Neuroscience 172 (2011) 329–341

TRANSCRIPTION FACTOR Sox11b IS INVOLVED IN SPINAL CORD REGENERATION IN ADULT ZEBRAFISH

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Abstract—Adult zebrafish have the ability to recover from spinal cord injury and exhibit re-growth of descending axons from the brainstem to the spinal cord. We performed gene expression analysis using microarray to find damage-induced genes after spinal cord injury, and found that Sox11b mRNA is up-regulated at 11 days after injury. However, the functional relevance of Sox11b for regeneration is not known. Here, we report that the up-regulation of Sox11b mRNA after spinal cord injury is mainly localized in ependymal cells lining the central canal and in newly differentiating neuronal precursors or immature neurons. Using an *in vivo* morpholino-based gene knockout approach, we demonstrate that Sox11b is essential for locomotor recovery after spinal cord injury. In the injured spinal cord, expression of the neural stem cell associated gene Nestin, and the proneural gene Ascl1a (Mash1a), which are involved in the self-renewal and cell fate specification of endogenous neural stem cells, respectively, is regulated by Sox11b. Our data indicate that Sox11b promotes neuronal determination of endogenous stem cells and regenerative neurogenesis following spinal cord injury in the adult zebrafish. Enhancing Sox11b expression to promote proliferation and neurogenic determination of endogenous neural stem cells after injury may be a promising strategy in restorative therapy after spinal cord injury in mammals. © 2011 Published by Elsevier Ltd on behalf of IBRO.

Key words: Sox11b, endogenous neural stem cells, spinal cord injury, nestin, Ascl1a (Mash1a), zebrafish.

In mammals, spinal cord injury destroys the cellular structures of the spinal cord causing cell death, inflammation, and secondary injury by necrosis, apoptosis, demyelination, and astrocytosis, all of which lead to loss of function (Silver and Miller, 2004; Karnezis et al., 2004; Thuret et al., 2006). There are no fully restorative therapies known for mammalian spinal cord injury (Thuret et al., 2006; Lu et al., 2004; Buchli et al., 2007; Cafferty et al., 2008; Bunge, 2008; Fawcett, 2009). Over the past few decades, the discovery of endogenous multipotent and pluripotent stem cell populations in specialized niches of the adult CNS that continually give rise to differentiating neural cells (Horner et al., 2000; Weiss et al., 1996; Shihabuddin, 2008) has led to advances in stem cell therapies for spinal cord injury (Agrawal and Schaffer, 2005; Bambakidis et al., 2005; Conti and Cattaneo, 2008). However, little is known about the mechanisms of activation and subsequent neural differentiation of endogenous neural stem cells by which we can gain further insights into the optimization of regenerative neurogenesis in mammals.

Adult zebrafish have an impressively high regenerative capacity and can recover from spinal cord injury almost to the extent of their original swimming ability before the injury (Becker et al., 1998, 2004; Bhatt et al., 2004; Fetcho et al., 2008). Therefore, zebrafish have become an attractive model for exploring the molecular mechanisms underlying regeneration and adult neurogenesis after spinal cord injury. To analyze the cellular and molecular mechanisms underlying this intrinsic ability to regenerate we identified damage-induced gene expression changes in the injured spinal cord caudal to the lesion site by microarray expression profiling. This strategy has been used to discover regulated genes in a successfully regenerating adult CNS of zebrafish (Veldman et al., 2007) as well as mammalian systems that do not regenerate (De Biase et al., 2005; Carmel et al., 2001). Using this system, we have identified a large set of genes regulated during spinal cord regeneration in zebrafish, including the transcription factor Sox11b, up-regulated in its expression after spinal cord injury. Since little is known of the role of Sox11b in CNS regeneration, we chose to investigate its function in zebrafish spinal cord injury.

The Sox family of transcription factors is characterized by the presence of a HMG (high mobility group)-box sequence motif (Pevny and Lovell-Badge, 1997; Bowles et al., 2000; Schepers et al., 2002). Sox proteins bind DNA sequences with an HMG domain, allowing them to function as transcription factors. According to the sequence homologies of the HMG domain, Sox proteins are divided into groups A–H (Bowles et al., 2000; Schepers et al., 2002) and are expressed in a tissue-specific manner during development, in which the C-group Sox11 (including two gene duplicates in zebrafish, Sox11a and Sox11b; De Martino et al., 2000) is mainly expressed in neural progenitor cells that have already been committed to neuronal

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Abbreviations: Ascl1a, achaete-scute complex-like 1a; bHLH, basic helix-loop-helix; BrdU, bromodeoxyuridine; HMG, high mobility group; MS222, aminobenzoic acid ethylmethylester; NBT/BCIP, nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate; qPCR, quantitative RT-PCR.

0306-4522/11 $ - see front matter © 2011 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2010.10.026

differentiation (Kamachi et al., 2000; Uwanogho et al., 1995). In chicken, maturing neurons express Sox11 transiently as they leave the neural epithelium (Uwanogho et al., 1995). In mice, the developing CNS prominently expresses Sox11 genes but the expression is down-regulated as neuronal development progresses (Hargrave et al., 1997; Southard-Smith et al., 1998). The expression of Sox11 remains at a very low level in adult mice, being prominently expressed in the neurogenic areas of the adult brain (Haslinger et al., 2009). The specific expression patterns of Sox11 genes suggest that they function at early stages of neural development, especially in the transition from immature to mature neurons. Much evidence shows that Sox11b expression is highly up-regulated in developing retinal ganglion cells of zebrafish during regeneration of the optic nerve (Veldman et al., 2007) and in dorsal root ganglion neurons after mouse sciatic nerve transection (Tanabe et al., 2003). Transient forebrain ischemia enhances Sox11 gene expression in the brain regions where neuronal progenitors and immature neurons are enriched (Kim et al., 2008). Given that specific developmental regulatory genes functioning during neural development are also present in endogenous neural stem cells and can be induced after injury (Veldman et al., 2007; Schepers et al., 2002; Kim et al., 2008; Bergsland et al., 2006), we wished to explore the function of Sox11b during endogenous regeneration after spinal cord injury.

Here, using a zebrafish spinal cord injury model, we report that Sox11b is predominantly expressed in ependymal cells lining the central canal and in newly-differentiating neuronal precursors or immature neurons of the gray matter. Moreover, Sox11b regulates the expression of neural stem cell associated gene Nestin and the proneural gene achaete-scute complex-like 1a (Ascl1a, also named Mash1a) in the injured spinal cord. Most importantly, morpholino-based suppression of Sox11b expression specifically impairs locomotor recovery after spinal cord injury. These data suggest that Sox11b is a critical transcription factor that contributes to regenerative neurogenesis via endogenous stem cells after spinal cord injury in the adult zebrafish.

EXPERIMENTAL PROCEDURES

Animals

Adult zebrafish (*Danio rerio*), 6 months old, were bought from Aquatica Tropicals (Plant City, FL, USA). The body lengths for all fish were 2–3 cm. Before surgery, the fish were kept in groups of 10 with a light cycle of 14 h light and 10 h dark at a temperature of 28 °C.

Spinal cord injury

All animal experiments were approved by the Rutgers University Animal Care Committee. Spinal cord transection was performed as previously described (Becker et al., 1997). Briefly, fish were anesthetized by immersion in 0.033% aminobenzoic acid ethylmethylester (MS222, Sigma-Aldrich, St. Louis, MO, USA) for 5 min. A longitudinal incision was made at the side of the fish to expose the vertebral column, which was then cut between two vertebrae at a level halfway between the dorsal fin and the operculum, about 4 mm caudal to the brainstem/spinal cord transitional zone. Wounds were sealed with Histoacryl (B. Braun, Melsungen, Germany), and the fish were kept individually at room temperature for up to 6 weeks following surgery. As control, a sham injury was performed by making the incision but without injury to the spinal cord.

Intraperitoneal BrdU application

Animals were anesthetized and i.p. injected with BrdU (bromodeoxyuridine) (Sigma-Aldrich) solution (2.5 mg/mL) in a volume of 50 μl immediately after the transection.

Microarray analysis

In total, we studied six groups of injured or sham-injured control fish for survival times of 4 h, 12 h, and 11 days. Total RNA was isolated from five pooled spinal cords (5 mm caudally away from the lesion site) per time point using Qiagen RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A total 200 ng of RNA was used for Agilent Zebrafish Oligo Microarray (Agilent Technologies, Santa Clara, CA, USA) analysis. Samples were labeled using the NuGEN (San Carlos, CA, USA) WT-Ovation kit and hybridized at the Bionomics Research and Technology Center (Rutgers University). Microarray assays were performed on three biological replicate spinal cord pools per time point. Gene expression results were imported and analyzed using Agilent GeneSpring GX software. Data were quantile normalized, filtered for detectable expression in 50% of the samples, and then tested by 2-way analysis of variance (ANOVA) (treatment, time) at 5% FDR. Hierarchical clustering and gene ontology analysis were performed using Agilent GeneSpring GX software or R/Bioconductor (http://www.bioconductor.org).

Real time quantitative RT-PCR (qPCR)

For qPCR analysis, first-strand cDNA was generated using random primers and Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The PCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The following primer sequences were used: Sox11b, 5′-cgagttcccggactattgca-3′ and 5′-tctcccgcgatcatctcact-3′; Ascl1a, 5′-cgccaagatggaaataagcg-3′ and 5′-caagcaggtggcatgaactg-3′; Nestin, 5′-cggcccttgatccgttataa-3′ and 5′-accgatgtggtgtgaggtttc-3′; Ribosomal protein P0, 5′-atcggctacccaactcttgct-3′ and 5′-tgtttcgacagtgacagccag-3′.

Reverse transcribed mRNA was amplified in the presence of SYBR green (Applied Biosystems). Accumulation of fluorescence was monitored during amplification in an Applied Biosystems 7900HT sequence detection system (Applied Biosystems), using 10 μl reactions in 384-well plates. All assays were performed on triplicate samples. All assay products were validated using melting curves to confirm the presence of single PCR products. Ribosomal protein P0 served as the internal control for qPCR.

In situ hybridization for Sox11b, Oct4, Nestin, Ascl1a

In situ hybridization probes (sense and antisense probes) for Sox11b (NM_131337.1), Nestin (XM_001919887.1), Ascl1a (NM_131219.1) and Oct4 (NM_131112) were transcribed *in vitro* from purified RT-PCR fragments. Each amplified probe was subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) containing the T7 promoter sequence: 5′-ccctatagtgagtcgtatta-3′ and SP6 promoter sequence: 5′-atttaggtgacactatagaa-3′. All four probes were transcribed *in vitro* using the SP6 MEGAScript Kit (Ambion, Austin, TX, USA) or T7 MEGAScript Kit (Ambion) as described previously (Tongiorgi et al., 1995).

Non-radioactive detection of mRNA in sections of adult zebrafish CNS was performed as published previously (Bernhardt et