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Activating transcription factor 3 promotes spinal cord regeneration of adult zebrafish

Lin-Fang Wang [a, 1], Shu-Bing Huang [a, 1], Hou-De Zhao [a, b, 1], Chun-Jie Liu [a, b], Li Yao [a], Yan-Qin Shen [a, b, *]

[a] *Medical School, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China*
[b] *Center for Neuroscience, Shantou University Medical College, 22 Xinling Road, Shantou, Guangdong 515041, China*

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A B S T R A C T

Zebrafish is an excellent model to study the mechanisms underlying successful central nervous system (CNS) regeneration. Previous study shows that activating transcription factor 3 (ATF3) promotes neurite outgrowth and is involved in optic nerve regeneration in zebrafish. Here, we used zebrafish model to investigate the role of ATF3 in regeneration following spinal cord injury (SCI). Quantitative polymerase chain reaction (qPCR) and *in situ* hybridization revealed that ATF3 mRNA levels increased at 12 h and 6 d following SCI. Double labeled immunofluorescence showed that ATF3 expressed in motoneurons. Treatment of anti-sense ATF3 morpholino (MO) inhibited locomotor recovery and decreased axon regeneration of spinal cord injured zebrafish. Further, inhibition of ATF3 up-regulated the expression of inflammatory factors tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). These data suggest that ATF3 could promote locomotor recovery and axon regrowth in zebrafish SCI model possibly by regulating inflammatory response.

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1. Introduction

In mammals, spinal cord injury (SCI) is primarily caused by the injury-induced interruption of axonal tracts and can be worsened by secondary injury related events including neuronal and glial loss, inflammatory response, which typically results in permanent neurological deficits [1,2]. In contrast to mammals, adult zebrafish can re-grow axons after SCI and re-establish connections to regain significant swimming ability [3,4]. Thus, finding beneficial molecules for SCI regeneration in zebrafish may support effective therapies for human SCI.

ATF3 is induced under a variety of stress signals including wounding and axotomy [5,6]. A report of genome-wide expression analysis finds that *atf-3* is up-regulated in injured spinal cord compared to uninjured group [7]. An increased expression of *atf-3* is demonstrated after injury in zebrafish optic nerve [8], in dorsal root ganglia (DRG) and motor neurons in sciatic nerve cut of rats [9]. In addition, ATF3 is required to strictly regulate immune function to prevent immune pathologies, for example, ATF3 negatively regulates production of pro-inflammatory cytokine like TNF-α and toll-like receptor-response genes [10,11]. The above-mentioned data highly suggest the role of ATF3 in neuroregeneration. Herein, we explore the role of ATF3 in recovery after SCI in adult zebrafish.

In the current study, we show that ATF3 is up-regulated following SCI as detected by qPCR and *in situ* hybridization. Immunofluorescence staining reveals ATF3 expression in motoneurons. Inhibition of ATF3 protein translation retards the recovery of both swimming ability and axonal regeneration across the lesion site of spinal cord. Besides, pro-inflammatory cytokines TNF-α and IL-1β mRNA are up-regulated after ATF3 MO treatment. These observations suggest that ATF3 may promote neuron repair after SCI in zebrafish via regulating inflammation.

2. Material and methods

2.1. Animals

Adult zebrafish (*Danio rerio*, 5 months old) were bought from Shanghai Jiayu Aquatic Company (Shanghai, China). Zebrafish were kept on a 14 h light and 10 h dark cycle at 28 °C and fed twice a day.

* Corresponding author. Medical School, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China.
E-mail address: shenyanqin@jiangnan.edu.cn (Y.-Q. Shen).
[1] These authors contributed equally.

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All animal experiments were approved by the Animal Ethics Committee of Jiangnan University.

2.2. Spinal cord injury

Zebrafish spinal cord transection was performed as previously described [12]. Briefly, adult zebrafish were anaesthetized in 0.033% aminobenzoic acid ethylmethylester (Sigma, St. Louis, MO, USA) dissolved in phosphate-buffered saline, pH 7.4. Zebrafish was placed on ice under a stereomicroscope for dissection. A pair of fine scissors was used to make a longitudinal incision through the muscle layer to expose the spine. The spinal cord was cut between two vertebrae, corresponding to the eighth vertebra (4–5 mm caudal to the operculum) of the spinal cord. Histoacryl (B. Braun, Melsungen, Germany) was applied to close off the wound, and the injured zebrafish were kept individually in water at 28 °C. Sham operations were identical except the spinal cord was not transected.

2.3. Quantitative real-time PCR

To study the expression of ATF3, TNF-α and IL-1β (NM_200964.1, AB183467.1 and AY340959.1), total RNA with 4 mm segment of lesion-caudal spinal cord was extracted following SCI with an EZgeneTM Tissue RNA Miniprep Kit (Biomiga, San Diego, CA, USA) according to the manufacturer's instructions. First-strand cDNA was generated using random primers and a PrimeScript™ RT Master Mix (Perfect Real Time) (TAKARA BIO, Otsu, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with a SYBR® Premix Ex Taq™ II (TliRNaseH Plus) (TAKARA BIO, Otsu, Japan) [13]. Fold-changes were estimated using $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001) using GAPDH as reference gene [14,15]. Primers for qRT-PCR were designed in Primer Express 5.0 software (Applied Biosystems, Foster City, CA, USA). All experiments were performed in duplicate, and PCR products were validated by melting curves to confirm single PCR products. The following primer sequences were used: ATF3 forward: 5′-GCTGTGGGCATCTGTGAATC-3′, reverse: 5′-GCACCCGTGTTTAGTCCTTT-3′.

GAPDH forward: 5′-GTGTAGGCGTGGACTGTGGT-3′, reverse: 5′-TGGGAGTCAACCAGGACAAATA-3′.

TNF-α forward: 5′-GCGCTTTTCTGAATCCTACG-3′, reverse: 5′-TGCCCAGTCTGTCTCCTTCT-3′.

IL-1β forward: 5′-GCTGGAGATCCAAACGGATA-3′, reverse: 5′-ATACGCGGTGCTGATAAACC-3′. The number of zebrafish for each group was eight.

2.4. In situ hybridization

Non-radioactive detection of mRNA in sections of the adult zebrafish was performed as previously described with small modifications [16]. Briefly, 4 mm segment of lesion-caudal spinal cords were fixed for 12 h in 4% paraformaldehyde at 4 °C, then was cryoprotected by incubation in 30% sucrose until the tissue sank.

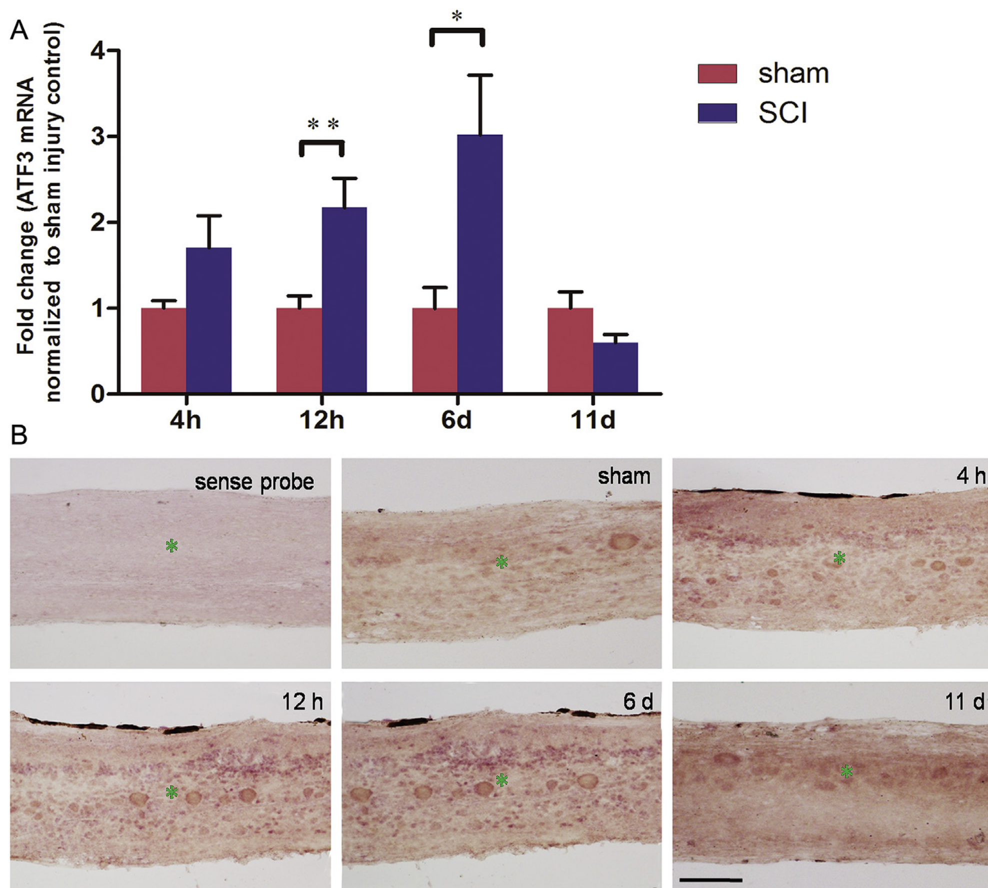

Fig. 1. Time course of ATF3 mRNA expression after SCI. A. qPCR showed expression of ATF3 mRNA in spinal cords at 4 h, 12 h, 6 d and 11 d after SCI. Significant upregulation was observed at 12 h and 6 d after SCI compared to the sham group (**$P < 0.01$, *$P < 0.05$, independent sample *t*-test; n = 8 fish/group). Values represent means ± SEM. B. *In situ* hybridization detection of ATF3 mRNA in spinal cords at 4 h, 12 h, 6 d and 11 d after SCI (n = 3 fish/group). Numbers of positive cells were increased along the central canal at 12 h and 6 d after SCI compared to the sham group. * indicates the central canal. Scale bars, 100 μm.

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