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Single-Cell Analysis Reveals Functionally Distinct Classes within the Planarian Stem Cell Compartment

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SUMMARY

Planarians are flatworms capable of regenerating any missing body region. This capacity is mediated by neoblasts, a proliferative cell population that contains pluripotent stem cells. Although population-based studies have revealed many neoblast characteristics, whether functionally distinct classes exist within this population is unclear. Here, we used high-dimensional single-cell transcriptional profiling from over a thousand individual neoblasts to directly compare gene expression fingerprints during homeostasis and regeneration. We identified two prominent neoblast classes that we named ζ (zeta) and σ (sigma). Zeta-neoblasts encompass specified cells that give rise to an abundant postmitotic lineage, including epidermal cells, and are not required for regeneration. By contrast, sigma-neoblasts proliferate in response to injury, possess broad lineage capacity, and can give rise to zeta-neoblasts. These findings indicate that planarian neoblasts comprise two major and functionally distinct cellular compartments.

INTRODUCTION

Adult stem cells play crucial roles in processes such as tissue turnover and regeneration, but regulatory mechanisms involved in the maintenance and lineage specification of stem cells remain poorly understood. Adult planarians maintain a population of dividing cells with broad differentiation potential, presenting the opportunity to study these processes in vivo.

The seemingly inexhaustible regenerative capacity of planarians has fascinated biologists for well over a century (Reddien and Sánchez Alvarado, 2004). Planarians can repair extensive wounds and replace entire organs, such as the brain. Planarian regeneration is mediated by a population of small, proliferative cells (~8 μm in diameter), termed "neoblasts," that are dispersed throughout mesenchymal regions of the body. The neoblast population is essential for regeneration and contains pluripotent stem cells ("cNeoblasts") that can give rise to all somatic cell types in the adult body (Wagner et al., 2011).

Studies using general mitotic markers (Newmark and Sánchez Alvarado, 2000; Wenemoser and Reddien, 2010), whole-body irradiation (Guedelhoefer and Sánchez Alvarado, 2012; Reddien et al., 2005b; Wolff and Dubois, 1948), and bromodeoxyuridine (BrdU) labeling (Eisenhoffer et al., 2008; Newmark and Sánchez Alvarado, 2000), have yielded population-level analyses of neoblast proliferation, dynamics, and collective cellular output. Furthermore, transcriptional analyses have identified many neoblast-enriched transcripts (Blythe et al., 2010; Eisenhoffer et al., 2008; Galloni, 2012; Labbé et al., 2012; Onal et al., 2012; Resch et al., 2012; Shibata et al., 2012; Solana et al., 2012; Wagner et al., 2012), including those encoding several Sox and Pou transcription factors, gene families with important roles in stem cell maintenance in other organisms. Neoblasts also express transcripts for the PIWI proteins Smedwi-1 and Smedwi-2 (Reddien et al., 2005b), the Bruno-like protein Bruli (Guo et al., 2006), and a Tudor protein (Solana et al., 2009). These proteins are typically found in association with nuage, an electron-dense perinuclear organelle present in germ cells, which plays a role in transposon silencing and maintenance of genome integrity (Voronina et al., 2011). Several of these nuage-related genes are required for maintenance of the neoblast population (Guo et al., 2006; Reddien et al., 2005b; Salvetti et al., 2005; Solana et al., 2009).

Previous studies have thus provided important insights into the general features of neoblasts. However, they also reinforced the historic notion that neoblasts represent a single homogeneous stem cell population. Recently, gene expression signatures specific to differentiated tissues were shown to mark small numbers of lineage-specified neoblasts during regeneration (Cowles et al., 2013; Currie and Pearson, 2013; Lapan and Reddien, 2011; Scimone et al., 2011) and in homeostasis (Hayashi et al., 2010; Moritz et al., 2012). In addition, neoblasts can be subdivided with respect to an otherwise uncharacterized surface antigen (Moritz et al., 2012) or a *trans*-splicing sequence (Rossi et al., 2014). However, as a consequence of small numbers and/or lack of functional characterization, it remains unknown whether the aforementioned phenomena reflect the presence of substantial cellular classes within the neoblast population.

Multidimensional single-cell transcriptional profiling is a powerful approach to distinguish true cellular heterogeneity from biological noise and has facilitated the deconvolution of heterogeneous cell populations across a wide range of biological systems. Here, we used this approach on neoblasts, comparing gene expression fingerprints from over a thousand individual

cells under different conditions. We show that neoblasts can be divided into multiple prominent classes, characterized by coregulated transcript sets and displaying distinct regenerative properties. We propose that neoblasts are a heterogeneous pool of cells, containing both pluripotent and prominent lineage-committed subsets.

RESULTS

Selection of Markers for Single Neoblast Profiling

To molecularly profile individual neoblasts of *Schmidtea mediterranea*, we performed highly parallel single-cell quantitative RT-PCR (qRT-PCR) on the Biomark (Fluidigm) platform (Figures S1A–S1F available online), analyzing 96 genes from each cell. Genes were selected from a de novo neoblast transcriptome (accession SRP042226) and included nuage-related neoblast markers (*smedwi-1*, *smedwi-2*, *smedwi-3*, *Smed-vasa-1*, *Smed-vasa-2*, *Smed-tdrd1L2*, *pumilio*, and *Smed-bruli* [Guo et al., 2006; Palakodeti et al., 2008; Reddien et al., 2005b; Salvetti et al., 2005; Solana et al., 2009; Wagner et al., 2012]); cell cycle regulators (*Smed-rb*, *Smed-pcna*, *Smed-mcm2*, and *Smed-cyclinB* [Reddien et al., 2005a; Salvetti et al., 2000; Zhu and Pearson, 2013]); markers of postmitotic planarian cell types (*Smed-prog-1*, *Smed-prog-2*, *Smed-porcn-a*, and *Smed-mhc1* [Eisenhoffer et al., 2008; Pearson et al., 2009; Wagner et al., 2012]); reference genes (*Smed-g6pd*, *Smed-ubiquilin*, *Smed-luc7-like*, and *Smed-clathrin*); and 16 other genes with enriched expression in neoblasts (Wagner et al., 2012) (Figure S1H; Table S1). The majority of selected genes consisted of the 55 most abundantly detected transcripts corresponding to transcription factors and transcriptional regulators in our neoblast transcriptome.

Global expression patterns of selected genes were assessed by whole-mount in situ hybridization and by RNA sequencing (RNAseq) analysis of isolated cell populations (Figure S1H). These analyses showed that although the selected transcripts were all present in neoblasts, they were not necessarily enriched in these cells.

Gene Expression Profiling Divides Neoblasts into Two Major Classes

We used fluorescence-activated cell sorting (FACS) (Hayashi et al., 2006) to isolate individual neoblasts with 4C DNA content (X1(4C)) from the prepharyngeal region of intact worms for single-cell transcriptional analysis (Figures S1A–S1D). Hierarchical clustering (HC) of the cells based on their gene expression profiles revealed that neoblasts comprise two major, roughly equally sized populations (Figure 1A; Figure S1G). One population, the zeta-class ("ζ-class"), marked in magenta, expressed high levels of a discrete set of genes (e.g., *Smed-zfp-1*, *Smed-g6pd*, *Smed-fgfr-1*, *Smed-p53*, *Smed-soxP-3*, and *Smed-egr-1*); the other population, the sigma-class ("σ-class"), marked in green, expressed low levels of those genes but had elevated expression of *Smed-soxP-1*, *Smed-soxP-2*, *Smed-soxB-1*, *Smed-smad-6/7*, *Smed-inx-13*, *Smed-pbx-1*, *Smed-fgfr-4*, and *Smed-nlk-1*. Within these two major classes, at least one subclass was identified: the gamma-class ("γ-class") marked in blue, was readily discerned as a subclass within the sigma-class and expressed high levels of *Smed-gata4/5/6*, *Smed-nkx2.2*, *Smed-hnf4*, and *Smed-prox-1* (see Figure S1G for description of further subclasses).

Feature reduction by analysis of variance (ANOVA) revealed a reduced set of markers (primarily transcription factors) with high differential expression between the classes (Figure 1B), and HC based on the 25 most discriminating genes correctly assigned the majority of cells to their classes. Principal component analysis (PCA) was used as an independent method to reduce data complexity and identified the differences between the sigma- and zeta-neoblasts as the primary source of variance in the expression data (Figure 1C). Moreover, the subset of transcripts contributing the majority of the variance was similar to that discovered by ANOVA (Figure 1D).

Fluorescence in situ hybridization (FISH) on FACS-isolated 4C cells, using *smedwi-1* as a ubiquitous neoblast marker, confirmed the largely overlapping expression of transcripts within each class, as well as the nonoverlapping expression of transcripts between classes (Figure 1E). Because many transcripts are expressed at low levels and because no single transcript can reliably label all members of a class, we assembled RNA probe pools for improved class detection by FISH. Balancing signal intensity, class specificity, and neoblast specificity, we pooled *zfp-1*, *fgfr-1*, *soxP-3*, and *egr-1* probes for zeta-neoblasts and *soxP-1* and *soxP-2* probes for sigma-neoblasts. Indeed, probe mixtures displayed nonoverlapping expression and improved overall class detection (Figure 1E).

Neoblast Classes Are Not Defined by Cell Cycle State

The two identified neoblast classes could reflect different cell cycle states within an otherwise homogenous 4C cell population, namely G2 phase and M phase. FISH analysis of animals treated with the M-phase blocker nocodazole (Figure S2D), however, showed that cells of each class were colabeled with the mitotic marker H3P (histone H3 phosphorylated on Serine 10), indicating that both classes are present among M-phase cells (Figure 2A; Figure S2E). Similarly, each class was rapidly colabeled with BrdU, a thymidine analog that is incorporated into newly synthesized DNA (Figures 2B and S2F), indicating that both classes are also present during S phase.

We next used FACS to isolate additional cell-cycle-restricted populations according to DNA content: 2C irradiation-sensitive cells ("X2 cells," which encompass a mixture of cells in G0 or G1 phase and early postmitotic cells) and cells with DNA content between 2C and 4C (cells in S phase, "X1(S)"; Figure S1D). Single-cell profiling showed that, although levels of several nuage-related neoblast markers (such as *smedwi-1*, *vasa-1*, *vasa-2*, *tdrd1L2*, and *bruli*) and all cell cycle markers (*rb*, *pcna*, *mcm2*, and *cyclinB*) were correlated with cell cycle stage, the sigma- and zeta-class remained prominent in all three stages analyzed (Figures S2G–S2I). In addition, PCA showed that the primary variance vector (PC1) of the gene expression fingerprints correlated with cell cycle stage, but the second, independent vector (PC2) correlated with class membership (Figure 2C).

Together, these data indicate that the σNeoblasts and the ζNeoblasts reflect two separate populations that remain present throughout the cell cycle. This represents the first identification of major cell-cycle-independent classes within the neoblast population.