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ATF3 is a novel nuclear marker for migrating ependymal stem cells in the rat spinal cord

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Abstract The present study identified ATF3 as a novel dynamic marker for ependymal stem/progenitor cells (nestin, vimentin and SOX2 positive) around the central canal of the neonatal or adult rat spinal cord. While quiescent ependymal cells showed cytoplasmic ATF3 expression, during 6–24 h in vitro these cells mobilized and acquired intense nuclear ATF3 staining. Their migratory pattern followed a centrifugal pathway toward the dorsal and ventral funiculi, reminiscent of the rostral migratory stream of the brain subventricular stem cells. Thus, the chain cell formation was, by analogy, termed funicular migratory stream (FMS). The FMS process preceded the strong proliferation of ependymal cells occurring only after 24 h in vitro. Pharmacological inhibition of MAPK-p38 and JNK/c-Jun (upstream effectors of ATF3 activation) prevented the FMS mobilization of ATF3 nuclear-positive cells. Excitotoxicity or ischemia-like conditions, reported to evoke neuronal and glial injury, did not further enhance migration of ependymal cells at 24 h, suggesting that, at this early stage of damage, the FMS phenomenon had peaked and that more extensive repair processes are delayed beyond this time point. ATF3 is, therefore, useful to identify activation and migration of endogenous stem cells of the rat spinal cord in vitro.
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Introduction

The ependymal region of the adult spinal cord in mammals harbors a pool of stem and progenitor cells (SPCs) readily activated and recruited by spinal damage (Weiss et al., 1996; Hugnot and Franzen, 2011). Even though their adult neurogenesis has not been observed (Sabourin et al., 2009; Hugnot and Franzen, 2011), the neural stem cells present in the adult spinal cord are recruited and proliferate after spinal cord injury (Yamamoto et al., 2001b), producing scar-forming astrocytes and myelinating oligodendrocytes (Meletis et al., 2008). The manipulation of endogenous spinal stem cells after injury could represent one valid alternative to stem cell

Abbreviations: ATF3, activating transcription factor; AU, arbitrary units; bHLH, basic helix-loop-helix; bZIP, Basic Leucine Zipper; CC, central canal; CREB, cAMP responsive element-binding; CSF, cerebrospinal fluid; DAPI, 4,6-diamidino-2-phenylindole; DCX, doublecourtin; EdU, 5-ethynyl-20-deoxyuridine; FMS, funicular migratory stream; GFAP, glial fibrillary acidic protein; MAPK, mitogen-activated protein kinases; PM, pathological medium; RMS, rostral migratory stream; SPCs, stem and progenitor cells; TBP, TATA binding protein.

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transplantation, since it is noninvasive and avoids the need for immune suppression (Meletis et al., 2008). Spinal stem cells are difficult to identify due to their heterogeneity and lack of specific expressional markers, since the ones currently used significantly overlap with those of mature astrocytes (McDonough and Martínez-Cerdeño, 2012). Furthermore, there is no specific marker to discriminate between quiescent and activated ependymal spinal cells, or to monitor migratory ependymal cells. Moreover, the signaling pathways and genes controlling the spinal SPC fate in normal and pathological conditions, remain largely unknown (Hugnot and Franzen, 2011). Brain transcription factors that regulate formation and proliferation of neural SPCs depend on the Sox family of genes, in particular Sox2 (Liu et al., 2013). Genomic and proteomic technologies have recently identified Wnt/beta-catenin, Notch, sonic hedgehog and growth factor networks as major signaling pathways involved in maintenance, self-renewal, proliferation and neurogenesis of the neural SPCs, and have demonstrated cross-talk between key molecules of these pathways and their modulations by transcription factors, miRNA and histone modifications (Yun et al., 2010).

At variance with the wealth of brain data, transcription factors controlling spinal cord stem cells remain incompletely understood as they have been studied with in vitro primary cultures, showing common expression of various homeodomain-type (Pax6, Pax7, Nkx2.2, and Prox1) and basic helix-loop-helix (bHLH)-type (Ngn2, Mash1, NeuroD1, and Olig2) regulatory factors in adult and embryonic rat spinal neural progenitors (Yamamoto et al., 2001a,b). In the course of our studies with biomarkers of neuronal damage (Kuzhandaivel et al., 2011) we serendipitously discovered intense immunostaining of ependymal cells for the Activating transcription factor 3 (ATF3): this observation led us to explore its expression in control or damage-induced protocols.

ATF3 belongs to the mammalian ATF/cAMP responsive element-binding (CREB) protein family of the Basic Leucine Zipper (bZIP) transcription factors (Hashimoto et al., 2002) that generate a wide range of either repressors or activators of transcription (Thompson et al., 2009). ATF3 is thought to be an immediate early gene, a stress inducible gene and an adaptive response gene, which, when activated by various stimuli, can control cell cycle and cell death machinery (Hunt et al., 2012). ATF3 promotes proliferation, motility and invasiveness of certain cancer cell lines (Wang et al., 2008; Thompson et al., 2009). ATF3 expression is normally very low in central neurons and glia, but it is markedly upregulated in response to injury and closely linked to survival and regeneration of peripheral axons (Hunt et al., 2012). Both cytoplasmic and nuclear ATF3 immunostaining has been reported with variations related to cell type, species, and injury state when it becomes prevalently nuclear (Hunt et al., 2012). ATF3 is supposed to have a role in neurite growth and regeneration (Moore and Goldberg, 2011) and it has been identified as a regulator of neuronal survival against excitotoxic and ischemic brain damage (Zhang et al., 2011). ATF3 knockout exacerbates inflammation and brain injury after transient focal cerebral ischemia (Wang et al., 2012). ATF3 has no known role in neuronal development of the intact nervous system, neither has its expression been reported in SPCs. The present study is the first report of ATF3 as a reliable marker of activated neuro-progenitor cells in the rat spinal cord.

Material and methods

Animals

The experiments were performed on neonatal or adult Wistar rats in accordance with the guidelines of the National Institutes of Health and the Italian act D.Lgs. 27/1/92 no. 116 (implementing the European Community directives no. 86/609 and 93/88), and with approval by the SISSA ethical committee for animal experimentation. All efforts were aimed at reducing the number of animals used and at minimizing their suffering. Spinal cords were dissected out from neonatal animals under urethane anesthesia (0.2 ml i.p. of a 10% w/v solution) with continuous superfusion with Kreb's solution containing (in mM): NaCl, 113; KCl, 4.5; $MgCl_2 7H_2O$, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2/5% CO_2, pH 7.4 at room temperature, as described previously (Mladinic et al., 2013). The adult spinal cord was dissected out from adult pregnant females, anesthetized with the 10.5% chloral hydrate, 0.4 ml/100 g i.m. and subsequently killed by an intracardiac injection (2 ml) of chloral hydrate.

Experimental protocols

The dissected spinal cords were immediately fixed in 4% paraformaldehyde or maintained for preset times in Kreb's solution at room temperature and then fixed. The p38 inhibitor SB203580 or the JNK/c-Jun inhibitor SP600125 (both from Calbiochem/Millipore, Milan, Italy) was added to the Kreb's solution for 12 or 24 h. To induce moderate or severe excitotoxic spinal cord injury, either 50 μM or 1 mM kainate was added to the Kreb's solution for 1 h and then washed out in Kreb's solution for further 24 h (this procedure has previously been described in detail by Taccola et al., 2008; Mladinic et al., 2013). The ischemia-like metabolic perturbation was induced as previously described (Taccola et al., 2008; Bianchetti et al., 2013; Mladinic et al., 2013) by incubating the tissue for 1 h in pathological medium (PM), namely Kreb's solution containing 10 mM H_2O_2, 500 μM sodium nitroprusside, lacking oxygen and glucose, with 6.75–6.80 pH and 230–240 mOsm osmolarity. When the spinal cord was maintained in vitro for 48–72 h, the tissue was kept in oxygenated (95% O_2/5% CO_2) Eagle's basal medium (Sigma-Aldrich, St. Louis, MI, USA) supplemented with 0.2% fetal calf serum, 30 ng/ml 7S nerve growth factor, 10 μg/ml insulin and 0.1 mg/ml gentamycin (Mladinic, 2007).

Fluorescence immunostaining procedure

The free-floating immuno-fluorescence protocol was used as previously described (Taccola et al., 2008; Mladinic et al., 2013). The primary antibodies (Supplemental Table 1) were visualized using appropriate secondary fluorescent Alexa Fluor 488 or 594 antibodies (1:500 dilution, Invitrogen, Carlsbad, CA, USA). Sections were stained in 1 μg/ml solution of 4,6-diamidino-2-phenylindole (DAPI) for 20 min to visualize cell nuclei and mounted on Superfrost Plus (Menzel-Glaser, Braunschweig, Germany) slides. The immunostaining signal was analyzed by Zeiss Axioskop2 microscope (Oberkochen, Germany) or TCS SP2 LEICA confocal microscope (Wetzlar,

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