 2014b; Lapan and Reddien 2012; Currie and Pearson 2013; Cowles et al. 2013; Adler et al. 2014; Vásquez-Doorman and Petersen 2014; Flores et al. 2016). Recently, Plass et al. (2018) performed highly parallel droplet-based single-cell transcriptomics and by applying a partition-based graph abstraction algorithm, combined with independent computational and experimental approaches, derived a consolidated lineage tree that includes all identified cell types rooted to a single stem cell cluster. In this tree, they identified gene sets that are co-regulated during the differentiation of specific cell types, thus providing a single tree that models stem cell differentiation trajectories into all identified cell types of adult planarians. According to the consolidated lineage tree, neoblasts differentiate into at least 23 independent cell lineages and several progenitors have been identified. In addition to all these putative subpopulations, in the planarian species, *D. japonica* a spatially well-defined abundant group of cells that show morphological features of neoblasts, are sensitive to irradiation, express *DjpiwiA* transcripts and genes involved in cell cycle progression, and is localized in the dorsal midline. This population is specifically identifiable by the expression of *DjPiwi-1* (Rossi et al. 2006, 2008), a *piwi*

homolog gene that has been found in planarians from the *Dugesia* genus and not in *S. mediterranea* and *Girardia dorotocephala* (Kashima et al. 2020). The function/fate of *DjPiwi-1*$^{+}$ cells is still unknown; however, we recently demonstrated that they are part of a population of *soxP-1*-negative lineage-committed neoblasts that, as a consequence of their very slow-cycling rate, are transiently resistant to continuous high-dose 5-fluorouracil (5FU) treatment (Gambino et al. 2020). In case of short low-dose 5FU treatment, cells of this dorsal midline subpopulation never disappear, activate proliferation after cutting but never change their expression pattern, remain negative for *soxP-1*, and do not seem to contribute to the repopulation process (Gambino et al. 2021). On the contrary, *DjPiwi-1*$^{+}$ expression appears to be associated with cells reentering the cell cycle at the ventral surface of the animal in challenging conditions, as demonstrated after a short 5FU low-dose and sublethal X-ray treatment (Gambino et al. 2021; Salvetti et al. 2009). The recent advances in single-cell transcriptomics in *D. japonica* species (García Castro et al. 2021) will allow more information to be obtained on *DjPiwi-1*$^{+}$cells of the dorsal midline, which may represent a valuable resource for the understanding of planarian stem cell biology.

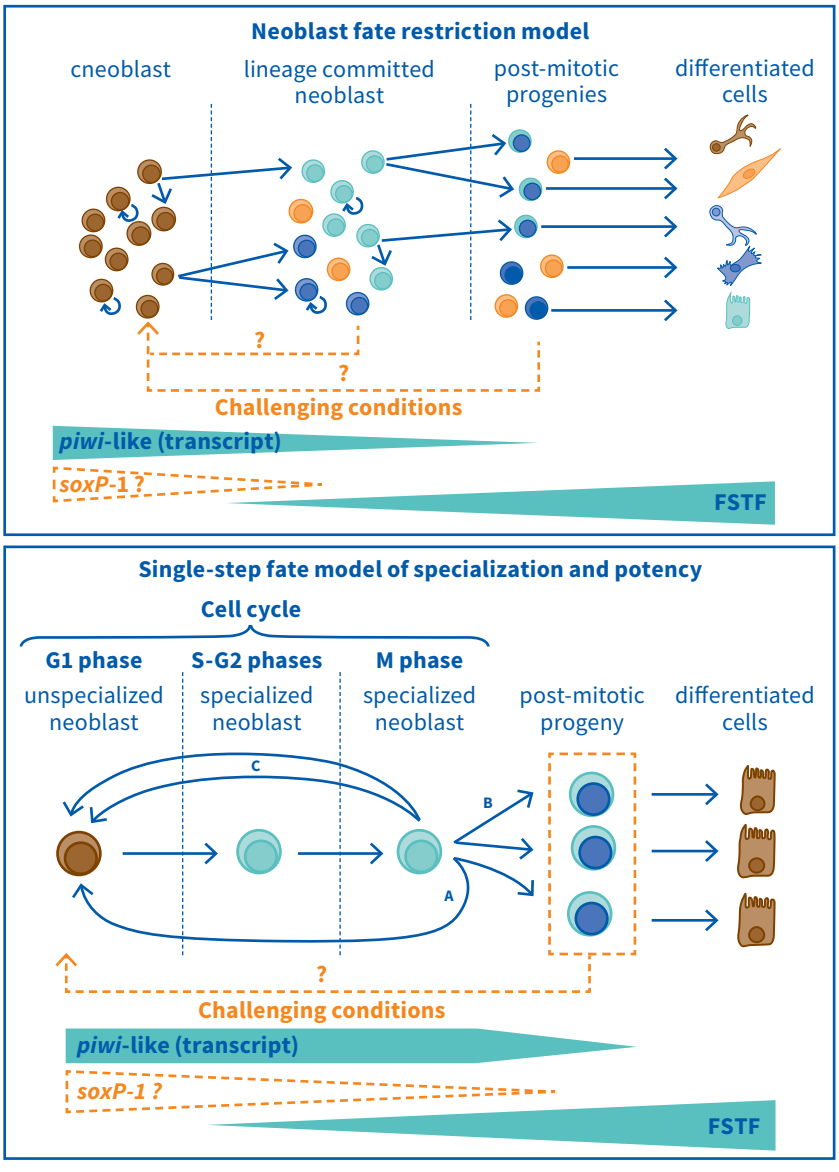

Figure 3. Scheme depicting the comparison between the neoblast fate restriction model and the single-step model of specialization and potency. In the first, the cNeoblasts can symmetrically self-maintain or asymmetrically divide to give rise to a daughter cNeoblast and a daughter specialized neoblast or even symmetrically divide to give rise to two specialized daughters. It is not clear how many times specialized neoblasts can divide, but in any case, they shortly produce postmitotic progenies, which gradually differentiate into a specialized cell. *piwi-like* gene transcripts are highly expressed in cNeoblasts, and their expression gradually declines in parallel

to fate restriction being very low or undetectable in postmitotic progenies and differentiated cells. Although *soxP-1* expression has been found to span several *piwi*-positive subclasses of neoblasts, several lines of evidence suggest that its expression is limited to pluripotent stem cells and declines in specialized neoblasts. FSTFs are specifically expressed in specialized neoblasts. In challenging conditions, including sublethal X-ray doses, short (5FU) treatment, and regeneration for some sexual planarians, bodies of evidence suggest that postmitotic cells or at least specialized neoblasts can revert their fate and acquire a wider differentiation potency. In the single-step model of specialization and potency, an unspecialized G1 neoblast become specialized, progressing through the cell cycle and starting to express FSTF from phase S. Concomitantly, a reduction in *soxP-1* expression could be hypothesized. Following the G2 phase, in many cases, an asymmetric division (A) generates an unspecialized neoblast, which will progress through a novel cell cycle, acquiring the same or a different specialization, and a postmitotic *piwi*-*soxP-1*-negative progeny that will differentiate. In other cases, a symmetric division can generate two unspecialized neoblasts (C) or even two postmitotic cells (B). It cannot be excluded that in challenging conditions, early postmitotic progenies might revert their fate and reenter the cell cycle. Source: Graphic by authors.

2.3. The Single-Step Fate Model of Specialization and Potency

An emerging viewpoint that opposes the historical idea that cNeoblasts are a subpopulation with a specific molecular signature refutes the existence of exclusive transcripts for pluripotent stem cells and accepts the concept of a modulation in the expression levels of neoblast specific transcripts. In this view, a recent paper by Gambino et al. (2021) demonstrated that following a short 5FU treatment, *soxP-1* expression is extensively downregulated below the detection limit of in situ hybridization. However, *soxP-1*-positive cells remain in the animal body, and only after a few weeks, some of these cells upregulate again *soxP-1* expression, restart proliferation and repopulate the entire planarian body. The idea that cell behavior is dependent on the transitory enrichment of specific transcripts invalidates the existence of clearly defined subpopulations organized in a strict hierarchy and opens to the concept of blurred borders between neoblast populations, with cells that possibly fluctuate from wider to restricted differentiative potential and "vice versa".

In this line of thought, a cutting-edge interpretation of the neoblast fate specification mechanism questions the idea of the existence of a limited population of cNeoblasts, and a strictly organized hierarchy of neoblasts with progressive restriction in differentiation potency has been recently published by Raz et al. (2021). They demonstrated, by single-cell transplantation in irradiated animals and colony assays, that no known neoblast subpopulation is exclusively pluripotent and neoblasts from different subpopulations can be clonogenic. They proposed a single-step fate model of specialization and potency: newly produced G1 neoblasts are pristine but become

specified by progressive enrichment in FSTFs during the progression through S/G2/M phases of cell cycle; a G2 specialized neoblast then asymmetrically divide to give rise to a non-neoblast daughter cell that will differentiate and a daughter cell that remains a neoblast and can again specialize to a different fate during the next cell cycle progression without progenies with intermediary potency. In other terms, this means that specialized neoblasts can return to pluripotency after cell division. This model fits well with the proposed switch from σ- to ζ-neoblasts in the S phase and is in line with some recent publications: (i) Gambino et al. (2021) demonstrated that in challenging conditions after 5FU treatment, neoblasts early postmitotic cells could modify their expression profile reacquiring a broader differentiative potential; (ii) Davidian et al. (2021) showed that subthreshold direct current stimulation rapidly restores pluripotent stem cell populations previously eliminated by lethal irradiation promoting cell cycle entry of postmitotic cells. However, even more remarkably, this new line of thought brings us back to findings obtained at the beginning of the 1980s from Gremigni's group (Gremigni and Miceli 1980; Gremigni et al. 1980a, 1980b, 1982) by using a triplo-hexaploid biotype of *D. polychroa* that provided a useful karyological marker because embryonic and somatic cells are triploid (3n = 12 chromosomes) and could be easily distinguished from male diploid (2n = 8 chromosomes) and female hexaploid (6n = 24 chromosomes) germ cells by their chromosome number. Gremigni et al. (1980a, 1980b, 1982) showed that a small percentage of male and (to a much lesser extent) female germ cells are involved in blastema formation and somatic tissue reconstruction, along with a large number of neoblasts, suggesting that germ cells, at the very beginning of their differentiation process, can interrupt their pathway toward specialization and return to the pluripotent state. This interpretation fits perfectly with the single-step fate model of specialization and potency proposed by Raz et al. (2021). In this case, germ cells can be interpreted as specialized neoblasts, which asymmetrically divide to give rise on the one hand, to a gamete precursor and, on the other hand, to a cell that specialized to a different fate. Unfortunately, the triplo-hexaploid biotype did not survive up to the molecular age, and thus, it was not possible to provide additional demonstrations (Salvetti and Rossi 2012).

A key event for the single-step fate model of specialization is the asymmetric division, which is still an unexplored field in planarian mainly due to technical difficulties. The first molecular evidence of asymmetric stem cell division has been provided by a comprehensive paper of the Sanchez Alvarado's group (Lei et al. 2016) in which, by applying a combined approach of RNAi and colony expansion assay after low-dose X-ray, they demonstrated that the epidermal growth factor pathway and its receptor *egfr-3* are involved in the expansion of neoblasts when their number is diminished by sublethal radiation. *egfr-3* is also fundamental in physiological conditions for the second peak of hyperproliferation at 48 h postamputation. Strikingly, *egfr-3* protein frequently shows an asymmetric

distribution on the neoblast membrane, and *egfr-3* distribution during mitoses was associated with symmetric/asymmetric distribution of *smedwi-1* transcripts and the chromatoid bodies. Thus, the authors hypothesize that *egfr-3* controls the repopulation of neoblast by regulating asymmetric versus symmetric cell division. Additional lines of evidence emerged from the analysis of the function of the planarian homolog of *mex*3 RNA-binding protein (*Smed-mex3-1*) that is expressed in both stem cell and immediate postmitotic progeny populations (Zhu et al. 2015). Knockdown of *mex*3-1 leads to a rapid decline of progenitor markers for multiple lineages but not of stem cells, suggesting its specific role in specifying committed progeny. Despite *Smed-mex3-1* mRNA showing no asymmetric distribution into stem cells, on the basis of its proven function in other model systems, the authors speculate that it may function to maintain asymmetry in stem cell lineage progression by promoting postmitotic fates and suppressing self-renewal (Zhu et al. 2015). Despite these pioneering papers, much research needs to be performed to demonstrate and understand asymmetric cell division in neoblasts.

In conclusion, the molecular classification of planarian neoblasts is still a work in progress, and although many efforts to link the molecular and functional definitions of cNeoblasts have been made, unambiguous cNeoblast markers have not been yet identified. Thus, we cannot picture cNeoblasts as a special subpopulation; on the contrary, we had to assume, according to the single-step fate model, that pluripotency is the consequence of transitory and cell-cycle related fluctuations in the quantitative transcriptional profile, rather than expression of specific genes; asymmetric distribution of cell fate determinants such as chromatin remodeling factors might be at the basis of self-maintenance mechanisms. In this view, all neoblasts are potentially clonogenic, and the expression of FSTFs is correlated with cell-cycle progression rather than limited to lineage-committed neoblasts with intermediate potency. This hypothesis is groundbreaking and relates to a previous probabilistic model that considers the possibility that pluripotency may be a transient, probabilistic state exhibited by stem cells. In this view, self-renewal becomes a feature not possessed by a discrete population of cells but transiently held by a small number of cells and arising depending on the demands of the animal (Adler and Sánchez 2015). However, many open questions still remain. For example, which precise changes in transcriptional profile drives the switch from G1 pluripotent state to S-G2 m lineage-committed state? Is the increase in the expression of FSTFs necessary and sufficient for the downregulation of self-renewal regulators such as members of the polycomb complex PRC2, the transcription factor *soxP-1* (Wagner et al. 2012), the RNA-binding proteins PIWI-like (*smedwi-2* and *3*), Bruno-like (*Bruli*), *Pumilio*, and *CIP29* (Reddien et al. 2005; Wagner et al. 2012; Guo et al. 2006; Salvetti et al. 2005; Rossi et al. 2007), histone-2B (Solana et al. 2012), the Retinoblastoma homolog (Zhu and Pearson 2013), and the epidermal growth factor receptor *egfr-3* (Lei et al.

2016)? What is the role of *p53* known to inhibit proliferation and stem cell identity and induce differentiation in the early progeny (Pearson and Sánchez 2010)? Can all the S-G2 m lineage-committed neoblasts reverse their differentiation fate with the same effectiveness? What is the role played by epigenetic inheritance? For example, the asymmetric inheritance of chromatoid bodies and *piwi* transcripts might bring into one daughter cell transcripts that, once translated, produce a specific chromatin condensation pattern that promotes self-renewal. Indeed, evidence that links PIWI proteins and chromatoid bodies to histone mRNA regulation in planarian stem cells has been provided (Rouhana et al. 2014). Finally, which positional information signals drive the decision of a G1 daughter cell to specialize toward a specific fate? Indeed, a classical niche, meant as a specific anatomical structure in which stemness is maintained, has not yet been proven in planarians. However, neoblast dynamics appear to be under the control of signaling from multiple tissues, suggesting that a global niche, a macroenvironment, comprehensive to the entire planarian body might exist (Rossi and Salvetti 2019).

3. From Lineage-Committed Neoblasts to Differentiated Cells: The Case of Epidermal Cell Differentiation

Independently from which specification model is valid, a committed neoblast should first become a postmitotic cell and then progress toward a fully differentiated fate. Several examples exist in the literature describing the role of molecular regulators in the differentiation of multiple planarian tissues including the eye (Lapan and Reddien 2012) and excretory system (Scimone et al. 2011). However, owing to the prolific production of some research groups in the last decade, the most comprehensive overview is available for the differentiation of epidermis. The Planarian epidermis is a monostratified tissue of multiple multiciliated and nonciliated cell types (Rompolas et al. 2010). However, despite similar morphological appearance, Wurtzel et al. (2017) identified eight different spatial transcriptional identities by the analysis of epidermis-enriched RNAseq libraries, demonstrating that planarian epidermis is a complex tissue with distinct cell types, all originating from the single lineage-committed class of ζ-neoblasts (van Wolfswinkel et al. 2014). ζ-neoblasts, characterized by the expression of a group of molecular markers such as *zfp-1*, divide and produce postmitotic progenitors that express the marker *prog-1 (NB.21.11e)*—the so-called early epidermal progeny. Early progeny cell identity is maintained for a short period of time; indeed, *prog-1*$^{+}$ cells disappear 2 days after lethal X-ray treatment and rapidly differentiate in the late epidermal progeny, characterized by the expression of *AGAT-1, AGAT-2*, and *AGAT-3* transcripts. The early growth response family transcription factor, *egr-5*, seems implicated in switching off the expression of *prog-1* and turning on the expression of genes necessary for the *AGAT-1*+ transition stage. *AGAT-1*$^{+}$ cells localize more distal with respect to *prog-1*$^{+}$ cells

and disappear 7 days after lethal irradiation (Tu et al. 2015; Eisenhoffer et al. 2008). The spatial expression pattern of *prog-1* and *AGAT-1* indicates that these progeny cells migrate to the outer surface of the animal during epidermal differentiation. Zhu and Pearson (2018) identified *myb-1* as a key regulator of the temporal phase of early progenitor specification during epidermal lineage differentiation. Indeed, *myb-1* (RNAi) resulted in a selective loss of the early progeny fate, causing *prog-1*$^{+}$ cells prematurely to adopt the late progeny transcriptional profile. Despite this heterochronic temporal shift, late progenitors resumed differentiation. The early transition state into the planarian epidermis is marked by the expression of *zpuf-6* transcript, which labels all the *AGAT-1*$^{+}$ cells, but also some *AGAT-1*$^{-}$ cells and some cells located into the epidermis monolayer. Final differentiation steps are then marked by *vimentin 3* and *rootletin* expression (Tu et al. 2015). Recent findings demonstrate that the terminal identity of epidermal cells is acquired early during the differentiation process, indicating that epidermal progenitors recognize their position and modulate their gene expression in a way to reflect the array of transcripts in the mature epidermis (Wurtzel et al. 2017). Strikingly, also the expression of cilia specific genes starts in early progenitors despite the fact that the formation of cilia is restricted to the mature epidermis, and accordingly, it has been demonstrated that genes involved in ciliogenesis that are inactive loci in the stem cell population are methylated in order to poise them for activation later in development (Duncan et al. 2015). This also demonstrates that the identity as a ciliated or not-ciliated epidermal cell is acquired in migratory progenitors before their terminal differentiation (Wurtzel et al. 2017). Interestingly, it has been demonstrated that terminal epidermal cell differentiation is finely regulated by the two specific components of the nucleosome remodeling deacetylase (NuRD) complex: the methyl-CpG-binding domain 2/3 (mbd2/3) gene (Jaber-Hijazi et al. 2013) and the GATA-type zinc-finger-domain-containing gene p66 (Vásquez-Doorman and Petersen 2016) revealing opening future avenues of research on how neoblast processes are coordinated at the epigenetic level (Dattani et al. 2019).

4. Embryonic Origin of Neoblasts

Where do cNeoblasts originate from? Are they the heritage of naïve embryonic stem cells? Or are they formed as a specific need for adult tissue maintenance? Yes and no! Planarians show an ectolecitic embryonic development in which blastomeres undergo dispersed cleavage among yolk cells, do not contact with one another, and divide asynchronously. During sphere formation, temporary embryonic tissues are formed and then degenerate as adult organs are shaped, owing to undifferentiated blastomeres remaining after sphere formation (Martín-Durán et al. 2012). Recently, a very comprehensive study on *S. mediterranea* embryonic development demonstrates that pluripotent neoblasts and lineage-dedicated progenitors arise when the morphogenesis of definitive organs begins (Davies et al. 2017). The authors

demonstrate that *smedwi-1* transcripts are expressed in the zygote and *smedwi-1*$^+$ cells, endowed with proliferative capability, are detectable throughout all embryogenesis. However, large-scale changes in gene expression occur in *smedwi-1*$^+$ cells, as definitive organogenesis begins (developmental stage S5). At this time, early embryo-enriched (EEE) transcripts, specifically expressed by blastomeres, dramatically decline and *smedwi-1*$^+$ cells start to be enriched in FSTF. Transplantation experiments of blastomeres collected from different developmental stages into lethally irradiated hosts demonstrate that the change in transcriptional profile reflects important functional differences. Indeed, *smedwi-1*$^+$ cells from S4 and S5 embryonic donor cells did not rescue lethally irradiated animals, while cells from S6–S8 embryos acted similarly to adult neoblasts and rescued the lethal phenotype. These findings suggest that cNeoblast specification occurs during S5. Considering that transcripts of genes previously implicated in neoblast maintenance such as *SoxP-1* or *bruli-1* show an expression profile similar to that of *smedwi-1* during embryogenesis, the authors suggest that the expression of pluripotency factors is probably necessary but not sufficient for the assumption of neoblast fate. Indeed, EEE transcripts downregulation in blastomeres is necessary for neoblast specification, suggesting that they might represent repressors of neoblast fate. This hypothesis is very intriguing; however, no direct proof has been provided such as analyzing the ability to rescue irradiated hosts after blocking the downregulation of EEE transcripts. Despite the significant advances in the comprehension of cNeoblast origin, several questions are still open—are EEE transcripts maternally deposited? If this is the case, which mechanisms affect maternal transcript degradation? When does zygotic genome activation occur? Further, how does it influence neoblast specification?

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