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Radial glial progenitors repair the zebrafish spinal cord following transection

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ABSTRACT

In mammals, spinal cord injury results in permanent sensory–motor loss due in part to a failure in reinitiating local neurogenesis. However, zebrafish show robust neuronal regeneration and functional recovery even after complete spinal cord transection. Postembryonic neurogenesis is dependent upon resident multipotent progenitors, which have been identified in multiple vertebrates. One candidate cell population for injury repair expresses *Dbx1*, which has been shown to label multipotent progenitors in mammals. In this study, we use specific markers to show that cells expressing a *dbx1a:GFP* reporter in the zebrafish spinal cord are radial glial progenitors that continue to generate neurons after embryogenesis. We also use a novel larval spinal cord transection assay to show that *dbx1a:GFP*$^+$ cells exhibit a proliferative and neurogenic response to injury, and contribute newly-born neurons to the regenerative blastema. Together, our data indicate that *dbx1a:GFP*$^+$ radial glia may be stem cells for the regeneration of interneurons following spinal cord injury in zebrafish.

Introduction

During vertebrate embryogenesis, neurons of the central nervous system (CNS) are initially derived from neuroepithelial progenitors, some of which transform into radial glia (Mori et al., 2005). By the end of embryogenesis most mammalian radial glia differentiate as astrocytes (Rakic, 2003). However in anamniotes radial glia persist widely in the CNS (García-Verdugo et al., 2002; Naujoks-Manteuffel and Roth, 1989; Zupanc and Clint, 2003), and their continued presence has been implicated in the striking ability of these animals to regenerate following injury (Chernoff et al., 2003; Hui et al., 2010; Rehermann et al., 2011). Thus radial glia have been suggested to represent an endogenous neural stem cell population.

In contrast to the permanent loss of sensory and motor function after spinal cord injury observed in mammals, urodele amphibians and teleost fish regenerate lost tissue and reestablish damaged connections, restoring function to nearly pre-injury levels (Chernoff et al., 2002; Kuscha et al., 2012). In addition to axonal regrowth after spinal cord transection in adult zebrafish (Becker et al., 1997, 2004; Goldshmit et al., 2012; Schweitzer et al., 2007), spinal lesion triggers generation of motoneurons and interneurons, with pre-injury levels restored by 6–8 weeks post injury (wpi, Reimer et al., 2008). While *olig2*$^+$ radial glia represent a pool of motoneuron progenitors that contribute to neurogenesis after lesion, the identity and behavior of other progenitor populations remain unknown.

In vertebrates, *Dbx* genes encode a family of homeodomain transcription factors expressed in the intermediate spinal cord that is necessary for spinal cord development (Jessell, 2000; Lu et al., 1992). *Dbx1*-expressing cells predominately produce Evx1/2$^+$ interneurons (Pierani et al., 2001), but also generate radial glia, astrocytes and oligodendrocytes (Fogarty et al., 2005). In mouse, *Dbx1* expression is not detectable beyond E16.5, suggesting that mammalian *Dbx1*$^+$ progenitors terminally differentiate (Fogarty et al., 2005). Zebrafish have two *Dbx1* orthologs, *dbx1a* and *dbx1b*, which are similarly expressed in the intermediate spinal cord (Gribble et al., 2007; Seo et al., 1999); however, their lineage is uncharacterized. Based on the multipotency of *Dbx1*$^+$ progenitors in amniotes, and the persistence of radial glia in zebrafish, we hypothesized that *Dbx1*-expressing cells might represent a population that could contribute to regeneration of the spinal cord following injury.

We previously generated a *dbx1a:GFP* transgenic reporter line (Gribble et al., 2009), and showed that GFP expression colocalized with endogenous *dbx1a* expression in embryonic spinal progenitors. In this study, we characterize the identity of *dbx1a:GFP* expressing cells and their progeny in the embryonic and larval zebrafish, and their response to spinal cord transection. We show that *dbx1a* mRNA expression persists beyond embryogenesis, and that the *dbx1a:GFP* reporter transgene labels a neurogenic spinal progenitor population. We also show that *dbx1a:GFP* expressing cells are slowly dividing neural progenitors that increase their rate of neurogenesis beyond basal levels in response to transection. Together, our data suggest that *dbx1a:GFP*$^+$

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radial glia may represent a neural stem cell population in the postembryonic spinal cord that can be activated in response to injury.

Materials and methods

Fish strains and staging

Embryos were obtained from wildtype (AB*), *Tg(olig2:dsRed)*^{vu19}, *Tg(elavl3:EGFP)*^{knu3}, and *Tg(-3.5 dbx1a:EGFP)*^{zd3} crosses (Gribble et al., 2009; Kucenas et al., 2008; Park et al., 2000, 2007), and staged according to Kimmel et al. (1995). Zebrafish were raised and bred according to standard procedures; experiments were approved by the University of Utah Institutional Animal Care and Use Committee.

In situ hybridization

Embryos were fixed in fresh 4% paraformaldehyde (PFA) overnight at room temperature, washed in PBS, decapitated and coarsely chopped. *In situ* hybridization was performed as described previously (Oxtoby and Jowett, 1993). For sectioning, embryos were cryoprotected in sucrose, embedded in OCT, and sectioned at 20 μm thickness on a Leica CM3050 cryostat. Images were taken on an Olympus BX51WI compound microscope using an Olympus Microfire camera. Images were processed using the GNU Image Manipulation Program (GIMP.org).

Immunohistochemistry

Embryos up to 48hpf (hours post fertilization) were fixed in fresh 4% PFA for 3 h at room temperature then overnight at 4 °C; embryos over 48hpf were fixed for 1 h at room temperature. After fixation, embryos were washed in PBS, cryoprotected in sucrose, embedded in OCT and sectioned at 12 μm or 50 μm thickness on a Leica CM3050 cryostat. For BrdU antigen retrieval, thick sections were incubated at room temperature for 90 min in 2 N HCl. For PCNA antigen retrieval, thin sections were incubated in 100 °C 10 mM sodium citrate buffer, pH 6.0. Primary antibodies used were: rabbit anti-GFP (1:5000, Invitrogen #A-11122), chicken anti-GFP (1:1000, Aves #GFP-1020), mouse anti-HuC/D (1:500, Invitrogen #A-21271), chicken anti-BrdU (1:500, ICL #CBDU-65A-Z), mouse anti-PCNA (1:1000, Sigma #p8825), rabbit anti-PCNA (1:100, Santa Cruz Biotechnology #F2212), rabbit anti-Sox3 (1:200, a gift from Dr. Mike Klymkowsky, University of Colorado-Boulder), rabbit anti-Sox3 (1:200, Pierce Custom Antibodies and Peptides), rabbit anti-DsRed (1:200, Clontech #632496), and mouse zrf-1 (1:200, Zebrafish International Resource Center #zrf-1). Secondary antibodies used were: goat anti-rabbit 488 (1:200, Invitrogen #A-11008), goat anti-rabbit 568 (1:200, Invitrogen #A-11041), goat anti-rabbit cy3 (1:200, Jackson ImmunoResearch #111-165-003), goat anti-mouse 633 (1:200, Invitrogen #A-21050), goat anti-mouse cy3 (1:200, Jackson ImmunoResearch #115-165-003), goat anti-chicken 488 (1:200, Invitrogen #A-11039), goat anti-chicken 633 (1:200, Invitrogen #A-21103), and donkey anti-chicken 488 (1:200, Jackson ImmunoResearch #703-485-155). Hoechst 33342 was added to secondary antibodies to visualize nuclei.

Confocal microscopy

Sections were imaged using an Olympus FV-1000XY confocal microscope using a 60x oil-immersion objective. Images were processed using ImageJ (http://rsbweb.nih.gov/ij) and GIMP (gimp.org). Projections were generated using FluoRender (Wan et al., 2012).

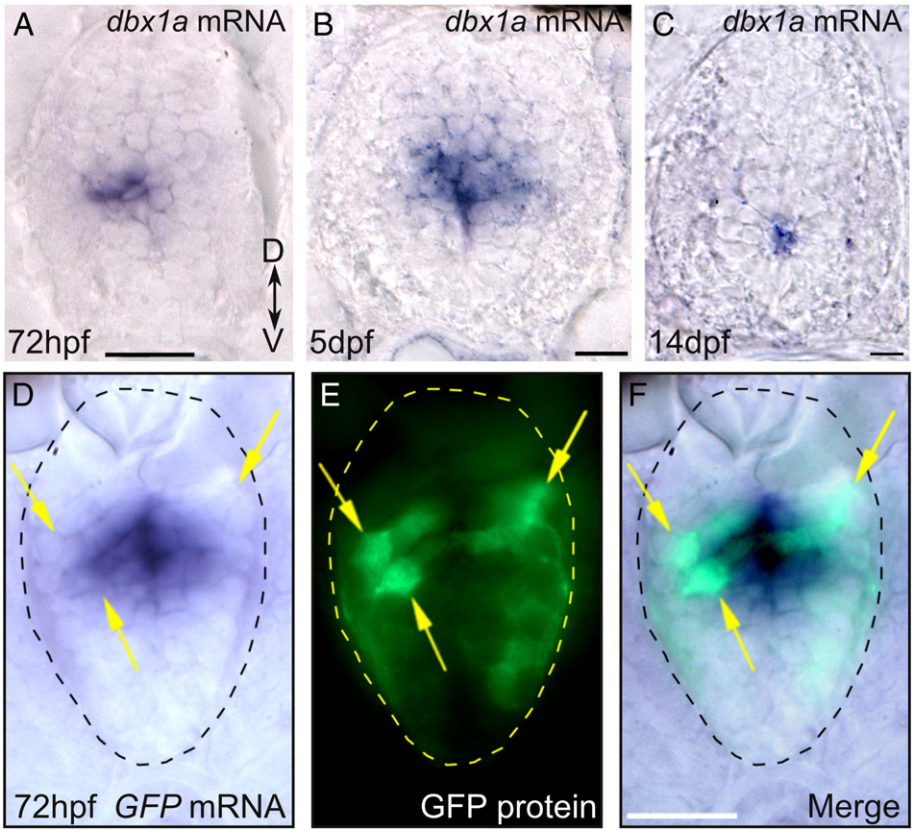

Fig. 1. Expression of *dbx1a* mRNA and *dbx1a:GFP* reporter. (A–C) *In situ* hybridization shows that *dbx1a* mRNA is expressed in the intermediate spinal cord through 14dpf. (D–F) In 3dpf *dbx1a:GFP* embryos, GFP protein expression is observed in lateral cells negative for *GFP* mRNA (arrows), suggesting that perdurance of protein can be used to trace the lineage of reporter-expressing cells. Scalebars = 10 μm.