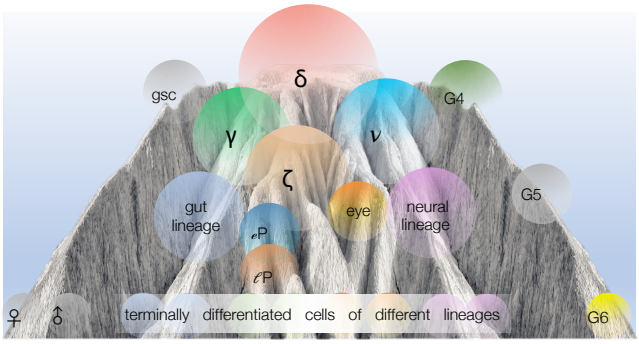
A lineage CLOUD for neoblasts

Visual Abstract



Visual abstract. The CLOUD-badlands landscape. Lineage specification is a continuous process, where cells gradually acquire features of multiple commitment. This counterposes with the rigid pyramidal organization of discrete progenitor cells, rather suggesting a Continuum of LOw-primed UnDifferentiated planarian stem and progenitor cells (CLOUD-PSPCs) that can plastically retune their fate commitment. Abbreviations in main text.

Title

A lineage CLOUD for neoblasts

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Abstract

In planarians, pluripotency can be studied *in vivo* in the adult animal, making these animals a unique model system where pluripotency-based regeneration (PBR) —and its therapeutic potential— can be investigated. This review focuses on recent findings to build a cloud model of fate restriction likelihood for planarian stem and progenitor cells. Recently, a computational approach based on functional and molecular profiling at the single cell level was proposed for human hematopoietic stem cells. Based on data generated both *in vivo* and *ex vivo*, we hypothesized that planarian stem cells could acquire multiple direction lineage biases, following a “badlands” landscape. Instead of a discrete tree-like hierarchy, where the potency of stem/progenitor cells reduces stepwise, we propose a Continuum of LOw-primed UnDifferentiated Planarian Stem/Progenitor Cells (CLOUD-PSPCs). Every subclass of neoblast/progenitor cells is a cloud of likelihood, as the single cell transcriptomics data indicate. The CLOUD-HSPCs concept was substantiated by *in vitro* data from cell culture; therefore, to confirm the CLOUD-PSPCs model, the planarian community needs to develop new tools, like live cell tracking. Future studies will allow a deeper understanding of PBR in planarian, and the possible implications for regenerative therapies in human.

Keywords

neoblast; potency state transitions; Stem cell heterogeneity; CLOUD; badlands landscape; live cell tracking

1. Introduction

Many animals have remarkable regeneration abilities. Salamanders can regenerate limbs, frogs can regrow tail, and zebrafish can restore fin and heart [1]. However, planarians are the almighty model of regeneration. These flatworms are virtually “immortal under the edge of knife”, as Lord Dalyell concluded in 1814, as their entire body can regenerate even from a tiny fragment [2]. Planarians lack circulatory and respiratory systems, but have a complex internal anatomy, with organs and tissues made by several different cell types [3-6]. And they have pluripotent stem cells. Pluripotency is the capacity of a cell to self-renew indefinitely, maintaining the ability to differentiate into any cell types. In triploblastic animals pluripotent stem cells disappear “as development proceeds, as more restricted somatic stem cells give rise to the tissues and organs” [7]. Planarians, however, maintain their pluripotent stem cells in the adulthood, and rely on them for tissue turnover, regeneration, growth-degrowth and to induce gonad formation [8].

When planarian is amputated, a non-pigmented tissue called blastema begins to form. Within the blastema, cells are patterned in such a way that any missing part is correctly regenerated [8, 9]. However, the regulation of cell fate determination in planarian is still largely unknown. For example, neoblasts were long known pluripotent as a population [3], but it was not until 2011 that Wagner and colleges demonstrated that certain individual neoblasts were able to rescue a stem cell-depleted animal [10]. These were named clonogenic neoblasts (cNeoblasts) and, as of today, they are only characterized functionally. Are cNeoblasts similar to mammalian embryonic stem cells? Do they follow a tree-like hierarchy of pluri- oligo- and uni-potent progenitors? Recently, single cell analysis individuated multiple prominent classes of planarian stem/progenitor cells with distinctive potencies [11-19]. These studies began to resolve the heterogeneity of the planarian stem cell population, but the shortage of methods like transgenesis, *in vivo* cell tracking and cell culture leaves us without a molecular insight about planarian stem cell commitment and fate decision.

In this review, we revised the data generated both *in vivo* (RNAi) and *ex vivo* (single-cell transcriptomics) to propose a novel way to see the relationship among planarian cells with different potency, based on the “Continuum of LOw-primed UnDifferentiated (CLOUD) stem/progenitor cells model recently suggested for human hematopoiesis [20]. Instead of a discrete lineage tree, the model considers the generation of multiple-direction lineage biases that generate clouds of likelihood for the commitment/fate restriction of each cell. Since planarians are animals that turned pluripotency into a resource also for adult individuals, they are the ideal model system for studying pluripotency-based regeneration (PBR) and its potential implications for human regenerative therapies.

2. The planarian adult stem cells

Planarian’s outstanding regeneration ability owes to a large resident population of stem cells that can differentiate into any cell type, including the germline [3, 8, 21, 22]. The planarian adult

stem cells, historically referred to as neoblasts, localize in the planarian parenchyma, literally "filling the gaps" among the organs with an archetypal pattern [8, 9]. Planarian neoblasts anarchically arise from an early cell population that express a unique set of embryo-enriched transcripts, distinct from the adult neoblasts [23]. Adult neoblasts are small roundish-to-ovoid cells ranging 5-10 μm, with high N/C ratio, abundant free ribosomes and few round mitochondria [24-26]. Bardeen and Baetjer showed in 1904 that neoblasts are required for the formation of the blastema [27], as planarians irradiated with 1750 rad failed to regenerate. Thanks to the use of cellular and molecular tools (*e.g.* WISH, RNAi, FACS, qPCR, BrdU labeling) neoblasts were further characterized over the last decade. Since long double-stranded RNA-mediated RNA interference (RNAi) proved successful in planarian [28], many key players that regulate both neoblast maintenance and differentiation were identified [15, 29-32]. The mRNA of *smedwi1*, a gene with no apparent RNAi phenotype, is expressed in cells that disappear within 1 day following irradiation [31, 33], which is why it is considered by many the canonical neoblast marker [33]. Neoblasts can be isolated by fluorescence-activated cell sorting (FACS), because of their sensitivity to irradiation and their cellular features (*e.g.* variable DNA content, sparse cytoplasm). Two populations of small cells sensitive to irradiation were defined as X ray-sensitive population 1 and 2 (X1 and X2), together with one large and heterogeneous population of X ray-insensitive cells (Xin) [12, 33, 34]. Neoblasts populate both the X1 (cells in S-G2/M phase of the cell cycle) and the X2 (cells in G1) gates. Following irradiation, the X1 population disappears within 24 hours, while the X2 population halves in approximately 5 days, as it contains a mixture of neoblasts and post-mitotic neoblast progeny. Although the X1 gate looked more homogenous than the X2, many studies have shown its heterogeneity, based on cell morphology and ultrastructure, the expression of specific markers [10, 12, 35, 36] and, more recently, based on single-cell transcriptomics signature [17, 37-40]. Hence, the term "neoblast", which defines a mixed population of pluri-, oligo- and uni-potent mitotic cells [1, 9, 12] that share a basic set of common features, is no longer sufficient to depict the complexity of the planarian stem cell system in absolute terms.

3. Conserved mechanisms govern the stem cell compartment in planarian

Among bilateral animals, planarians are considered unique, owing to their extreme tissue plasticity and their remarkable regenerative ability, both hinging on the presence of adult pluripotent stem cells. Yet, several studies over the last decade suggested that planarians might be unique for the presence of embryonic-like stem cells in the adult, but not for the molecular mechanisms that govern them, which do not differ dramatically from those that regulate mammalian embryonic stem cells, from both cell autonomous and non-cell autonomous perspectives [41-44]. Planarian's pluripotency network share remarkable similarities with mouse and human ones [41]. During eye development, the eye specialized genes *six-1/2* and *eya* are

involved in the early specification of eye precursor cells in planarian as well as in vertebrates and other animals [37, 45, 46]. Moreover, numerous genes have a fundamental role in the specification of the germline in many animals. *Vasa* is essential for the formation of the germ cells in Drosophila [47]; PIWI genes are conserved function also in non-bilateral metazoans [48, 49]; the anti-apoptotic gene *Bcl2* plays a key role in regulating (germ) stem cell maintenance in planarians and mammals alike [50-53]. Non-cell autonomous mechanisms are also conserved. The group of Peter Reddien recently identified muscle cells as the conserved source of the patterning signals in planarians (*e.g.* WNT, BMP, notum, frizzled, ndk, sFRP, netrin-1) [54]. Such a positional control mechanism is ancient, as also acoels, which separated from planarians about 550 million years ago [55-57], rely on it [54]. Even though it is still unclear whether the stem cell response downstream of the signaling is similar by homology or convergence [56], the striking similarity in positional patterning between planarians and acoels suggests that such a mechanism was a common trait in all bilaterians [54, 55]. From insects to mammals, Wnt signaling mediates axial polarity during embryo- and organogenesis [58, 59] and during heart regeneration in zebrafish [60]. Other conventional pathways were also found to play important roles in tissue maintenance and regeneration of planarian, such as BMP [61, 62], ERK [63], Akt [64], JNK [65], and EGFR [30].

Altogether, this striking similarity between planarian and other animals implies that the stem cell governance across bilaterians is potentially preserved along evolution. As a unique system that allows investigating pluripotent stem cells *in vivo*, planarian emerges as an ideal paradigm to study the cellular mechanisms that tune pluripotency-based regeneration, and turn them into human therapies.

4. More than one neoblast

The neoblast population is heterogeneous. In 2011, Peter Reddien coined the term cNeoblast to describe the planarian pluripotent stem cell from the functional perspective, as a single cNeoblast is able to restore both homeostasis and regeneration competence after transplantation into a stem cell-depleted host [10]. The cNeoblast is likely the keystone of the planarian stem cell system, but proving its clonogenicity and looking for its molecular signature at the same time was not possible. One year later, however, the first evidence of the existence of a committed proliferating cell was shown in the frame of eye regeneration. *Smedwi1*$^{+}$*/h2b*$^{+}$ cells were found posterior to the eyes in intact animals, which also expressed the pan-eye marker *ovo* [37]. Further evidences of lineage-restricted planarian stem/progenitor cells were proposed recently [11-19]. Nine bHLH (basic helix-loop helix) genes were found specifically expressed in a subset of neural (stem) cells that are needed for regeneration [15]. Rossi and colleagues found a novel SL RNA which is highly enriched in a subset of neoblasts [66]. Our group discovered that a large portion of X1 cells co-express the markers of the epidermal

lineage (*Prog-1*, *Agat-1*) together with the 6/9.2 surface antigen [12]. Recently, 3 classes of neoblasts were defined using high-dimensional single cell transcriptomics [17]. Comparing the gene expression profiles of a thousand individual cells, van Wolfswinklel and colleagues suggested the existence of two prominent classes of Neoblasts, namely δ (sigma) and ζ (zeta), and at least one additional subclass γ (gamma) within the δ class. In spite of the lack of univocal markers that defined the neoblasts subclasses, they proposed discrete sets of transcripts that largely overlap with *smedwi1* expression (Table 1). They also found that neoblast subclasses are cell cycle-independent. Interestingly, the expression of the 6/9.2 antigen, and therefore the expression of the epidermal lineage markers in X1 cells, are also cell-cycle-independent [12]. Both δ and ζNeoblasts are found in the animal during homeostasis and regeneration. However, the early cellular wound response is dominantly controlled by the δNeoblasts, suggesting that these stem cells maintain the long-term self-renewal capacity. Moreover, ablation of ζNeoblasts via *zfp-1* RNAi showed that δNeoblasts could reestablish the ζ class. Therefore, δNeoblasts are pluripotent stem cells that lay upstream of ζNeoblasts and, most likely, encompass the population of cNeoblast. Zeta-neoblasts are multipotent stem cells required for the maintenance and regeneration of the epidermis. Also the γ subclass of neoblasts express a specific set of genes (Table 1), which includes genes related to the planarian gastrovascular system, like *nkx-2.*2, *hnf-4* and *gata-4/5/6* [17]. Using single-cell transcriptomics, the group of Bret Pearson recently identified the planarian neural stem cell, the *ν*Neoblast. According to *in silico* lineage tracking, *ν*Neoblasts derive from δNeoblasts, co-express the neoblast markers *smedwi1* and *smedwi2* with neuron-specific genes (Table 1) and give rise to *pc-2*$^{+}$/*synapsin*$^{+}$ neurons [39]. Neoblasts have also been described for pharynx and protonephridia, as defined by specific sets of transcripts (Table 1) [11, 67]. Although there is no evidence that these two multipotent cell populations originate from δNeoblasts, the co-expression of their specific sub-sets of genes with the pan-neoblast marker *smedwi1* strongly suggests that they are committed neoblasts, analogous to ζ, γ and *ν*Neoblasts.

Classically, the distinction between stem and progenitor cells was based on step-wise decisions where pluripotent stem cells gradually restrict their potency throughout discrete intermediate stages, in a tree-like structure. Do planarian stem cells follow a similar model? Worth mentioning is that neither the δ nor the ζNeoblasts are homogenous cell populations. Transcriptomic data suggest that single neoblasts, rather than being fully characterized by the expression of a set of markers, are clouds of individual cells with distinctive signatures. For example, a subset of the ζ cells express *AbdBb* together with *six-6*, whereas another subset express high levels of *AbdBa* together with *meis-2* and *gata1/2/3* [17]. The group of Bret Pearson also provided the first insight into the pigment cell lineage. They suggested that a still unknown pigment cell progenitor, distinct from the ζ cells, may stem from the endodermal γNeoblasts and give rise to

both dendritic and punctate cells [19]. The relationship among neoblast subclasses —and between these and their respective progeny— is far from being clear. For example, γNeoblasts express *hnf-4* and *gata-4/5/6* together with *egfr-1*, which is required for the differentiation of the gut. Whether *hnf-4*$^{+}$/*gata-4/5/6*$^{+}$/*egfr-1*$^{+}$ cells stem directly from δNeoblasts or γNeoblasts express *egfr-1* one step later, as committed gut progenitors, is currently unknown. Based on the data from Barberán and colleagues, the inhibition of *egfr-1* blocked the differentiation of new gut cells but significantly increased the expression of *hnf-4* and *gata-4/5/6* [30], maybe in consequence of the accumulation of γNeoblasts incapable to proceed in the differentiation path. Interestingly, *egfr-1* RNAi also resulted in the hyper-proliferation of ζ and δNeoblasts. A similar scenario was shown in *mex3-1*-deficient planarians, where the commitment of new cells into brain, intestine, and pharynx was severely impaired [68]. Altogether, this indicates that factors expressed in stem cells may exert their control on the differentiation of post-mitotic lineages from an upstream level.

If it is proved that some of the δNeoblasts are pluripotent, there are currently no evidences to infer about the potency of the other subclasses. Although de-differentiation of post-mitotic cells was never observed in wild-type planarians, it was recently shown that, following the downregulation of *Hippo*, some post-mitotic cells can reacquire *smedwi1* expression [69]. It is therefore possible that lineage-restricted ζ, γ and ν*Neoblasts could also regain pluripotency, under certain circumstances. We know from other animal models that the stem cell identity is not strictly cell autonomous, and there is plasticity for certain cells to regain stemness [70]. For example, progenitor cells in Drosophila can take up stem cell functions when germline stem cells are lost [71, 72]. Transient-amplifying progenitor cells in the mouse testis have the potential to become spermatogonial stem cells [70]. In Xenopus, dedifferentiation was shown during limb regeneration, which requires the regulation of the reprogramming factor *Sall4* [73]; *Oct4* and *Sox2* are instead required for heart regeneration in zebrafish, suggesting that reprogramming *in vivo* could re-induce pluripotency [74]. More evidences need to be collected in order to define how the different subclasses of neoblasts relate to each other and how and when potency state transitions take place.

5. Pathways underlying pluripotent state transition

Neoblasts have been recently ranked into different subclasses according to their differentiation potential, but the stem cells within each individual subclass do not share a univocal molecular signature. Since the function of one cell type depends also on its location [75], we can assume that cells that belong to the same lineage might differ because of their localization in the body of the animal. [32, 76]. For examples, *smedwi1*$^{+}$/*ovo*$^{+}$ neoblasts are only found in the prepharyngeal area [37], and intestinal neoblasts usually locate close to the gut [10]. The gradients of morphogens that are responsible of generating the positional cues are secreted by

the muscle cells, that express discrete sets of positional control genes (PCGs) according to the orientation of the muscle fibers and their localization within the body of the animal [77]. Moreover, both proliferation and differentiation cues depend on the absence of certain body parts, like the head or the flank, but not of specific tissues, like the eyes [78]. Henceforth, planarians regeneration seems to follow a “target-blind” mode. Upon injury, generic wound signals and specific positional gradients induce neoblasts to generate diverse cell types, to create more opportunities for cell fate decision to occur. For example, parenchymal ζNeoblasts exit cell cycle following amputation, and start differentiating. While differentiating, epidermal progenitors activate a specific set of transcripts according to the BMP gradient generated by the dorsal muscle cells, which is responsible for the regional identity of the future epidermal cells [31, 40, 79]. It is tempting to think that it is not the intrinsic regulation of pluripotency, but the way that pluripotent cells are controlled within the animal’s body that makes the difference in terms of regenerative ability among animals.

Conventional lineage tree models of commitment/differentiation are based on discrete and definable stages where each cell has a precise molecular and epigenetic signature that correspond to a precise function, as proposed by Waddington in 1942 [80]. The “fluidic” identity that planarian stem/progenitor cells acquire, however, raises the question whether such a tree-like model is suitable for describing the planarian stem cell system. Seminal is the example of eye regeneration. During head regeneration, some *smedwi1*$^{+}$/*h2b*$^{+}$ neoblasts start to express the early eye lineage markers *six-1/2 eya* and *ovo*. Later on, the expression of *sp-6/9* and *dlx* induces the differentiation into *tyro*$^{+}$ cells of the optic cup, where *otxA* specifies the retinal cell fate [13, 37]. According to the t-distributed stochastic neighbor embedding (t-SNE) [38], *tyrosinase*-positive cells localize almost exclusively in the epidermal lineage (Figure 1A), while mature retinal cells expressing the Transient Receptor Potential Cation family genes (*TRPC4*, *5*, *6*) locate almost exclusively in the neural lineage (Figure 1B). This raises the question about the relationship among the eye neoblast (which generates cells of both the epidermal and the neuronal clusters), the νNeoblast (which supposedly generates only neuronal cells) and the ζNeoblasts (which supposedly only generates epidermal cells [13, 37, 39, 57].

The idea that cell differentiation follows a rigid Waddington’s-like landscape was recently challenged for human hematopoietic cells [20]. In its stead, the authors proposed a layered multi-step model, similar to a badlands landscape, where the stem cells, defined by single-cell transcriptomics, acquire multiple directions and the barriers between individual lineages gradually deepen. In the upper part of the badlands, the difference between contiguous lineages may be very small in terms of gene expression, at the point that two cells with similar molecular profile may virtually locate in two different portions on the landscape. Downstream, differences in the molecular signature and potential increase, so that the border between

lineages becomes impassable. Rather than a discrete tree-like hierarchy, where the potency of stem/progenitor cells reduces stepwise, we propose a Continuum of LOw-primed UnDifferentiated planarian stem/progenitor cells (CLOUD-PSPCs), where specific stem or progenitor cells can undergo multiple transitory states that gradually restrict their potential (Figure 2). Each transitory state is defined as a cloud of likelihood where cells have a higher probability to be found. Therefore, there are no exact boundaries to distinguish a stem from an early-committed cell, and lineage restrictions emerge directly from the CLOUD without undergoing a strict progenitors' hierarchy. However, the lower is the degree of priming, the more fluid is the fate that a cell could take, which means that ζ, γ and νNeoblasts are likely more prone to change their fate than their progeny cells.

6. Future steps: the need of *in vivo* tool

The recent studies on planarian stem cell subclasses represent a big step in understanding how the planarian stem cell system is organized; however, the long-standing question of how individual neoblasts behave still remains to be answered. Although technical progresses were made, such as planarian cell transplantation [10] and immobilization for imaging [81-83], the in depths understanding of planarian stem cell biology requires the possibility of tracking the fate of individual transplanted cells. In turn, this requires the labeling of the transplanted cells, which can either be genetic (transgenesis) or non-genetic (surface antibodies, supra/intravital markers). Although the proof of principle of planarian transgenesis has been produced in *Girardia* (*Dugesia*) *tigrina* [84], several attempts to achieve transgenesis in *S. mediterranea* failed. Hence, the labeling strategies we consider in the following paragraphs for tracking the fate of individual planarian cells *in vivo* are based on non-genetic cell labeling.

1. Supravital dyes

Being the option of using fluorescently labeled transgenic neoblast to trace the fate of individual cells not currently in the menu, alternatives should be adopted. Live and retrograde tracking was successfully achieved by labeling cell subtypes with different fluorescent labels, such as FluoSpheres [85, 86], CellTracker [87, 88] and Mitotracker [89] dyes, both *in vitro* and *in vivo*. Preliminary data showed that FluoSpheres can be effectively used to label planarian cells *ex vivo* and trace them following transplantation into an immobilized planarian. FluoSpheres are equally shared between the daughter cells after each cell division, allowing tracking of the cell progeny over 3-4 generations. This approach may reveal powerful, especially once coupled with *in situ* hybridization against markers of lineage-specific differentiation. However, to be informative the FluoSphere-based labeling requires either an upstream selection of a subclass of cells, or the transplantation of a single labeled cell.

2. Surface antigens

Alternative to live cell tracking based on supravital markers, antibody-based labeling offers the advantage of specifically stain a subset of cells. In 2012, our group raised a library of monoclonal antibodies specific for planarian plasma membrane proteins. Several antibodies recognized subpopulations of stem cells of the X1 gate [12]. *Ex vivo* live immunostaining with one of these antibodies (6/9.2), coupled with fluorescence-activated cell sorting (FACS) and qPCR, revealed that 6/9.2^{+} cells of the X1 gate expressed low levels of the early and late epidermal progeny markers *prog-1* and *agat-1*, besides the pan-stem cell markers *smedwi1*, *pcna* and *cycB* [12]. After the identification of discrete neoblast subpopulations based on single-cell transcriptomics [17], we discovered that 6/9.2^{+} cells express high levels of ζ and γNeoblast markers (*egr-1*, *fgr-1*, *soxP-3*, *zfp-1*, *gata-4/5/6*, *nkx-2.2*), while 6/9.2^{-} cells express markers specific of δNeoblasts *(inx-13, SoxP-1, SoxP-2, smad-6/7)* (unpublished observation). These data suggest that the cells that do not express the 6/9.2 antigen are probably uncommitted, pluripotent stem cells and that the 6-9.2 antigen marks the commitment towards epidermal and gut lineages. Even though the characterization of the 6/9.2 surface antigens is desirable, the use of unknown plasma membrane antigens has been a useful tool to classify and select stem cell populations in other model systems [90, 91] and so even the difficult-to-characterize 6/9.2 antigen shall perform to shed some light into the planarian stem cell compartment dynamics.

3. Immobilization for long-term imaging

One of the challenges of live tracking method is the lack of a proper methodology to immobilize planarian for long-term imaging at cellular or subcellular resolution, especially because planarians are negatively phototactic over the entire visible spectrum [92]. Current methods for planarian immobilization include anesthetizing with chloretone (1,1,1-trichloro-2-methyl-2-propanol) [82] or low-percentage ethanol [81], or embedding in low melting point agarose, as recently shown by the group of Eva-Maria Collins [83]. They developed the Planarian Immobilization Chip (PIC), a microfluidic system that allows high resolution imaging and high-throughput screens of a variety of organisms [83]. However, this device can immobilize planarians for approximately 5 hours without causing injury, which is highly limiting for stem cell lineage tracing. To overcome this limitation, other natural or synthetic materials should be investigated that offer advantages for planarian immobilization. A promising candidate is the alginate hydrogel. As other protein products that are routinely used in biomedical applications, alginate is natural and non-toxic, but it offers additional advantages. It is biomimetic, gelates almost instantaneously and has excellent chemical and physical characteristics, like chemical inertia, tunable elasticity and mechanical stability [93, 94]. Ultra-high viscosity alginate hydrogels were successfully applied to cell growth, encapsulation and transplantation [95, 96]. Hence, the application of alginate hydrogel for planarian immobilization could prove the perfect tool for a high-resolution long-term imaging.

7. Conclusions

Like an *in vivo* cell culture dish, the planarian system uniquely allows elucidating how pluripotency copes with adulthood. Various approaches have been developed to unravel the complexity of the planarian stem cell system, combined with bioinformatics tools to provide an extensive knowledge database to rise and validate hypotheses. Planarian stem cells have a pluripotency network similar to that of mammalian embryonic stem cells, and similarly to these, they are under the tight control of a developmental-like patterning. In planarians, however the non-cell autonomous regulation depends on permanent signals secreted by the muscle cells, rather than by transient ones. This feature is shared with ancient bilateral animal – the acoels – which strongly suggests that the boundary between regenerative and non-regenerative animals may not owe to the intrinsic regulation of pluripotency, but more likely to the overall control that the adult body produces over the stem cells. Such a control coordinates the planarian stem cell compartment, modulating the fate decisions via the generation of multipotent stem cells. Recent studies individuated at least 5 subpopulations of *smedwi1*$^{+}$ proliferating cells, each characterized by the expression of a set of specific markers and the restriction to a specific cell fate. However, whether ζ, γ and *ν*Neoblasts, and other fate-restricted cells are or are not pluripotent – and if not, whether they can de-differentiate into a more naïve state – remains an open question. Currently, we cannot depict a model of planarian stem cell commitment; moreover, it appears that the coarse positional information that promotes commitment has the potential to generate a multitude of lineage-restricted stem/progenitor cells, rather than a single one, to provide more opportunities for differentiation to occur. Consequently, a rigid tree-like hierarchy, like the traditional Waddington's landscape, may fall short in portraying the potency state transitions in planarians, as recently shown for the human hematopoietic stem cell system. In this review, we proposed a badlands landscape, instead, where a Continuum of LOw-primed UnDifferentiated Planarian Stem/Progenitor Cells (CLOUD-PSPCs) could acquire multiple direction lineage biases. Every subclass of neoblast/progenitor cells is a cloud of likelihood, rather than the linear product of a stepwise potency reduction process, as the single cell transcriptomics data indicate (Figure 3).

To understand further how pluripotency state transitions are regulated in planarian, proper tools are needed, like live cell tracking. The more knowledge we gain on the planarian stem cell system and on the way pluripotency-based regeneration is regulated, the better chances we have to apply such a knowledge to the field of regenerative medicine.

Table 1. Subclasses of Neoblasts and progenitors in *S. mediterranea*

Neoblast subclasses	Specific markers	Give rise to:	References

cNeoblasts	uncharacterized	every cell types	[10]
δNeoblasts	*soxP-1*, *soxP-2*, *smad-6/7*	brain, photoreceptors, protonephridia, pharynx, muscles; ζ, γ, νNeoblasts	[17]
ζNeoblasts	*zfp-1*, *soxP-3*, *egr-1*	epidermal lineage	[17]
γNeoblasts	*hnf4*, *gata4/5/6*, *nkx2.2*	intestinal cells	[17]
νNeoblasts	*ston-2*, *elav-2*, *ptprd-9*, *msi-1*	neurons	[39]
Pigment progenitor	*foxF-1*, *albino*, *fgfrL-1*, *ets-1*	pigment cells	[19]
Pharynx neoblasts	*foxA*	pharynx cells	[67]
Protonephridia neoblasts	*pou2/3*, *six1/2*, *eya*, *Osr*, *Sall*	excretory system	[11]
Eye progenitor	*ovo*, *eya*, *six-1/2*, *sp6/9*	photoreceptors and optic cup cells	[13, 37]
6/9.2^{+} progenitor	*6/9.2*, *prog-1*, *agat1*	N/A	[12]

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Declarations of interest

The authors declare no competing interests

Submission declaration

The work described has not been published previously, it is not under consideration for publication elsewhere, that its publication is approved by all authors and, if accepted, it will not be published elsewhere in the same form

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List of abbreviations

RNAi	RNA interference
WISH	Whole-mount *In Situ* Hybridization
qPCR	quantitative Polymerase Chain Reaction
FACS	Fluorescence-Activated Cell Sorting
X1, X2	FACS-defined, X-ray sensitive cell populations 1 and 2
Xin	FACS-defined, X-ray insensitive cell population
PBR	Pluripotency Based Regeneration
CLOUD-PSPC	Continuum of Low-primed UnDifferentiated Planarian Stem/Progenitor Cell
cNeoblasts	clonogenic neoblasts
dsRNA	long double stranded RNA
PIC	Planarian Immobilization Chip
BrdU	Bromodeoxyuridin

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Figure 1

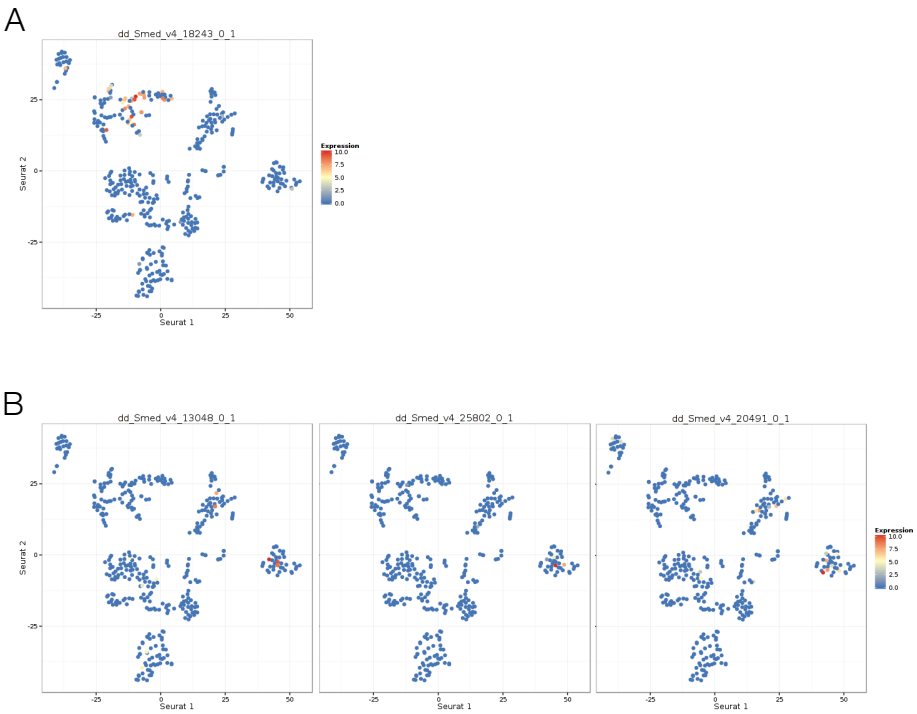

Figure 1. t-distributed stochastic neighbour embedding (t-SNE) for eye markers. According to the whole-transcriptome landscape generated on single planarian cells [17], different planarian cell types cluster according to the expression of specific genes. Localization of the *tyrosinase*-positive cells of the optic cup (A) and the *TRPC4*, *5*, *6*-positive cells of the retina (B)

Figure 2

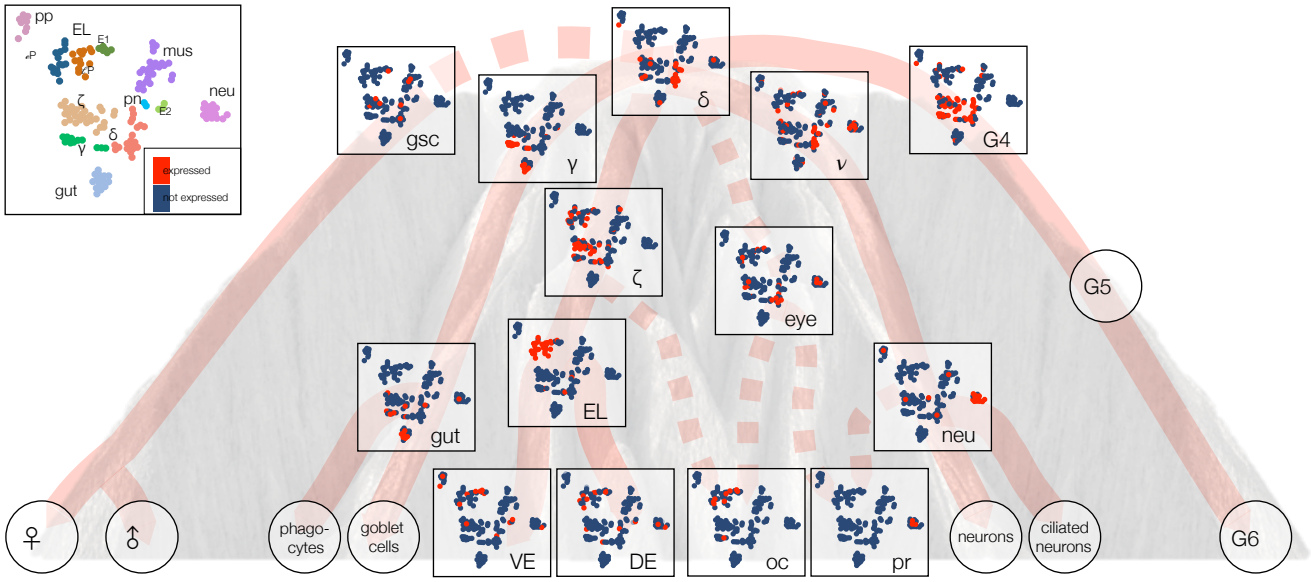

Figure 2. Neoblasts' heterogeneity modelled on the badlands landscape. Four subclasses of neoblasts have been described so far, each identified by the expression of a specific set of genes and by the commitment towards one or more cell lineage. Additional stem/progenitor cells have been proposed so far, like the eye-Neoblast and the Group 4 neoblasts; A germ stem cell population is also postulated. Instead of by a discrete set of markers, planarian stem/progenitor cells are depicted as cloud of likelihood within the t-SNE plot generated via single-cell whole transcriptomics. Individual plots are idealized based on the expression of the proper set of specific markers, as follows: δ (δNeoblast): *soxP-2*, *smad-6/7*, *inx-13*; γ (γNeoblast): *nkx-2.2*, *hnf-4, gata-4/5/6*, *prox-1*; ζ (ζNeoblast): *egr-1*, *soxP-3*, *zfp-1*; ν (νNeoblast): *elav-2*, *msi-1*; eye (eye neoblast): *ovo-1*, *eya*, *six-1/2*; gsc (germ stem cell): *nanos*; EL (epidermal lineage, early+late): *prog-1*, *prog-2*, *agat-1*, *agat-3*; gut (gut lineage): *gata-4/5/6*; neu (neural lineage): *pc-2*, *synapsin*; DE (dorsal epidermis): *ovo-2*, *prdm-1*; VE (ventral epidermis): *kal-1*, *foxJ-1*; oc (optic cup cells): *tyrosinase*; pr (photoreceptors): *TRPC4*, *5*, *6*. Other abbreviation used in the figure: G4/5/6: Group 4/5/6 [39]; ♀/♂: female/male gametes.

Figure 3

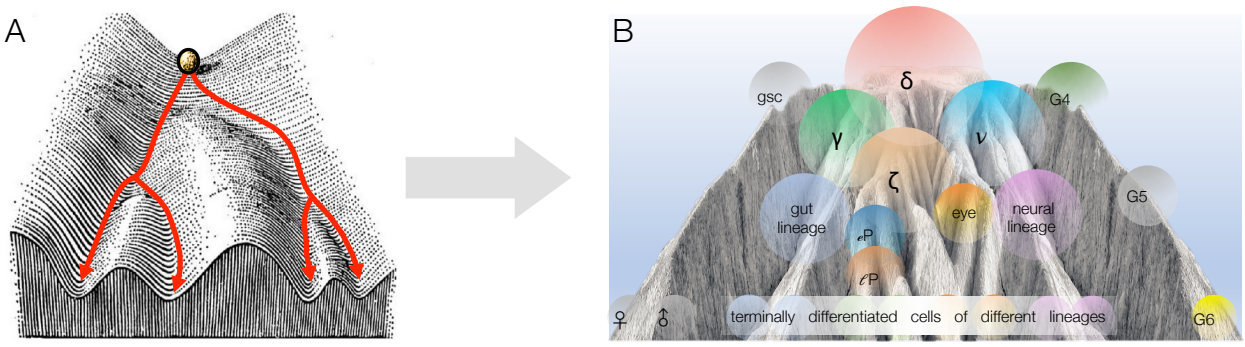

Figure 3. From Waddington's to CLOUD-badlands landscape. As recently proposed for human hematopoietic stem cells [20], lineage specification is a continuous process, where cells gradually acquire features of multiple commitment. This counterposes with the picture of a rigid pyramidal organization of discrete hierarchically organized progenitor cells (**A**), rather suggesting a Continuum of LOw-primed UnDifferentiated planarian stem and progenitor cells (CLOUD-PSPCs) that can plastically retune their fate commitment (**B**). The missing tiles of the final picture have to be experimentally ruled out, for example coupling multichannel fluorescent-activated cell sorting using specific surface antigens to single-cell transcriptomics and transplantation into irradiated animals. Abbreviations as for figure 2, except: ℯP: early epidermal progeny; ℓP: late epidermal progeny; E1: epidermis 1; E2: epidermis 2.