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Myths vs. FACS: what do we know about planarian stem cell lineages?

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ABSTRACT Historically, planarian neoblasts were thought to be a homogeneous population of pluripotent stem cells; however, recent population and single-cell level analyses have refuted this idea. Evidence for lineage commitment at the neoblast level has been provided via a number of independent studies using a variety of methods. *In situ* hybridization experiments first demonstrated the co-expression of lineage-specific markers in neoblasts (marked by *piwi-1* expression) isolated by FACS. Subsequently, single cell transcriptomic analyses of FACS-isolated neoblasts uncovered broad lineage-primed neoblast classes based on the clustering of transcriptional profiles and expression of known tissue-specific markers. Additionally, single neoblast pluripotency (and fate restriction) has been demonstrated by single cell transplantation experiments into neoblast-void animals. Here we look to recount the current status of the planarian neoblast field and offer discussion on the caveats of neoblast biology and how to address them experimentally.

KEY WORDS: *stem cell, neoblast, planarian, clonogenic, lineage*

Introduction

Humans replace billions of cells every day through the process of normal physiological cell turnover (homeostasis), and all of the new cells are ultimately the product of adult stem cells (ASCs) (Pellettieri and Alvarado, 2007). ASCs are central to several aspects of human biology such as tumor suppression, regeneration (or lack thereof), and aging, yet ASCs are extraordinarily difficult to study in the lab (with few exceptions, such as hematopoietic stem cells). *In vitro,* ASCs have highly asymmetric cell lineage kinetics, so they are difficult to expand in culture (Sherley, 2002). *In vivo* models either tend to have few ASCs or have ASCs that are very difficult to visualize or manipulate. For these reasons, ASCs remain understudied.

The freshwater planarian is a free-living member of the phylum Platyhelminthes in the Lophotrochozoan superphylum and has recently gained traction as an *in vivo* system to study ASC biology. Planarians are one of the best regenerators in nature. In just 7 days, a tiny planarian body fragment can fully regenerate and correctly pattern all missing tissues to create a functional, proportional worm (Cebrià, 2007; Newmark and Alvarado, 2002; Reddien and Alvarado, 2004). Impressively, the asexually reproducing strain of *Schmidtea mediterranea* has been propagated in laboratories for over a decade by repeated cycles of amputation and regeneration,

highlighting the essentially immortal nature of these flatworms. This regenerative ability is supported by an abundant population of ASCs, called neoblasts, present throughout the planarian body (Dubois, 1949; Pellettieri and Alvarado, 2007; Randolph, 1892; Randolph, 1897; Rink, 2013; Zhu and Pearson, 2016). Neoblasts were historically considered a homogeneous population of pluripotent stem cells, but the dawn of single-cell transcriptomic analysis has brought with it evidence of heterogeneous, lineage-primed neoblast classes as well as a myriad of committed progenitor cells (Cowles *et al.*, 2013; Currie *et al.*, 2016; Currie and Pearson, 2013; Lapan and Reddien, 2012; Molinaro and Pearson, 2016; Scimone *et al.*, 2014; van Wolfswinkel *et al.*, 2014).

As the only cycling cells in planarians, neoblasts can be identified as cells with >2C DNA content (i.e. cells in the S/G2/M phases of the cell cycle), which are rapidly lost following lethal irradiation due to DNA damage (Eisenhoffer *et al.*, 2008; Newmark and Alvarado, 2000; Reddien *et al.*, 2005). These properties enable the prospective isolation of cycling neoblasts using fluorescence activated cell sorting (FACS). Developed by Hayashi *et al.*, this technique relies on the irradiation sensitivity of neoblasts combined with cell

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cycle analysis using the DNA dye Hoechst (Hayashi *et al.*, 2006). With this strategy, a population of Hoechst^{hi} cells is reproducibly lost within 24 hours post-irradiation (hpi) (Fig. 1 A,B). Referred to as the X1 FACS gate, these cells exhibit enriched expression of cell cycle markers and at least 90% express *piwi-1*; therefore, this gate is considered to be the "stem cell" gate (Eisenhoffer *et al.*, 2008; Hayashi *et al.*, 2006; Reddien *et al.*, 2005). Due to the lack of transgenesis and absence of cell-surface antibodies in planarians, this Hoechst-based FACS isolation strategy remains the only method for prospective isolation of neoblasts. Thus, in the experiments leading to the major neoblast class discoveries discussed below, the definition of these cells as neoblasts was based initially on their isolation from the X1 gate, with subsequent molecular verification using known neoblast markers such as the Argonaut-family homologs *piwi-1* and *piwi-2* (Reddien *et al.*, 2005).

In a landmark study from almost a decade ago, Eisenhoffer *et al.*, demonstrated the enrichment of "progeny markers" in another irradiation-sensitive population of cells referred to as the "X2" FACS gate (Fig 1 A,B) (Eisenhoffer *et al.*, 2008). The X2 gate resembles a classic Hoechst side population as these cells register as having <2C DNA content, which is due to Hoechst efflux (Hayashi *et al.*, 2006; Pearson and Alvarado, 2010). This can be demonstrated by flooding the cells with 5-times the normal Hoechst concentration, which results in a shift of this gate toward 2C DNA content. This indicates that the low Hoechst fluorescence normally observed is due to efflux of the dye, which is commonly seen in classic side populations (Fig 1C). Following a lethal dose of irradiation, the actively cycling neoblasts of the X1 gate are quickly eliminated, followed closely by depletion of cells in the X2 gate and leaving behind the X-insensitive (Xins) gate, which contains irradiation-insensitive, mature cell types from most tissues (Fig 1 A,B) (Eisenhoffer *et al.*, 2008; Hayashi *et al.*, 2006). From this observation, the authors reasoned that irradiation-depletion kinetics could serve as a proxy for cellular differentiation state, and thus the X2 gate is often considered the "progeny" gate. That said, how neoblasts progress through these FACS gates after cell division is not precisely known. Interestingly, the expression level of some Xins-enriched genes has been observed to decrease after irradiation, a phenomenon that does not entirely fit with its accepted description as the "mature cell" gate (Zhu *et al.*, 2015).

It is also unclear whether G1 neoblasts reside in the X2 gate (in which *piwi-1* expression is detectable, albeit at much lower levels than in the X1 gate (Labbé *et al.*, 2012)) or in between the X1 and X2 gates in an understudied region of the FACS plot that also contains irradiation-sensitive cells. To date, nearly all transcriptomic work on planarian neoblasts has focused on the X1 gate.

This review will summarize what is known about the neoblast classes and explore some interesting caveats of planarian neoblast biology. Are the lineage-primed neoblast classes *bona fide* stem cell populations? What exactly are the cell types in each FACS gate? If it is a distinct cell type, how do we find the pluripotent neoblast in the FACS cloud? What are the techniques and tools needed in the future to answer questions definitively about neoblast identity and cellular potential? Here we speculate about the answers to these questions and discuss how they might be addressed experimentally.

Lineage-primed neoblast classes

The original morphology-based characterization of the seemingly homogeneous neoblast population obscured a plethora of underlying molecular heterogeneity for decades, but recent advances in molecular techniques such as *in situ* hybridization (ISH) have been exceptionally revealing. The last 10 years have seen tremendous progress in the field of planarian neoblast biology, and thus far, there have been five major neoblast classes described in *S. mediterranea*: zeta neoblasts of the epithelial lineage, gamma neoblasts of the gut lineage, nu neoblasts of the neural lineage, the pluripotent sigma class, and finally, an experimentally verified clonogenic neoblast class (cNeoblasts) (Molinaro and Pearson, 2016; Wagner *et al.*, 2011; van Wolfswinkel *et al.*, 2014). With the exception of cNeoblasts, these neoblast classes were identified through single-cell transcriptomic analyses and some putative molecular markers have been described. The cNeoblast was identified through a series of elegant cell transplantation experiments, in which a small percentage of transplanted cells were able to clonally expand and rescue lethally-irradiated hosts (Wagner *et al.*, 2011). Molecular markers for cNeoblasts have not yet been identified, and prospective isolation using FACS has not yet been achieved for any neoblast class. Thus, studying the roles
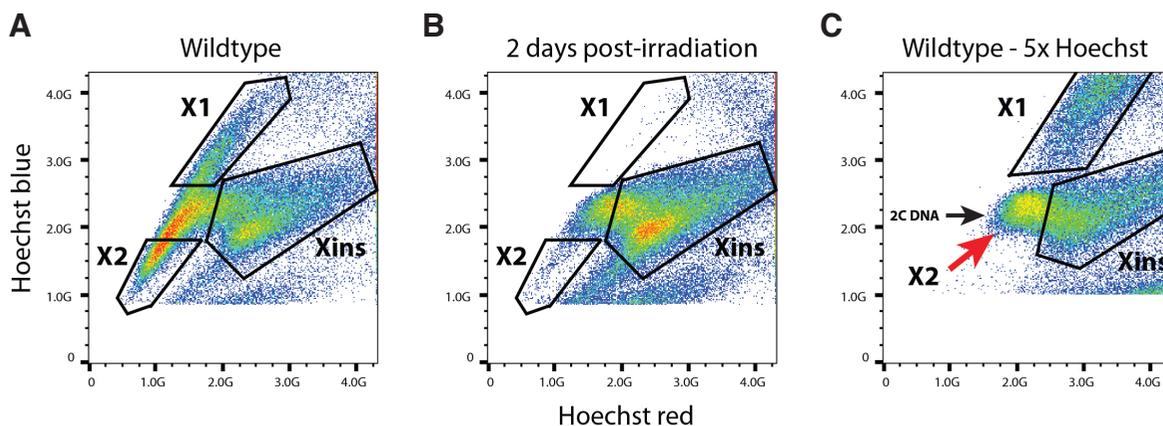
Fig. 1. Fluorescence activated cell sorting (FACS) strategy for planarian neoblasts and early progeny. *FACS plots of Hoechst-stained cells in* **(A)** *unirradiated and* **(B)** *lethally irradiated animals using a previously published staining protocol (Pearson and Alvarado, 2010).* **(C)** *Increasing the Hoechst concentration 5-fold (to 125μg/ml) results in a shift of the X2 gate into the 2C DNA content region of the plot, demonstrating that these cells do contain 2C DNA content and normally register as Hoechst-low due to efflux of the Hoechst dye.*

and dissecting the cellular potential of any neoblast class during regeneration remains a challenge.

The detection of a wide range of tissue-specific genes within *piwi-1+* neoblasts by double fluorescent ISH (dFISH) established that lineage specification is likely to occur at the neoblast level. *piwi-1+lineage-marker+* cells have now been identified for many different tissues. For example, *sp6-9*, a transcription factor required for optic cup regeneration, was observed to be expressed in a trail of eye precursor cells originating in *piwi-1+* neoblasts (Lapan and Reddien, 2011; Lapan and Reddien, 2012). Likewise, the expression of transcription factors required for the regeneration of various neural subtypes has been observed in *piwi-1+* neoblasts (e.g. the *pitx+piwi-1+* precursors of serotonergic neurons) (Cowles *et al.*, 2013; Currie and Pearson, 2013). More recently, a large-scale ISH study by Scimone *et al.*, demonstrated the non-overlapping expression of a wide variety of tissue-specific markers within *piwi-1+* neoblasts isolated from the X1 FACS gate (Scimone *et al.*, 2014). These and other such studies led to the hypothesis that there may be broad populations of lineage-committed neoblasts present in adult animals. Conversely, ideas regarding the concept of neoblast "types" versus neoblast "states" have also been articulated. These ideas postulate that, rather than maintaining lineage-committed neoblast populations, the specification of any given neoblast is stochastic and may change with the changing tissue needs of the animal (Adler and Alvarado, 2015).

The development of single-cell transcriptomics has facilitated the search for broad neoblast heterogeneity; however, further studies will likely require the establishment of *in vivo* lineage tracing technologies in order to rule out the stochastic model of neoblast specification. Nevertheless, putative epithelial, gut, and neural neoblast populations have already been described by a variety of methods (see below), and we will refer to them here as "lineage-primed neoblast classes". While much functional characterization remains to be performed, recent *in silico* evidence suggests that lineage-primed neoblast populations likely arise from a pluripotent neoblast class, termed the sigma neoblasts (Molinaro and Pearson, 2016; van Wolfswinkel *et al.*, 2014). Unfortunately, genes originally thought to be specific to the sigma neoblast class have more recently also been detected in lineage-primed neoblast classes by single cell RNA-sequencing (scRNAseq) (Wurtzel *et al.*, 2015). Because cell-lineage relationships are primarily assayed by RNA-interference (RNAi) knockdown of cell type-specific transcripts, it remains difficult to demonstrate conclusively that sigma neoblasts reside upstream of the lineage-primed classes. Indeed, it is possible that sigma neoblasts represent a heterogeneous mix of currently unresolved neoblast classes that may even include cNeoblasts.

Epithelial-primed zeta neoblasts

The epithelial lineage from cNeoblast → *zfp-1+* (zeta neoblast) → *prog-1+* (early progenitor) → *AGAT-1+* (late progenitor) → mature epithelial cells is perhaps the best-understood planarian lineage, passing through multiple progenitor cell states over the course of 7 days of differentiation (Eisenhoffer *et al.*, 2008; Pearson and Alvarado, 2010; Tu *et al.*, 2015; van Wolfswinkel *et al.*, 2014; Wurtzel *et al.*, 2015; *et al.*, 2017; Zhu *et al.*, 2015). Prior to the discovery of zeta neoblasts, the work of Eisenhoffer *et al.*, led to the discovery of the X2-enriched *prog-1* and *AGAT-1* neoblast progeny markers. These authors first proposed a lineage relationship from *prog-1+* early neoblast progeny to *AGAT-1+* late

progeny based on irradiation depletion and BrdU incorporation kinetics (Eisenhoffer *et al.*, 2008). Soon after, RNAi knockdown of another X2-enriched gene, *p53*, resulted in dysregulated neoblast proliferation followed by successive loss of the *prog-1+* and *AGAT-1+* progenitors, further supporting this lineage progression (Pearson and Alvarado, 2010). Finally, using single-cell qPCR of 96 candidate genes, van Wolfswinkel *et al.*, identified *zfp-1* as a specific marker of a subset of neoblasts, which they named the zeta neoblasts (van Wolfswinkel *et al.*, 2014). RNAi knockdown of *zfp-1* resulted in animal lethality caused by a failure in epithelial maintenance, and epithelial regeneration did not occur; although, remarkably, a regeneration blastema containing all other tissues still formed. Molecular analyses revealed the consecutive loss of *prog-1* and *AGAT-1* in *zfp-1(RNAi)* worms, thereby supporting the model that *prog-1+* cells and *AGAT-1+* cells serve respectively as early and late progenitors of the epithelial lineage (although it remains technically possible that *prog-1+* cells may progress down other differentiation paths, given difficulties in specifically removing *prog-1+* cells and the lack of a *prog-1(RNAi)* phenotype).

Whether zeta neoblasts are *bona fide*, self-renewing stem cells is unclear. Although zeta neoblasts were initially isolated from the X1 FACS gate and take up BrdU following a short 4-hour chase period, it is possible that they immediately exit the cell cycle following mitosis to begin the terminal differentiation program toward epidermis. To address the self-renewal potential of zeta neoblasts, van Wolfswinkel *et al.*, turned to the X2 FACS gate. While often regarded as the "progeny" gate, the X2 gate is known to contain *piwi-1+* cells, which are assumed to be G1 neoblasts (Labbé *et al.*, 2012; Molinaro and Pearson, 2016; Zhu *et al.*, 2015). Indeed, van Wolfswinkel *et al.*, were successful in identifying zeta expression profiles in X2 cells. In this case, co-expression of post-mitotic epithelial lineage markers, such as *prog-1*, in these cells led the authors to conclude that X2 zeta neoblasts had likely exited the cell cycle, suggesting that zeta is a transient epithelial progenitor population incapable of self-renewal (van Wolfswinkel *et al.*, 2014).

The epidermis is a heterogeneous tissue, raising the question of how zeta neoblasts become specified toward specific, spatial epithelial fates. This is a difficult question to answer given current technical limitations, but a recent scRNAseq study has uncovered a role for positional information in epithelial fate determination. In this study, single cells from the epithelial lineage were collected from dorsal, ventral and lateral regions in the animal. Differential expression analyses identified several transcripts with spatially restricted expression domains. For example, expression of *PRDM1-1* within zeta neoblasts was restricted to the dorsal side of the animal, and *PRDM1-1* expression in maturing cells is also restricted to the dorsal epithelium. Likewise, expression of *kal1* was found in zeta neoblasts and mature epithelial cells only on the ventral side. Interestingly, expression of dorsal *(PRDM1-1)* and ventral *(kal1)* markers within zeta neoblasts was mutually exclusive (Wurtzel *et al.*, 2017). These findings indicate that spatial cues are sensed at the earliest stage of epithelial commitment.

How positional signals are relayed back to zeta neoblasts remains to be fully elucidated, but BMP signaling has been demonstrated to play a role. Loss of BMP signaling by RNAi knockdown of *bmp4* results in animal-wide ventralization. Upon *bmp4* knockdown, the normally ventral *kal1+* cells were observed dorsally. Intriguingly, dorsal *kal1* expression was dependent on the presence of zeta neoblasts, as animals exposed to lethal irradiation prior to

bmp4(RNAi) did not exhibit mislocalized *kal1* expression (Wurtzel *et al.*, 2017). This result suggests that at least some terminal fate decisions are made at the zeta neoblast level (Fig 2).

Neural-primed nu neoblasts

The fact that planarians can regenerate a fully functional brain in only 7-10 days following decapitation, coupled with constant homeostatic neural turnover, led to the hypothesis that neural lineage-committed neoblasts exist in the animal (Ross *et al.*, 2017). While the presence of neoblast descendants (*piwi-1⁻* PIWI-1⁺) expressing neural markers have been described for multiple neural fates (Cowles *et al.*, 2013; Currie and Pearson, 2013; März *et al.*, 2013), more upstream, multipotent neural neoblasts were more difficult to identify *in vivo*. Using scRNAseq of X1 cells from the planarian head, our group recently described a putative neural neoblast population based on detection of a neural gene expression signature (Molinaro and Pearson, 2016). These cells, which we named nu neoblasts, expressed the neoblast marker *piwi-2* as well as several known neural markers such as *pc-2* and *chat,* and were surprisingly *piwi-1ˡᵒ.* Similar to zeta neoblasts, the self-renewal potential of nu neoblasts is currently unknown. In BrdU pulse-chase experiments, nu neoblasts incorporate BrdU within 4 hours of administration. This, coupled with the fact that these cells were isolated from the X1 FACS gate, indicates that nu neoblasts are present during the S/G2/M phases of the cell cycle. Whether nu neoblasts subsequently exit the cell cycle to terminally differentiate or enter the G1 phase to begin a new cycle is unknown, as is what happens to neurogenesis if nu neoblasts are ablated.

Differential expression analysis comparing nu neoblasts with the remaining X1 cells sequenced in our study demonstrated a clear enrichment of neural genes and identified several new candidate markers of this class. These markers appear to also be expressed in the brain proper by ISH and some of them are predicted to be transcription factors, perhaps suggesting a role in terminal fate selection. Functional characterization of nu neoblasts by RNAi knockdown of nu-enriched genes is a necessary next step for understanding the process of neural lineage progression in planarians. Focusing on the incorporation of new neurons into the brain in intact worms (via BrdU pulse-chase experiments) and during brain regeneration under RNAi conditions will provide important insights about the roles of nu neoblast-enriched genes in specifying neural fates.

In a separate study, our group also demonstrated a role for hedgehog signaling, in conjunction with the transcription factors *nkx2.1* and *arx*, in the specification of ventral-medial neurons in the brain (Currie *et al.*, 2016). dFISH experiments demonstrated expression of the *hedgehog* (*hh*) ligand in the ventral-medial region of the brain and expression of its receptor *patched-1* (*ptc-1*) in neighbouring neoblasts. RNAi of *hh* resulted in a significant decrease in the production of several ventral-medial neural populations. The differentiation of planarian glia was recently reported to depend on hedgehog signaling as well. Planarian glia marked by expression of *if-1* and *cali* are normally located within the neuropil (Wang *et al.*, 2016). Following knockdown of *ptc-1* (i.e. increasing hedgehog signaling), a dramatic increase in *if-1⁺/cali⁺* cells was observed outside of the neuropil. Interestingly, *ptc-1* expression was not detected in nu neoblasts by scRNAseq (Molinaro and Pearson, 2016). These observations raise interesting questions regarding the specification of different neuronal fates: Do other neural lineage-primed neoblast classes exist that give rise to ventral-medial brain fates? Does a dedicated neoblast class serve to maintain glial populations? Are nu neoblasts the source of new neurons primarily in non-ventral-medial brain regions? While it remains difficult to answer these questions conclusively without tracing cells *in vivo*, additional scRNAseq of neoblasts from various regions surrounding the brain may provide insights on the existence of distinct neural stem cell populations, analogous to the study of spatial heterogeneity in zeta neoblasts described above. Interestingly, in a recent study that demonstrated a role for *tcf-1* in the specification of dorsal-lateral neural subtypes, several nu neoblast-markers were found to be co-expressed in *tcf-1ʰⁱ* cells, perhaps suggesting a link between nu neoblasts and dorsal-lateral brain fates (Fig 3) (Brown *et al.*, 2018).

Gut-primed gamma neoblasts

Originally identified as a subclass of the sigma class, the gamma neoblasts are predicted to be intestinal progenitors due to their expression of several known gut markers (Barberán *et al.*, 2016; Flores *et al.*, 2016; Forsthoefel *et al.*, 2012; González-Sastre *et al.*, 2017; Wagner *et al.*, 2011; van Wolfswinkel *et al.*, 2014). The differentiation potential of gamma neoblasts has not been directly assessed, but expression of the conserved, endodermal transcription factors *hnf4, gata456a,* and *nkx2.2* in these cells is highly suggestive of intestinal fate commitment. All three factors are robustly expressed in the gut by ISH (Forsthoefel *et al.*, 2012; Wagner *et al.*, 2011). Knockdown of either *nkx2.2* or *gata456a* by RNAi results in hindered gut regeneration, however, in the case of nkx2.2 the gut regeneration phenotype is likely secondary to a neoblast proliferation defect (Forsthoefel *et al.*, 2012; González-Sastre *et al.*, 2017). Like zeta and nu neoblasts, their X1 origin confirms that gamma neoblasts pass through the S/G2/M phases of

Fig. 2. Proposed zeta neoblast model. *Cycling sigma (σ) neoblasts give rise to zfp-1⁺ zeta (ζ) neoblasts, which respond to BMP signaling and other positional cues to become specified toward dorsal or ventral epidermis. Yellow, dorsal epidermal fate; purple, ventral epidermal fate.*

planarian gut cells and supports a role for gamma neoblasts in gut maintenance and regeneration (Fig 4).

cNeoblasts: balancing adult pluripotency with controlled regeneration

The presence of pluripotent stem cells in an adult organism is peculiar, but irrefutable evidence demonstrates this to be the case in planarians. Transplantation of a single neoblast into a lethally irradiated host, in which homeostatic turnover and regeneration cannot occur due to the lack of proliferating neoblasts, led to the clonal expansion of the donor neoblast and differentiation of its progeny along lineages from all three germ layers (Wagner *et al.*, 2011). Further, transplantation of an asexually-derived cNeoblast into a lethally irradiated sexual host resulted in the complete replacement of host tissues, thereby switching the host genotype to that of the donor cNeoblast (Wagner *et al.*, 2011). Thus, cNeoblasts must reside at the top of the planarian stem cell hierarchy.

At present, cNeoblasts have only been identified retrospectively by the observation of clonal expansion following sub-lethal irradiation experiments or in single cell transplants, and molecular markers have not been identified. Thus, many questions remain to be answered regarding this unusual adult stem cell population. Are cNeoblasts a maintained population (are they sigma neoblasts?) or do they arise stochastically via the de-differentiation of another neoblast class only when required (e.g. following tissue loss)? What regulatory mechanisms are in place to prevent aberrant proliferation and the development of tumors? How can we learn more about cNeoblasts without molecular markers?

cNeoblasts as a discrete cell type or cell state?

The isolation of individual cNeoblasts for transplantation experiments was performed by FACS, however, the cytotoxic effects of Hoechst meant that cells could not be sorted using the classic Hoechst-staining strategy. Instead, cNeoblasts were collected from the "X1(FS)" FACS gate, which contains cells that display forward scatter and side scatter properties similar to X1 neoblasts (Wagner *et al.*, 2011). Due to the crudeness of this sorting strategy, the cells collected were even heterogeneous in morphology, and so the frequency of cNeoblasts is difficult to decipher. Nevertheless, because cNeoblasts were initially obtained from intact animals, it is likely that they represent a homeostatically-maintained neoblast population.

Given the abundance of multipotent neoblasts in adult planarians, the presence of actively cycling pluripotent stem cells seems to present a needless risk for the occurrence of tumorigenic mutations and subsequent teratoma formation, and such cells are not known to be common in adult organisms of other metazoans. Why, then, would

Fig. 3. Proposed nu neoblast model. *Because nu (ν) neoblasts do not express* ptc, *and nu neoblast markers are expressed in* tcf-1+ *cells by scRNA-seq, we propose that nu neoblasts may represent a dorsal-lateral neural progenitor. Thus, cycling sigma (σ) neoblasts can give rise to dorsal-lateral nu neoblasts, which may give rise specifically to dorsal-lateral neurons, or to* ptc+ *ventral-medial neural neoblasts that respond to Hh signaling from the brain to give rise to ventral-medial neurons. It is currently unclear whether these* ptc+ *neoblasts also produce glia, or if a separate glial progenitor exists in the planarian. DL, dorsal-lateral brain; VM, ventral-medial brain.*

the cell cycle, however, previous work on *hnf4* and *nkx2.2* indicate that these cells may not be self-renewing. While dFISH experiments have demonstrated mesenchymal expression of *hnf4* and *nkx2.2* in putative gamma neoblasts neighbouring the gut, these cells persist for several days following lethal irradiation, suggesting that they are downstream of cycling neoblasts (Forsthoefel *et al.*, 2012). Conversely, irradiation does cause a loss of *gata456a+piwi-1+* cells within 24 hours, and *nkx2.2* and *hnf4* expression levels were reduced following *gata456a* knockdown, suggesting that *gata456a+piwi-1+* gamma neoblasts may represent the earliest stage of gut commitment (González-Sastre *et al.*, 2017). In addition, RNAi knockdown of the gut-enriched EGF receptor *egfr-1* resulted in an accumulation of putative gamma neoblasts around the gut and a decrease in the number of terminally differentiated intestinal cells (Barberán *et al.*, 2016). Interestingly, *egfr-1* expression was also detected in PIWI-1+ cells next to the gut (Barberán *et al.*, 2016). This finding implicates a role for EGF signaling in the terminal differentiation of

Fig. 4. Proposed gamma neoblast model. *Cycling sigma (σ) neoblasts give rise to* gata456a+ *gamma (γ) neoblasts adjacent to the gut, which turn on expression of gut markers* nkx2.2 *and* hnf4 *and begin to terminally differentiate toward intestinal fates in response to EGF signaling. Green, gut cells.*

the planarian contain such cells? The unprecedented regenerative ability of planarians perhaps justifies the necessity of cNeoblasts. If lineage-primed neoblasts are analogous to the tissue resident multipotent stem cells of vertebrates, then these neoblasts would be expected to take care of homeostatic tissue needs and minor wounds but could conceivably require back-up when regenerating entire tissues *de novo*. In this context, cNeoblasts would serve to replenish the lineage-primed neoblast classes during regeneration. A role for perpetually cycling cNeoblasts during homeostasis, on the other hand, is more difficult to rationalize; however, there is currently no experimental evidence to discount the possible co-existence of quiescent and actively cycling cNeoblasts.

cNeoblasts as a quiescent population?

This leads to the hypothesis that cNeoblasts may be maintained as a reserved, or quiescent, stem cell population. The non-existence of quiescent neoblasts in planarians is largely accepted, but an argument can be made that this conclusion has not been sufficiently tested. To date, the only experiments to directly ask whether slow-cycling neoblasts exist in planarians consisted of continuous BrdU administration and analysis of the fraction of labeled mitotic neoblasts (Newmark and Alvarado, 2000). With the hypothesis that slow-cycling neoblasts would not take up the BrdU label, the authors subjected worms to several BrdU injections per day for 3 days and observed that ~99% of neoblasts were labeled by this strategy. However, only cell morphology of dissociated animals was used to determine whether a cell was a neoblast (i.e. no molecular markers). This result indicated that all neoblasts pass through S phase of the cell cycle at least once every 3 days. In addition, the authors found that by 12 hours post-BrdU administration ~96% of mitotic cells (marked by H3P) were also BrdU$^+$, indicating that all G2/M-labeled neoblasts had recently passed through S phase of the cell cycle. From this the authors reasoned that there was unlikely to be a G2-arrested population of neoblasts. What was not considered in these experiments, however, was the effect of continuous injections and repeated wound healing on neoblasts. It is plausible that this repeated wounding could provide a sufficient injury signal to activate a reserved neoblast population (Wenemoser and Reddien, 2010). The repeated incorporation of BrdU into actively cycling neoblasts would also likely result in highly cytotoxic conditions, which could conceivably lead to a depletion in the active neoblast pool by cell death and subsequent quiescent neoblast activation. To more directly test for the presence of slow-cycling, quiescent neoblasts, a pulse-chase-pulse assay using two different thymidine analogs (such as BrdU and EdU) would be more appropriate. The first pulse would be used to assay long-term label retention, while incorporation of the second pulse would confirm that these label-retaining cells are still capable of proliferation. By allowing a long chase period (perhaps several weeks) between pulses, any double-positive cells present following the second pulse must not have cycled during the chase and, therefore, would represent a quiescent population.

Support for the quiescent cNeoblast hypothesis

Interestingly, there are several examples in the planarian literature that, if viewed under the hypothesis that cNeoblasts can maintain a quiescent state, would lend support to this idea. Here we focus on two specific examples: EGF and p53 signaling.

EGF signaling promotes proliferation

The role of EGF signaling in promoting quiescent stem cell proliferation is well established in other systems. For example, quiescent neural stem cells (qNSC) in the mouse subventricular zone do not normally express the EGF receptor (EGFR) (Daynac *et al.*, 2013). These cells are much more resistant to gamma-radiation than their actively proliferating counterparts. Following irradiation-induced depletion of the active NSC pool, qNSCs have been shown to exit dormancy and acquire EGFR expression, consistent with a role for EGF signaling in promoting qNSC activation and proliferation (Daynac *et al.*, 2013). In a similar manner, EGFR inhibition in highly proliferative Lgr5$^+$ adult intestinal stem cells negated their ability to form intestinal organoids in culture. Molecular analysis revealed that, in contrast to wildtype Lgr5$^+$ cells, those treated with the EGFR inhibitor lacked the general cell cycle marker Ki67 and did not incorporate EdU, indicating an induction of quiescence (Basak *et al.*, 2017). Thus, EGF signaling is likely involved in quiescent stem cell activation.

In planarians, sub-lethal irradiation results in the loss of most neoblasts. Rare surviving neoblasts then proliferate and eventually repopulate the entire neoblast compartment, restoring homeostatic cell turnover and regenerative ability (Wagner *et al.*, 2011; Wagner *et al.*, 2012). Using a repopulation assay, Lei *et al.,* recently demonstrated a role for EGF signaling in neoblast repopulation. Following sub-lethal irradiation, *egfr-3(RNAi)* animals were unable to repopulate the neoblast compartment (Lei *et al.*, 2016). Importantly, homeostatic defects were not observed following *egfr-3* knockdown. This suggests that the rare surviving neoblasts require EGF signaling to proliferate and act primarily during regeneration.

Several lines of evidence suggest that these rare surviving neoblasts may be quiescent cNeoblasts. First, the ability to resist higher doses of gamma-radiation is a common feature of quiescent stem cells. Second, surviving neoblasts clonally expand to repopulate the entire neoblast compartment and restore normal tissue production, indicating that the surviving neoblasts are pluripotent.

In this way, the *egfr-3* RNAi phenotype could be interpreted as a defect in quiescent neoblast activation. In planarians, the regenerative response is characterized by two peaks in mitosis, the first occurring at 6 hours post-injury (hpi) and the second occurring around 48 hpi (Wenemoser and Reddien, 2010). Analysis of the mitotic response during regeneration in *egfr-3(RNAi)* animals revealed a slight decrease in the magnitude of the first peak and almost a complete lack of a second peak (Lei *et al.*, 2016). The first mitotic peak, which also occurs following minor wounding and feeding (Wenemoser and Reddien, 2010), is likely the responsibility of the active neoblast compartment, possibly including the lineage-primed neoblast classes. It is conceivable that following tissue loss this first mitotic peak largely exhausts the active neoblast compartment. For successful regeneration to occur, neoblasts of all lineages must then be replenished by a reserved pluripotent neoblast population. If reserved neoblasts cannot receive activating signals, the mitotic response would be mitigated, as observed following *egfr-3* knockdown (Fig 5A). This is consistent with the idea that cNeoblasts are reserved for replenishing the active neoblast compartment as it becomes depleted due to the demanding tissue requirements of regeneration. Importantly, in the case of *egfr-3* knockdown, the repopulation and regeneration defects were due to a decrease in proliferation, not

an increase in cell death (Lei *et al.*, 2016). This supports the notion that irradiation-resistant pluripotent cNeoblasts require EGF signaling to allow proliferation.

p53 maintains quiescence

p53, primarily known for its tumor suppressor activity, is an important negative regulator of proliferation (Rivlin *et al.*, 2011). A role for p53 in maintaining quiescence in hematopoietic stem cells (HSC) has been described. p53 is highly expressed in HSCs, which, as a population, remain quiescent (Fleming *et al.*, 1993; Foudi *et al.*, 2009; Trumpp *et al.*, 2010; Wilson *et al.*, 2008; Wilson and Trumpp, 2006). While microenvironmental factors arising from the bone marrow niche likely contribute to the maintenance of HSC quiescence (Wilson and Trumpp, 2006), p53 expression has also been shown to play a role (Liu *et al.*, 2009). In *p53-/-* mice, the proportion of HSCs in G0 is significantly reduced while the number of cycling HSCs is increased, indicating premature activation of these cells (Liu *et al.*, 2009). Similar findings from human fibroblasts, in which abrogation of p53 function results in activation of the normally quiescent population, further support a role for p53 in maintaining quiescence (Itahana *et al.*, 2002).

In intact planarians, knockdown of *p53* by RNAi results in animal-wide neoblast hyperproliferation followed by eventual neoblast exhaustion (Pearson and Alvarado, 2010). Intriguingly, this hyperproliferation was not observed during regeneration. The original interpretation of this result was that the level of proliferation achieved during regeneration is the highest possible in planarians. An alternative interpretation revolves around p53's role in maintaining quiescence. Let us hypothesize that, in intact animals, pluripotent cNeoblasts are not required to maintain tissue homeostasis and are maintained in a quiescent state by p53. Under this hypothesis, upon *p53* knockdown, these cells would become prematurely activated, causing the observed hyperproliferation and eventually leading to neoblast exhaustion. During regeneration, reserved neoblasts would normally be activated to replenish the active neoblast compartment, and so loss of quiescence at this stage should not influence proliferation levels compared to controls. Thus, the observed p53 phenotype is consistent with the expected effect of premature activation and differentiation of quiescent cNeoblasts (Fig. 5A). Interestingly, *p53* expression was found to be enriched in the X2 FACS gate, where it was detected in approximately 14% of cells (Pearson and Alvarado, 2010). This is notable considering that the X2 gate resembles a Hoechst side population, which have classically been found to be highly enriched for stem cells in many tissues, including multipotent quiescent stem cells (Alvi *et al.*, 2003; Bhatt *et al.*, 2003; Bhattacharya *et al.*, 2003; Goodell *et al.*, 1996; Wulf *et al.*, 2003).

An interesting caveat of the *p53* phenotype is revealed at a low RNAi dose, which results in the formation of teratoma-like outgrowths (Pearson and Alvarado, 2010). It is plausible that lowering the RNAi dose limits the phenotypic effect to a small number of cells, thereby resulting in only localized hyperproliferation. These outgrowths were found to contain all major tissue types, suggesting that an aberrantly proliferating cNeoblast may be the founding cell.

In a recent study by Cheng *et al.*, a transcriptional hierarchy controlling epithelial lineage commitment and progression was described, at the top of which sits p53. This was not surprising, as p53 had previously been shown to be co-expressed with and affect other epithelial lineage markers (*prog-1* and *AGAT-1*), and was detected in *zfp-1*[+] zeta neoblasts by single cell qPCR and scRNAseq (Pearson and Alvarado, 2010; van Wolfswinkel *et al.*, 2014; Wurtzel *et al.*, 2017). What was interesting, however, was the discovery by Cheng *et al.*, of *p53*[+]*piwi-1*^{+} cells located adjacent to the gut. These cells were found to express various combinations of zeta, gamma and sigma neoblast markers, leading the authors to speculate that these may be pluripotent neoblasts. While work regarding the planarian stem cell niche is scant and the proliferative potential of *p53*[+]*piwi-1*^{+} cells is yet to be assessed, it is intriguing to consider that the gut epithelium might provide niche signals important for maintaining quiescent cNeoblasts (Cheng *et al.*, 2017).

FACS strategies moving forward

Strategies for studying lineage-primed neoblast classes

In the case of the neoblast classes discussed above, we recognize these cell groups as neoblast populations primarily because they were originally isolated from the X1 gate, however, it is difficult to say with certainty whether these populations truly fit the definition of stem cells. While BrdU incorporation into zeta and nu neoblasts has been demonstrated after very short chase periods, it remains entirely possible that these cell types are specified during S-phase and do not re-enter the cell cycle. Indeed, a recent study by Lai *et al.*, suggested that sigma neoblasts may be the only cycling neoblast class based on the expression of sigma-enriched genes in what were described as "giant" endocycling neoblasts, which appeared following knockdown of the condensin I subunit *Smed-NCAPG* (Lai *et al.*,
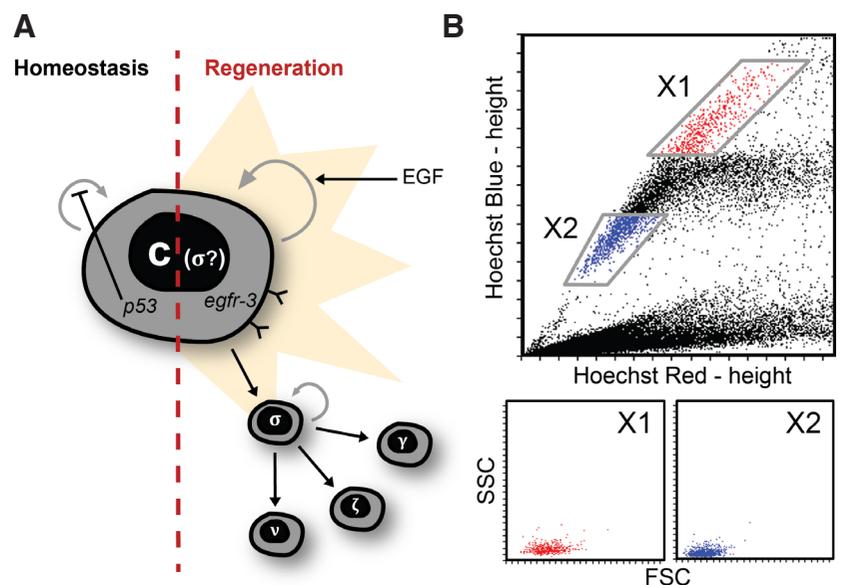
Fig. 5. Proposed cNeoblast model. (A) *During homeostasis,* p53 *acts to inhibit the cell cycle and maintain cNeoblasts (c) in a quiescent state. During regeneration, expression of* egfr-3 *is increased and cNeoblasts begin to proliferate in response to EGF signaling originating from the niche. cNeoblasts reside at the top of the stem cell hierarchy in planarians and replenish all downstream neoblast populations.* **(B)** *FACS plots demonstrating the similar forward scatter (FSC) and side scatter (SSC) properties of X1 and X2 cells.*

2017). Without the ability to prospectively isolate specific neoblast classes or trace cells *in vivo*, it will be difficult to show definitively whether these cells are capable of self-renewal.

Closer examination of the X2 gate may prove to be informative for evaluating the self-renewal potential of the neoblast classes. Because G1 neoblasts likely reside in the X2 (or non-X1) gate, lineage-primed neoblasts capable of self-renewal should transition through this gate during the G1 phase of the cell cycle. As discussed above, transcriptional analysis of X2 zeta neoblasts has provided some insights regarding their self-renewal potential. Additional scRNAseq of the X2 gate may reveal zeta neoblasts that retain a general G1 neoblast expression profile. Similar analyses for the gamma and nu classes will be necessary to begin elucidating the self-renewal capacity of these cells.

As a work-around solution for the lack of prospective isolation of lineage-primed neoblasts, several studies have used qPCR to check for the presence of tissue-specific gene expression in single-cell cDNA libraries prior to sequencing (Scimone *et al.*, 2016; Wurtzel *et al.*, 2017). Libraries that lack expression of the gene are immediately removed from the experiment, resulting in a highly purified collection of cDNA libraries for sequencing. While effective, this approach is laborious and incompatible with newer high throughput technologies, such as Drop-seq (Macosko *et al.*, 2015). As an alternative approach, FACS purification of fluorescently labeled cells may prove useful. Although transgenic labeling is not currently possible and cell-surface antibodies have proven hard to come by, FISH is a very well-established technique in the planarian field. Fortunately, several protocols exist describing methods for reverse-crosslinking formaldehyde fixed samples and subsequently preparing cDNA libraries for RNA-seq. One such protocol, FRISCR, is specifically designed to recover high quality RNA from single fixed cells (Thomsen *et al.*, 2015). Granted, prospective isolation of live lineage-primed neoblasts is desirable, this FISH/FACS strategy for scRNAseq would provide an in-depth look at the transcriptional events regulating neoblast specification.

Strategies for studying cNeoblasts

Because molecular markers have not yet been identified for cNeoblasts, the techniques for neoblast class enrichment described above are not possible for this class. However, once again the X2 gate may hold some answers. As a pluripotent stem cell, cNeoblasts presumably reside at the top of the stem cell hierarchy and therefore represent *bona fide* stem cells. As such, cNeoblasts must also exist outside of the S/G2/M phases of the cell cycle. Although they were described to have similar physical properties as cells from the X1 gate, cells of the X2 gate display very similar forward and side scatter properties as X1s (Fig. 5B), therefore, it is difficult to conclude from these metrics alone whether cNeoblasts primarily reside in the X1 or X2 gate. Considering that bona fide stem cells are often found within the Hoechst side population and that current evidence cannot rule out the hypothesis that cNeoblasts are homeostatically quiescent, the X2 gate seems a likely source of cNeoblasts.

Future outlook

Understanding planarian stem cell lineages is an important prerequisite for elucidating the robust regenerative mechanisms employed by these animals. While single-cell transcriptomic analysis has proven valuable, it will be necessary to establish other tools to accomplish detailed lineage analyses. Given the prior success of FACS strategies for live neoblast and progeny isolation, the production of cell surface antibodies that are compatible with live cells would be invaluable for prospectively isolating different classes of neoblasts, similar to what is done for human HSC lineage isolation. Once cells are isolated, their potency could be tested *in vitro* by cell culture or *in vivo* by transplantation into irradiated hosts. An even better approach would involve transgenically marking cells prior to transplant so all of a cell's descendants can be assayed as well. This combination of subtype isolation with lineage tracing would give definitive answers regarding the potency of a given cell, as well as whether the lineage-primed classes are competent to respond to niche signals and go back up the hierarchy to pluripotency.

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