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An RNAi Screen Reveals Intestinal Regulators of Branching Morphogenesis, Differentiation, and Stem Cell Proliferation in Planarians

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SUMMARY

Planarians grow and regenerate organs by coordinating proliferation and differentiation of pluripotent stem cells with remodeling of postmitotic tissues. Understanding how these processes are orchestrated requires characterizing cell-type-specific gene expression programs and their regulation during regeneration and homeostasis. To this end, we analyzed the expression profile of planarian intestinal phagocytes, cells responsible for digestion and nutrient storage/distribution. Utilizing RNA interference, we identified cytoskeletal regulators required for intestinal branching morphogenesis and a modulator of bioactive sphingolipid metabolism, *ceramide synthase*, required for the production of functional phagocytes. Additionally, we found that a gut-enriched homeobox transcription factor, *nkx-2.2*, is required for somatic stem cell proliferation, suggesting a niche-like role for phagocytes. Identification of evolutionarily conserved regulators of intestinal branching, differentiation, and stem cell dynamics demonstrates the utility of the planarian digestive system as a model for elucidating the mechanisms controlling postembryonic organogenesis.

INTRODUCTION

Many animals replenish the cells of internal organs in response to physiological turnover, but only some can regenerate organs in response to traumatic injury. For example, in widely studied vertebrates and invertebrates, the epithelial lining of the digestive tract is renewed continuously, and some molecular pathways utilized in the context of tissue homeostasis are also employed in response to infection and cytotoxic damage (Faro et al., 2009; van der Flier and Clevers, 2009; Jiang and Edgar, 2012). By contrast, other animals are capable of de novo morphogenesis of gastrointestinal (GI) organs: sea cucumbers regenerate their digestive tract after spontaneous evisceration (Mashanov and García-Arrarás, 2011); several annelids regrow missing portions of their GI organs during asexual reproduction (Takeo et al., 2008; Zattara and Bely, 2011); and the ascidian *Polyandrocarpa misakiensis* regenerates its esophagus, stomach, and intestine after amputation (Kaneko et al., 2010). Among vertebrates, the ability to recover from intestinal transection has been identified only in amphibians (Goodchild, 1956; O'Steen, 1958). Because animals capable of GI regeneration have not been accessible to molecular genetic approaches, the mechanisms underlying GI regeneration remain obscure.

The planarian *Schmidtea mediterranea* has emerged as a useful model for studying organ regeneration. Planarians regenerate in response to nearly any type of amputation; even small tissue fragments are capable of regenerating into complete animals (Newmark and Sánchez Alvarado, 2002; Reddien and Sánchez Alvarado, 2004). Pluripotent somatic stem cells called neoblasts are distributed throughout most regions of the planarian body (Newmark and Sánchez Alvarado, 2000; Wagner et al., 2011). After amputation, neoblasts proliferate and differentiate, regenerating a variety of organs, including the nervous system (Cebrià, 2007; Agata and Umesono, 2008), excretory system (Rink et al., 2011; Scimone et al., 2011), and intestine (Forsthoefel et al., 2011). As the only dividing somatic cells, neoblasts also supply new tissue in response to cellular turnover and during growth (Newmark and Sánchez Alvarado, 2000; Eisenhoffer et al., 2008; Wagner et al., 2011).

The planarian intestine provides an attractive system in which to examine mechanisms of stem-cell-based organogenesis, including the regulation of differentiation, the maintenance and remodeling of organ morphology by postmitotic cells, and the influence of differentiated tissue on stem cell dynamics. Neoblasts differentiate into enterocytes during both growth and regeneration (Forsthoefel et al., 2011; Wagner et al., 2011). Furthermore, postmitotic enterocytes remodel during the addition of intestinal branches in growing animals and the re-establishment of gut morphology after amputation (Forsthoefel et al., 2011). However, aside from the potential influence of a number of axial polarity cues (Forsthoefel and Newmark, 2009; Reddien, 2011), the mechanisms that regulate differentiation and remodeling of enterocytes are unknown. Similarly, the role of differentiated cells in influencing neoblast dynamics is poorly understood. Because neoblast proliferation is upregulated after feeding (Baguñà, 1974, 1976a; Baguñà and Romero, 1981), and neoblasts associate with the severed ends of intestinal

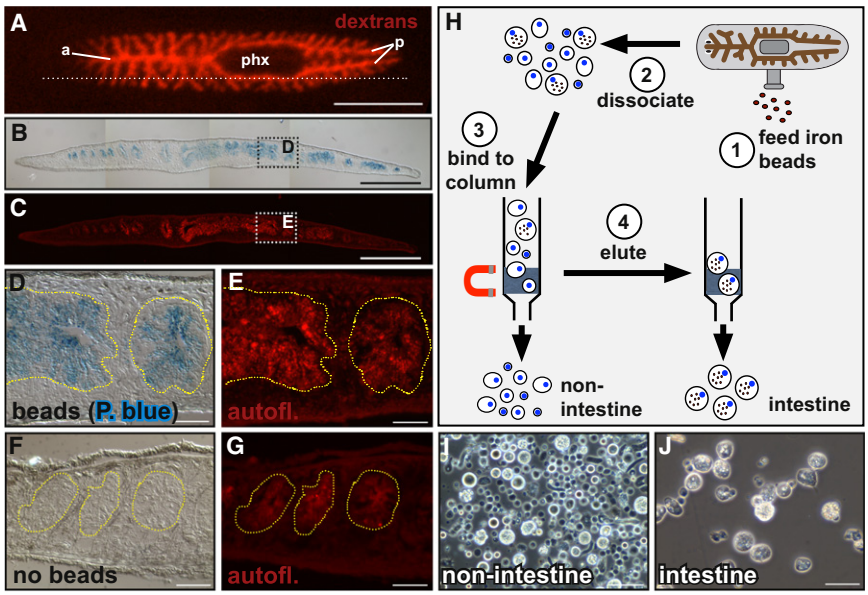

Figure 1. Isolation of Planarian Intestinal Phagocytes

(A) Intestinal branches in a live planarian fed Alexa 568-conjugated dextrans. One primary anterior branch (a) extends to the head; two posterior branches (p) project around the pharynx (phx); secondary and tertiary branches extend laterally. Dotted line indicates sectioning plane in (B) and (C).

(B) Prussian Blue-labeled parasagittal section of a planarian fed iron beads.

(C) Correlation with intestinal autofluorescence (red) demonstrates intestine-specific iron uptake.

(D and E) Magnification of boxed regions in (B) and (C); yellow dashed lines delineate gut branches.

(F and G) Animals fed liver only do not label with Prussian Blue.

(H) Schematic depicting intestinal phagocyte purification.

(I and J) Nonintestinal ("flow-through," I) and purified intestinal cells (J) obtained by magnetic sorting.

(A) Anterior is to the left, dorsal view. (B–G) Anterior is to the left; dorsal is up. Scale bars: 1 mm (A); 500 μm (B and C); 50 μm (D–G, I, and J).

branches after amputation (Wenemoser and Reddien, 2010), the intestine could serve as a source of signals that regulate neoblast dynamics.

Expression of genes required for organ morphogenesis is often maintained by differentiated cells in fully developed organs (Cebrià, 2007; Zorn and Wells, 2009; Scimone et al., 2011; Lapan and Reddien, 2012). Thus, expression profiling of postmitotic tissues is an important step in elucidating the regulation of organ growth and regeneration. Here, we have developed a protocol for isolating planarian intestinal phagocytes, enabling us (1) to characterize the gene expression profile of this cell type and (2) to perform a targeted RNAi screen to identify genes required for intestinal morphogenesis and function.

RESULTS

Isolation of Intestinal Phagocytes

The planarian intestine is an extensively branched system of epithelial tubes (Figure 1A) comprised of a single layer of columnar cells resting on a basement membrane and encircled by enteric muscles (Willier et al., 1925; Ishii, 1965). As a "blind" gut, food enters the intestine and waste is excreted through a centrally located muscular pharynx (Figure 1A) (Hyman, 1951). Two intestinal cell types have been identified histologically: absorptive phagocytic enterocytes that engulf food particles for intracellular digestion, and secretory goblet or "gland" cells that release enzymes (Ishii, 1965; Garcia-Corrales and Gamo, 1986, 1988).

Phagocytes retain fluorescent conjugates and other molecules after feeding and digestion (Figure 1A) (Morgan, 1900; Saló and Baguñà, 1985; Forsthoefel et al., 2011). The intestine also retains "Feridex," an aqueous colloid of superparamagnetic iron oxide associated with dextran (Figures 1B–1E). Prussian Blue stains intestinal branches in Feridex-fed animals (Figures 1B–1E) but not in control animals fed only liver (Figures 1F and 1G), demonstrating the specificity of iron uptake. We developed a purification strategy in which we dissociated Feridex-fed planarians into single cell suspensions and separated phagocytes from other cell types via magnetic column (Figures 1H–1J).

Global Analysis of Phagocyte Gene Expression

We compared the gene expression profiles of purified phagocytes (Figure 1J) to other planarian cells (Figure 1I) using microarrays (Table S1 available online). Of 11,521 transcripts represented on the arrays, 1,514 were upregulated in phagocytes at levels $\geq$1.5-fold than in other planarian cell types (Figures S1A–S1F; Table S1). Of these, 1,152 genes (~76%) are predicted to encode homologs of proteins found in other organisms (Table S1). For example, the top BLASTX hits for 30 genes matched proteins from the parasitic flatworm *Schistosoma japonicum* (Table S1).

To characterize the phagocyte expression profile, we assigned gene ontology (GO) terms (Ashburner et al., 2000) and determined which biological process, molecular function, and cellular component categories were overrepresented in intestinal cells (Table S2; Figures S1G–S1I). Many enriched categories are consistent with a polarized cell type that remodels after injury and is responsible for intracellular digestion, nutrient distribution, and storage. For example, the most highly represented biological process categories include: regulation of cell shape; secretion; transport; tube development; and morphogenesis of an epithelium (Figure S1G). Within the cellular component hierarchy, gene products are predicted to localize to membrane-based organelles and the actin cytoskeleton (Figure S1I).

Validation Reveals Dynamic Expression during Regeneration and Distinguishes Subpopulations of Intestinal Phagocytes

In order to validate the microarray results, we analyzed the expression of a subset of upregulated genes using whole-mount in situ hybridization. We focused on genes predicted to regulate several aspects of intestinal biology, including: remodeling or branching morphogenesis (cytoskeletal regulators); gene expression or differentiation (transcription factors, RNA binding