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HMGB1 Contributes to Regeneration After Spinal Cord Injury in Adult Zebrafish

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Abstract High mobility group box 1 (HMGB1, also called amphoterin) facilitates neurite outgrowth in early development, yet can exacerbate pathology and inhibit regeneration by inducing adverse neuroinflammation when released from dying cells, suggesting that HMGB1 plays a critical, yet undefined role in neuroregeneration. We explored whether HMGB1 contributes to recovery after complete spinal cord transection in adult zebrafish. Quantitative PCR and in situ hybridization revealed that HMGB1 mRNA levels decreased between 12 h to 11 days after spinal cord injury (SCI), then returned to basal levels by 21 days. Western blot and immunohistological analyses indicated that the time course of HMGB1 protein expression after SCI parallels that of mRNA. Immunofluorescence staining revealed that HMGB1 translocates from nuclei into the cytoplasm of spinal motoneurons at 4 and 12 h (acute stage) following SCI, then accumulates in the nuclei of motoneurons during the ensuing chronic stage (after 6 days following SCI). Immunohistology of transgenic zebrafish, expressing green fluorescent protein in blood vessels, showed enhanced HMGB1 expression in blood vessels in the vicinity of motoneurons. Application of anti-sense HMGB1 morpholinos inhibited locomotor recovery by 34 % and decreased axonal regeneration by 34 % compared to fish treated with a control morpholino. The present study shows that HMGB1 expression increases in both endothelial cells and motoneurons, suggesting that HMGB1 promotes recovery from SCI not only through enhancing neuroregeneration, but also by increasing angiogenesis. The inflammatory effects of HMGB1 are minimized through the decrease in HMGB1 expression during the acute stage.

Keywords HMGB1 · Amphoterin · Spinal cord injury · Regeneration · Neurovascular · Zebrafish

Introduction

The development of the central nervous system is highly similar between mammals and lower vertebrates, yet the ability of higher vertebrates to recover from central nervous system injury, which in lower vertebrates recapitulates central nervous system development, is absent. Spinal cord recovery in lower vertebrates involves both neurogenesis and inflammation. However, due to the potential of inflammation to inhibit neurogenesis, to successfully accomplish neuroregeneration the processes of neurogenesis and inflammation must be highly coordinated. HMGB1 is a protein central to both neurogenesis and inflammation. We previously showed that, HMGB1 mRNA in the nucleus of the medial longitudinal fascicle (NMLF) decreases to 87 % at 12 h and by 11 days after SCI, HMGB1 is increased to 113 % compared with the sham injury group [1]. HMGB1, containing two DNA binding domains and a C-terminal tail, has been identified both as a non-histone

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chromosomal protein in nuclei, and as a cytokine in the extracellular environment, and plays an important role in both mammalian and zebrafish nervous systems [2]. During early development, HMGB1 is widely expressed in both the nuclei and cytoplasm of cells in neurogenic brain structures, including the hippocampal dentate gyrus, olfactory bulbs and cell lining of the telencephalic ventricles [3], and is detectable in the nuclei of adult neurons and astrocytes [4], spinal cord oligodendrocytes [5], choroid plexus endothelial cells [6], and in vitro in microglia [7]. Knock-down of HMGB1 in zebrafish embryos leads to a marked reduction in numbers of tyrosine hydroxylase-expressing neurons in the forebrain and a reduction in brain size [8], and, in vitro, HMGB1 promotes neurite outgrowth of N18 neuroblastoma cells, cortical neurons and cerebellar granule cells [9–11]. HMGB1 is upregulated in rat spinal cord at 72 h after injury, and increases in rat brain by 3 days following ischemia [12, 13]. This broad distribution in the nervous system, involvement in neurogenesis, and upregulation following spinal cord injury suggests that HMGB1 plays a prominent role in recovery from central nervous system injury.

HMGB1 released from microglia and/or degenerating neurons produces multiple inflammatory and neurotoxic factors [7]. In contrast to the neurogenic properties of HMGB1, HMGB1 is highly inflammatory upon release from cells [14], and can contribute to neuropathology and inhibition of neurogenesis through secondary tissue damage. During pathological changes in mammals, HMGB1 is released into the extracellular environment and mediates inflammation through binding to the receptor for advanced glycation end products (RAGE), and toll-like receptors (TLR)-2, TLR-4, and TLR-9 [15–18]. In a rabbit SCI model, the level of HMGB1 in serum increases at 72 h after injury and leads to elevation of TNF-α and apoptosis of motoneurons, and reduction of HMGB1 serum concentrations is associated with better survival of neurons in the spinal cord [19, 20]. In the rat spinal nerve ligation model, HMGB1 expression is strongly enhanced at 7 days after ligation and induces neuropathic pain by neuroinflammation, with the pain being alleviated after application of HMGB1 antibody [21]. In addition, HMGB1 overexpression in astrocytes promotes neurovascular remodeling following ischemia, and inhibition of HMGB1 reduces density of microvessels in the peri-infarct area and impairs recovery of locomotor ability in ischemic mice compared with non-ischemic control mice [22]. Together, these observations suggest that HMGB1 plays dual and antagonistic roles during regeneration after central nervous system injury.

To delineate the roles of HMGB1 in recovery following SCI, we used an adult zebrafish model in which nearly full locomotor activity is recovered within 6 weeks after complete transection of the thoracic spinal cord. We show that HMGB1 expression rapidly decreases within 12 h after spinal cord injury, and that decreased HMGB1 levels persist for 11 days, followed by a full recovery by 21 days. These changes in HMGB1 expression were observed in both motoneurons and the spinal cord vascular system. Further, inhibition of HMGB1 expression by anti-sense morpholinos retarded the recovery of locomotor ability and axonal regeneration across the lesion site. These results are consistent with a model for recovery from spinal cord injury in which an HMGB1 is initially released to promote neurogenesis and cell survival, then downregulated to minimize inflammation during recovery.

Materials and Methods

Animals

Wild-type adult zebrafish (*Danio rerio*, 6 months old) were purchased from the Huiyuan Aquatic Animals Company (Shantou, Guangdong, China). Fli1a::EGFP transgenic fish, in which EGFP is driven by the endothelial cell-specific Fli1 promoter [23], were kindly provided by Professor Zilong Wen (Hong Kong University of Science and Technology). All fish used in the experiments were kept on a 14-h light and 10-h dark cycle at 28 °C, and fed twice a day. All animal experiments were approved by the Animal ethics Committee of Shantou University Medical College.

Spinal Cord Injury

Spinal cord transection was performed as described [24]. Briefly, fish were anaesthetized by immersion in 0.033 % aminobenzoic acid ethylmethylester (MS222, Sigma, St. Louis, MO, USA) in phosphate-buffered saline, pH 7.4 (PBS) for 5 min. A longitudinal incision was made at the side of the fish to expose the vertebral column. The spinal cord was cut between two vertebrae, approximately 4 mm caudal to the brainstem/spinal cord transitional junction. Wounds were sealed with Histoacryl (B. Braun, Melsungen, Germany), and the injured fish were kept individually at 28 °C. A sham control lesion was performed by making the incision, but omitting the spinal cord transection. All surgical procedures were performed on ice under a microscope.

Real-Time Quantitative RT-PCR

To study the expression of HMGB1 mRNA (NM_199555) in the spinal cord caudal to the lesion site, total RNA was extracted from the 4 mm segment of spinal cord directly caudal to the lesion site at different time points after SCI. First-strand cDNA was generated using random primers and a ReverTra Ace^{R} qPCR RT Kit (TOYOBO, Osaka, Japan). Quantitative real-time polymerase chain reaction (qPCR) was performed with a SYBR^{R} Green Real-time PCR Master Mix (TOYOBO) as previously described [25]. Primers for qPCR were designed using Primer Express 5.0 software (Applied

Biosystems, Foster City, CA, USA). All assays were performed in duplicate, and assay products were validated using melting curves to confirm the presence of single PCR products. GAPDH served as the internal control [26]. The following primer sequences were used: zebrafish HMGB1 forward: GAGCGATGGAAGACTATGT, reverse: TCGGAACTCGG AGCAGA. GAPDH forward: GTGTAGGCGTGGACTGT GGT, reverse: TGGGAGTCAACCAGGACAAATA [27]. A no-template control group was performed in each experiment using the same reaction conditions at different time points. The number of fish for this assay was six for each group.

In Situ Hybridization

In situ hybridization probes (sense and anti-sense) for HMGB1 mRNA were transcribed in vitro. Purified PCR fragments were cloned into the pGM-T vector (Tiangen, Beijing, China), and the sequences were verified by sequencing. Digoxigenin (DIG)-labeled sense and anti-sense RNA probes were generated using the Megascript system (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Non-radioactive detection of mRNA in sections of the adult zebrafish was performed as described with small modifications [28, 29]. Spinal cords were fixed for 24 h in 4 % paraformaldehyde in PBS at 4 °C, followed by incubation in 15 % sucrose in PBS overnight at 4 °C. Then, 16-μm-thick sections of spinal cords were cut from fresh-frozen tissue on a cryostat, pre-hybridized for 2 h at 55 °C, and hybridized with the DIG-labeled probes at 55 °C overnight. After extensive washing (at 50 °C), alkaline phosphatase-coupled anti-DIG fragment antibodies (Fabs, Roche, Indianapolis, IN, USA) were applied at room temperature for 1 h. Antibody binding was detected using an alkaline phosphatase reaction with nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche) as substrates. As a negative control, sense probes were developed in parallel under the same conditions as the anti-sense probes. Sections from sham control and injured fish were analyzed on the same slides. All sections were viewed and photographed using an epifluorescence microscope (Axio Imager Z1, Zeiss, Oberkochen, Germany). Three fish were analyzed for each group.

Immunohistochemistry

All tissues were processed for immunofluorescence after fixation in 4 % formaldehyde in PBS at 4 °C overnight. Serial sections (16 μm in thickness) from the spinal cord (longitudinal, 0–4 mm caudal to lesion site) were used. Sections were prepared as described above and processed for immunostaining [30]. The primary antibodies against zebrafish HMGB1 were raised in rabbits [8] (1:200), and against zebrafish anti-Islet-1 in mice (1:200, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). Secondary antibodies were Alexa Fluor 594- or CY3-conjugated goat anti-rabbit IgG (1:800, Jackson ImmunoResearch, West Grove, USA) and FITC-conjugated goat anti-mouse IgG (1:800, Jackson ImmunoResearch). Fluorescence images were collected using an epifluorescence microscope (Axio Imager Z1, Zeiss, Oberkochen, Germany) [31]. Motoneuronal cell bodies were identified as Islet1-immunopositive cells. Motoneurons touching the endothelial cells of blood vessels were counted in longitudinal sections of fli1a::EGFP transgenic and wild-type zebrafish. Sections were obtained from eight randomly chosen sites on the spinal cord 0–4 mm in length caudal to the original spinal lesion site. The number of fish assayed was three for each group.

Western Blot Analysis

Total protein was extracted by homogenizing spinal cord tissue in RIPA buffer (Soliba, Beijing, China). After centrifugation of the homogenate at 14,000×*g* and 4 °C, protein concentration in the supernatant was determined with a BCA kit (Pierce, Rockford, USA). The supernatant was denatured by boiling for 5 min in SDS sample buffer. Fifty micrograms of total protein was then subjected to SDS-PAGE on 10 % gels. Proteins were transferred to a polyvinylidene difluoride membrane and probed with the following antibodies: rabbit anti-zebrafish HMGB1 (1:2,000) and mouse anti-zebrafish GAPDH (1:1,000, Beyotime, Haimen, China). Goat anti-rabbit IgG and goat anti-mouse IgG (1:1,000, Jackson ImmunoResearch) conjugated to horseradish peroxidase were used as secondary antibodies. The membrane with proteins was incubated with BeyoECL Plus (Beyotime) for 1 min and imaged by a Gel Image System (Alpha Innotech, San Leandro, USA). The gray value of each band was measured and normalized to that of the GAPDH band using ImageTool software (San Antonio, Texas, USA). Five fish were assayed in each group.

Morpholino Treatment

HMGB1 anti-sense morpholino (MO) (5′-GATCCTTCCCC ATCTTTGCCTAAAT-3′, vivo-porter-coupled) and standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′, vivo-porter-coupled) (Gene Tools, Philomath, OR, USA) were dissolved in Danieau solution [32] and soaked in small pieces of Gelfoam (Upjohn, Kalamazoo, MI, USA). These pieces of Gelfoam were divided into smaller pieces to yield 800 ng of MO (approximately 0.2 μl MO per piece). One piece was applied to the transection site immediately after spinal cord transection. Each animal was allowed to survive the surgery for 6 weeks [29].

Swim Tracking

Swimming capabilities of injured fish were assessed each week after MO application for 4 weeks in two trials of 5 min each (trial interval, 4 h). In each trial, a zebrafish was placed in a brightly illuminated (100 lx) tank (42×30×30 cm) filled with aquarium water (5 cm deep) at 25 °C. A video camera recorded the trials above the tank. Swim paths were tracked with ethovision software (Noldus, Wageningen, The Netherlands). Mean lengths of the swim paths (total distance moved) of the two trials were used for graphic presentation and statistical analysis. The experimenter was blinded to the treatment of the animals. The number of fish analyzed for this assay was nine for each group.

Anterograde Tracing

To study whether regenerated axons had passed the lesion site, biocytin was applied via a second surgery at the site of the brainstem/spinal cord junction at 6 weeks after SCI [33–35]. After 24 h, spinal cords were removed, and blocks of tissue from 0–4 mm caudal to the original spinal lesion site were fixed in 4 % formaldehyde in PBS at 4 °C overnight, embedded in 15 % sucrose, and sectioned coronally (in 25-μm-thick sections) or longitudinally (in 16-μm-thick sections) on a cryostat. Biocytin labeling was detected with Streptavidin-Cy3 (1:400, Bioss). Three animals were in each group (control MO- or HMGB1 MO-treated). Fluorescence intensity was presented as the mean intensity evaluated by ImagePro plus software (Media Cybernetics) from 12 randomly selected spinal cord sections 0–4 mm caudal to the lesion site from each group. Four animals from each group were analyzed.

Statistical Analysis

Using SPSS 13 software, the independent sample t test was used for comparing the two treatment groups, and one-way ANOVA was used for multiple group comparisons. Data are presented as means±SEM. $P<0.05$ was set as the threshold for significance ($^*P<0.05$, $^{**}P<0.01$). All experiments were performed in triplicate.

Results

HMGB1 mRNA Levels Along the Caudal Part of the Central Canal Change with Time After SCI

To explore the involvement of HMGB1 in the regeneration of spinal cord-injured adult zebrafish, expression of HMGB1 mRNA in the spinal cord was examined by real-time qPCR and in situ hybridization at different time points after complete spinal cord transection. Time points at 4 and 12 h represent the acute response phase of the injury, and those at 6, 11, and 21 days represent the chronic response phase. HMGB1 mRNA levels were increased by twofold at 4 h after SCI, but were decreased by 12 h after SCI compared with the sham injury group. From 12 h to 11 days after SCI, HMGB1 mRNA expression was maintained at a low level ($^*P<0.05$, independent sample t test; $n=6$), then gradually returned to the sham injury group level at 21 days ($P>0.05$) (Fig. 1a). The polymerization and Ct value of the no-template control group were undetectable. The qPCR results were confirmed by in situ hybridization. In the sham injury group (4 h after sham injury), HMGB1 mRNA-positive signals were mostly in neurons and some in ependymal cells lining the central canal. At 4 h after SCI, HMGB1 mRNA-positive cells were primarily ependymal cells along the central canal caudal to the lesion site, indicating that injured neurons had lost HMGB1 expression. At 12 h, 6 days and 11 days after SCI, a significant decrease in HMGB1 mRNA levels was detected in both the ependymal cells and in motoneurons, when compared to the sham injury group (Fig. 1b). However, by 21 days after SCI, HMGB1 mRNA signals, along the central canal and in motoneurons, were similar in both SCI and sham groups. No signal was detected in sections incubated with sense probes, and no significant difference was found between non-injured zebrafish and the sham injury group (data not shown). Therefore, HMGB1 levels rapidly decrease and are maintained at low levels following SCI.

Acute Stage Downregulation and Chronic Stage Recovery of HMGB1 Protein Levels Following SCI

To investigate the effects of SCI on HMGB1 protein expression and distribution, Western blot analysis and immunohistology were performed at different time points after SCI. Zebrafish spinal cord tissues were harvested at 4 and 12 h, and at 6, 11 and 21 days after SCI. HMGB1 protein levels decreased by 12 h after SCI compared to sham-injured and uninjured fish. At 6 days after SCI, the level of HMGB1 protein levels had declined by 25 %, and continued to decrease to 50 % of the sham injury group until 11 days after SCI ($P<0.05$, one-way ANOVA with Tukey's post-hoc test; $n=5$). By 21 days after injury, HMGB1 protein returned to levels similar to those of the sham injury group ($P>0.05$) (Fig. 2a, b). Therefore, similar to HMGB1 mRNA, HMGB1 protein levels also decreased following SCI, although the decrease in protein quantitatively lagged behind the decrease in mRNA levels.

Immunohistology was performed to locate the intracellular distribution of HMGB1. In the sham injury group, HMGB1 was mainly localized to cell nuclei in the ependymal cells lining the central canal. At 4 h after SCI, HMGB1 was not only detectable in nuclei, but was also detectable in the cytoplasm. From 12 h to 11 days post-injury, HMGB1 protein levels decreased and increased again by 21 days in both

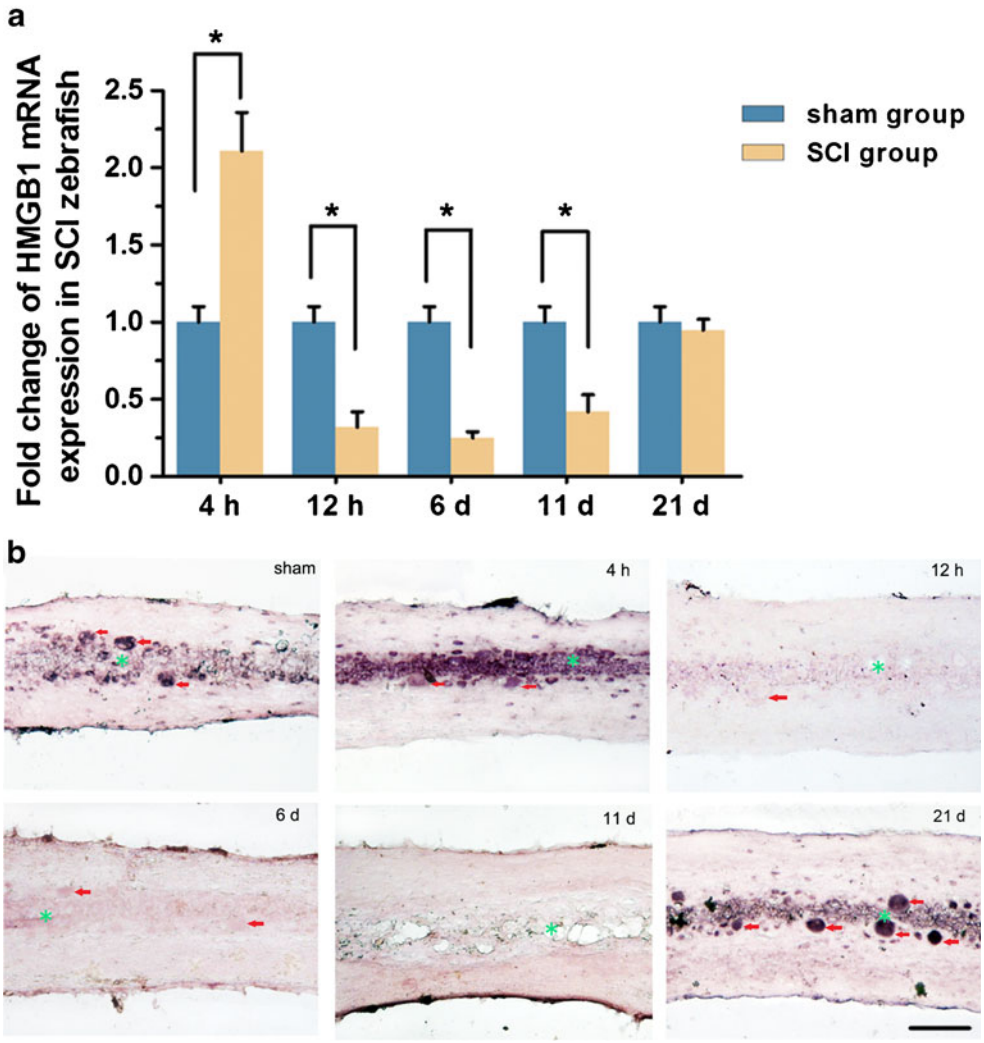

Fig. 1 Time course of HMGB1 mRNA expression after SCI. **a** qPCR of HMGB1 mRNA in the spinal cord caudal to the lesion site at different time points after SCI. Significant upregulation was observed at 4 h, followed by downregulation at 12 h, 6 days, and 11 days after SCI compared to the sham-injured group (*$P<0.05$, **$P<0.01$, independent sample *t* test; $n=6$ fish/group). Values represent means±SEM. **b** In situ hybridization detection of HMGB1 mRNA in longitudinal sections of the spinal cord 4 mm caudal to the lesion site at 4 and 12 h, and 6, 11, and 21 days after SCI ($n=3$ fish/group). Numbers of positive cells are higher along the central canal at 4 h and lower at 12 h, 6 days, and 11 days after SCI compared to the sham-injured group at 4 h. At 21 days after SCI, numbers of positive cells returned to basal levels. *Arrows* mark motoneurons of spinal cord. *Asterisks* indicate the central canal. *Scale bar* 100 μm

cytoplasm and nuclei in cells in the vicinity of the central canal (Fig. 2c). Since ependymal cells of the spinal cord form a stem cell niche which promotes regeneration after SCI [36], this possibly reflects changes in HMGB1 levels within the neural stem cell population.

HMGB1 Localizes in Both Nuclei and Cytoplasm of Motoneurons After SCI

To investigate whether motoneurons express HMGB1, double-immunostaining was performed for HMGB1 and Islet-1 (a marker for motoneurons) in uninjured, sham-injured, and injured zebrafish at varying times after SCI. In the spinal cords of sham-injured zebrafish, most of the HMGB1 staining was present in motoneuron nuclei. At 4 h after SCI, localization of HMGB1 became cytoplasmic. By 12 h after SCI, HMGB1 decreased in the cytoplasm of motoneurons. However, by 6 to 11 days after SCI, HMGB1 could again be detected in both the nuclei and the cytoplasm of motoneurons. In the 21-day SCI group, HMGB1 levels declined in the cytoplasm but were again found in the nuclei (Fig. 3). There was no difference in the co-localization of HMGB1 and Islet-1 between sham-injured and uninjured zebrafish, and at the time points investigated, there was no difference between sham-injured and uninjured animals (data not shown). These results show that HMGB1 is present in motoneuron nuclei, and is translocated to the cytosol and downregulated following injury. Similar to the localization observed in endothelial cells, recovery initially involves re-expression of HMGB1 in both cytosol and nuclei, followed by a gradual return to exclusive nuclear localization.

HMGB1 Is Expressed in Endothelial Cells and Associated with Motoneurons After SCI

We investigated the relationship between HMGB1 expression in endothelial cells and the localization of motoneurons along the central canal by using Fli1a::EGFP transgenic fish, which

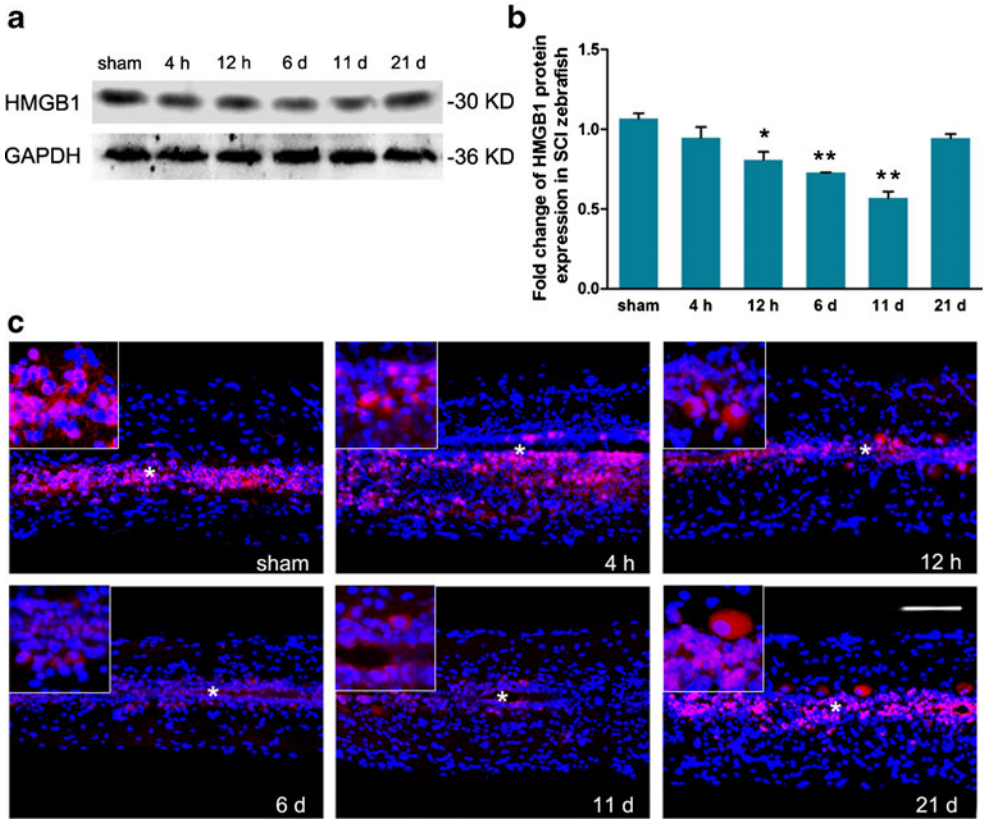

Fig. 2 Time course of HMGB1 protein expression following SCI. **a** Western blot analysis shows HMGB1 protein expression in the spinal cord caudal to the lesion site at different time points after SCI. Significant downregulation was detected at 12 h, and 6, and 11 days after SCI compared to the sham injury group at 4 h. At 21 days after SCI, the level of HMGB1 protein returned to the level of the sham injury group. **b** The intensity of bands was quantified with ImageTool software. The fold change compared with the sham injury group shows the reduction of HMGB1 protein levels achieved significance at 12 h, and 6, and 11 days after SCI (*$P<0.05$, **$P<0.01$, one-way ANOVA with Tukey's post-hoc test; $n=5$ fish/group). Values represent means±SEM. **c** HMGB1 expression in the spinal cord 4 mm caudal to the lesion site was examined by immunofluorescence in longitudinal sections at different time points after SCI. HMGB1-positive cells were reduced along the central canal from 12 h to 11 days after SCI. The number of HMGB1-positive cells returned to basal levels at 21 days after SCI. *Asterisks* indicate the central canal. HMGB1 (*red*), nuclei (*blue*). *Scale bar* 100 μm

express EGFP in endothelial cells, and analyzed the expression pattern of HMGB1 in the vasculature over time after SCI by examining the cell bodies of motoneurons adjacent to blood vessels. Interestingly, HMGB1 was strongly upregulated in blood vessels at 6 days and decreased at 11 and 21 days after SCI compared to the sham injury group (Fig. 4a). In parallel, the numbers of motoneurons adjacent to blood vessels increased by 242 % over the sham injury group at 6 days after SCI, and by 168 % at 21 days after SCI (Fig. 4b and c) ($P<0.05$). Thus, recovery is marked by an increased density of motoneurons adjacent to blood vessels.

Inhibition of HMGB1 Protein Expression After SCI Retards Locomotor Recovery After SCI

Application of an HMGB1 anti-sense MO at the time of SCI reduced levels of HMGB1 protein by 49 %, as judged by Western blot analysis of spinal cord tissue below the lesion site, when compared to the standard control MO, when measured at 4 weeks after SCI (*$P<0.05$, independent sample t test; $n=5$) (Fig. 5a, b). HMGB1 MO also reduced functional recovery after SCI, as quantified by the distance swum within a given amount of time. At 1 week after SCI, no difference between the groups treated with HMGB1 MO and standard control MO was observed (Fig. 5c). However, HMGB1 MO-treated fish showed retardation of locomotor recovery at 2, 3, and 4 weeks post-injury. At 2 weeks after SCI, the total distance moved by fish treated with HMGB1 MO (855.7±178.3 cm, $n=9$, *$P<0.05$) was reduced by 64.6 % compared to that of fish treated with control MO (1,365.2±123.7 cm, $n=9$). At 3 weeks after SCI, the distance moved in 5 min of HMGB1 MO-treated fish (986.6±82.8 cm, $n=9$, *$P<0.05$) was 34 % less than that of the control MO group (1,493.6± 85.6 cm, $n=9$). Similarly, at 4 weeks after SCI, the total distance moved by fish treated with HMGB1 MO (1,144.1± 116.4 cm, $n=9$, *$P<0.05$) was 33 % less than that of the control group (1,711.8±228.4 cm, $n=9$). Thus, HMGB1 MO treatment strongly reduces locomotor recovery, indicating a requirement for HMGB1 in spinal cord regeneration.

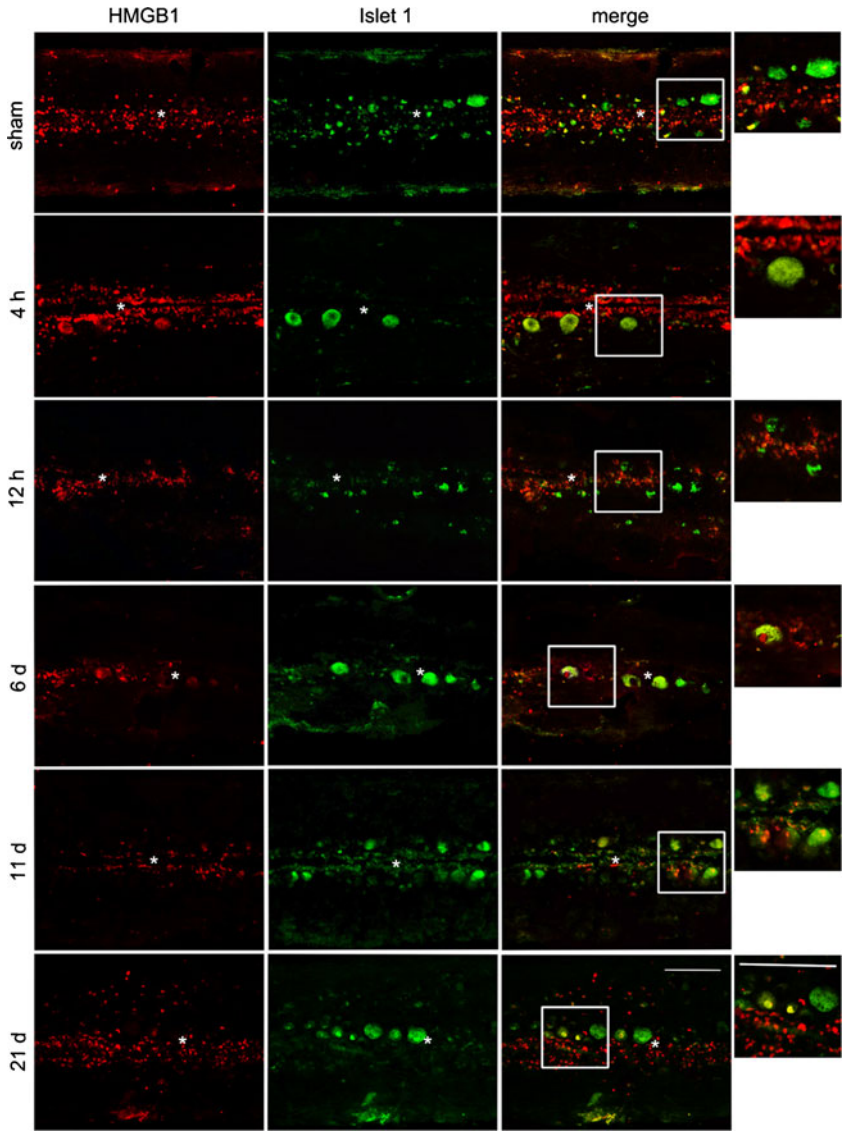

Fig. 3 HMGB1 protein is expressed in nuclei and cytoplasm of motoneurons following SCI. Double-immunofluorescence staining of HMGB1 with Islet-1 in longitudinal sections (4 mm caudal to the lesion site) shows the post-SCI changes in HMGB1 localization in motoneurons along the central canal. HMGB1 is mainly present in nuclei of motoneurons in the sham injury group. After SCI, it is expressed in the cytoplasm during the acute stage (4 and 12 h). By 6 and 11 days after SCI, a low level of HMGB1 was maintained in the cytoplasm, and HMGB1 becomes expressed again mainly in the nuclei. At 21 days after SCI, HMGB1 expression was restored in the nuclei and retained at a low level in the motoneuron cytoplasm. *Asterisks* indicates the central canal. *Scale bar* 100 μm

Reduction of HMGB1 Protein Levels Impairs Axon Regrowth Beyond the Lesion Site After SCI

To further elucidate the protective role of HMGB1 in spinal cord regeneration, we studied axonal regrowth/sparing beyond the lesion site by anterograde tracing. Biocytin was applied at the brainstem/spinal cord junction of fish at 6 weeks after SCI [37]. After 24 h, biocytin was detected with Streptavidin-Cy3 at a site 4 mm distal to the first lesion site. Axon regrowth/sparing beyond the first lesion site is illustrated in coronal and longitudinal sections. The numbers of biocytin-positive axons in coronal and longitudinal sections from HMGB1-inhibited SCI zebrafish were considerably fewer than those from SCI zebrafish treated with control MO. An uninjured group was used as a normal control. Axonal signals in the uninjured group were considerably stronger than either the HMGB1- or control MO-treated groups (Fig. 6a). Immunofluorescence intensity analysis of biocytin in coronal sections of zebrafish treated with HMGB1 MO showed a 34 % decrease in axonal regrowth/sparing compared with zebrafish treated with standard MO after SCI ($P<0.05$) (Fig. 6b). These observations support the view that HMGB1 is involved in axonal regrowth, allowing locomotor recovery during regeneration after SCI.

Discussion

HMGB1 Expression is Downregulated in the Acute Stage and Restored in the Chronic Stage After SCI

Most published studies on traumatic nervous system injury focus on rodents and on short periods of observation after the injury. HMGB1 expression in the rat is enhanced at 48 h after

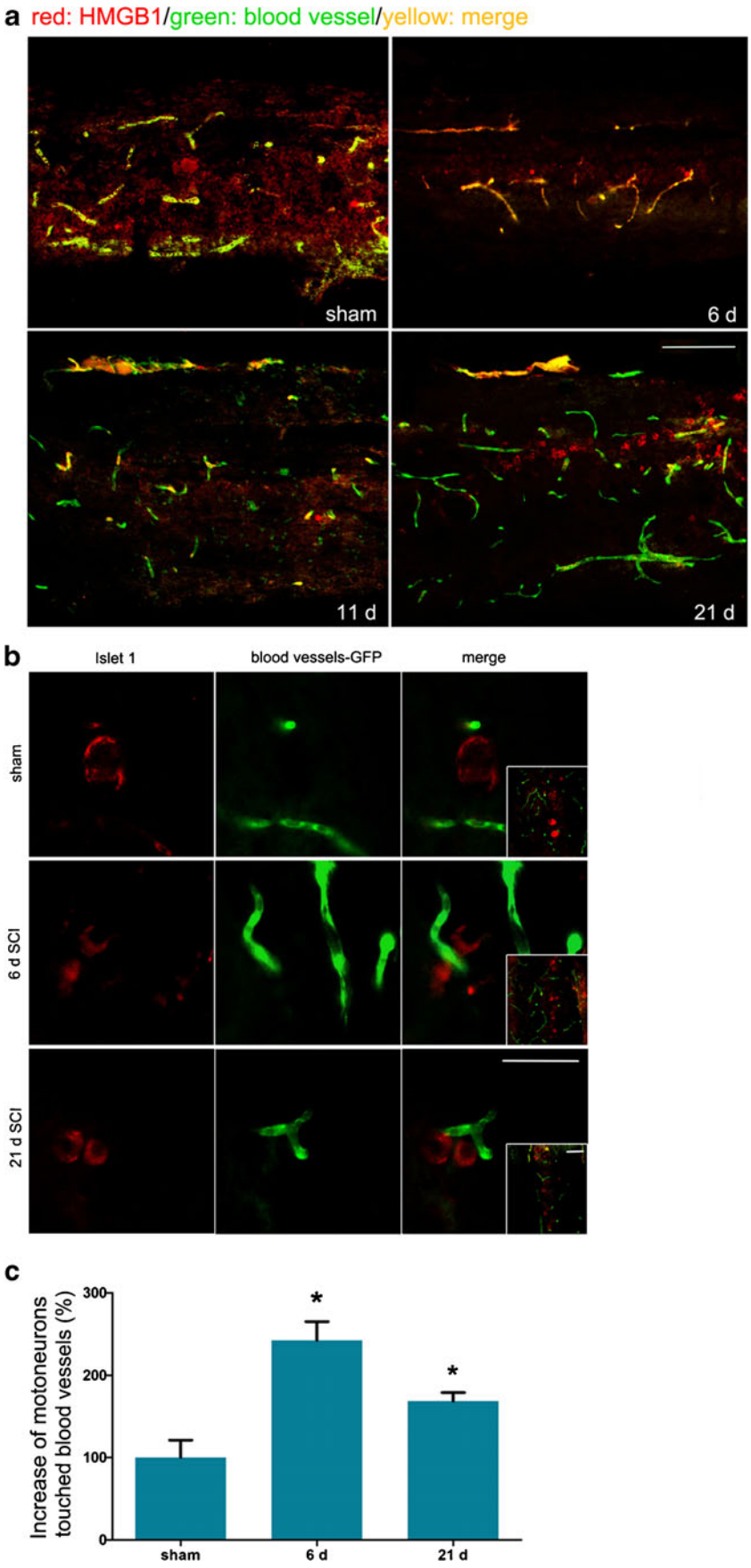

Fig. 4 HMGB1 protein is expressed in vasculature and motoneurons with increased association with blood vessels after SCI. **a** Immunofluorescence staining of HMGB1 in longitudinal sections (4 mm caudal to the lesion site) of (fli1a::EGFP)y1 transgenic zebrafish shows that HMGB1 is highly expressed in blood vessels at 6 days and decreased at 11 and 21 days after SCI when compared with the sham injury group. *Scale bar* 100 μm. **b** Motoneurons marked by Islet-1 (*red*) in longitudinal sections (4 mm caudal to the lesion site) of (fli1a::EGFP)y1 transgenic zebrafish shows increased association with blood vessels (*green*) after SCI. *Scale bar* 50 μm. **c** The number of motoneurons associated with blood vessels is increased at 6 and 21 days after SCI compared with the sham injury group (measured with ImagePro Plus software, $*P<0.05$, one-way ANOVA with Tukey's post-hoc test, $n=3$). Values represent means±SEM

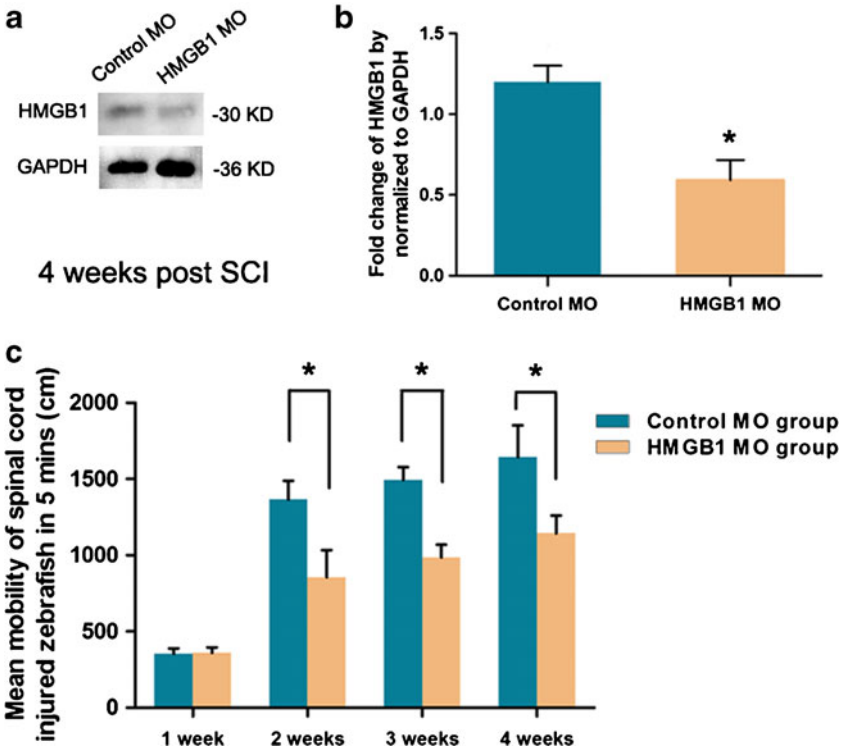

Fig. 5 Reduction of HMGB1 expression after spinal cord injury retards locomotor recovery. HMGB1 protein expression is reduced at 4 weeks after SCI by HMGB1 MO, as detected by Western blot analysis (**a** and **b**). Intensity of the bands was quantified by ImageTool software. HMGB1 protein levels at 4 weeks after MO treatment are decreased by 50 % by application of HMGB1 MO as compared with the control MO group (*$P<0.05$, independent sample t test; $n=5$ fish/group). Values represent means±SEM. **c** Total distance moved in 5 min of zebrafish treated with HMGB1 MO or control MO ($n=9$ fish/group) after SCI. From 2 to 4 weeks after SCI, the total distance moved was reduced by treatment with HMGB1 MO compared to fish treated with control MO (*$P<0.05$, **$P<0.01$, independent sample t test; $n=9$). Values represent means±SEM

SCI and parallels increased inflammation that occurs through upregulation of TNF-α [12]. In a rat spinal nerve ligation model, HMGB1 mRNA levels are high at 7 days after the lesion [21], and HMGB1 protein levels in adult rats are increased in serum at 10 days after brain ischemia [13]. In mice and humans afflicted with stroke, HMGB1 protein is

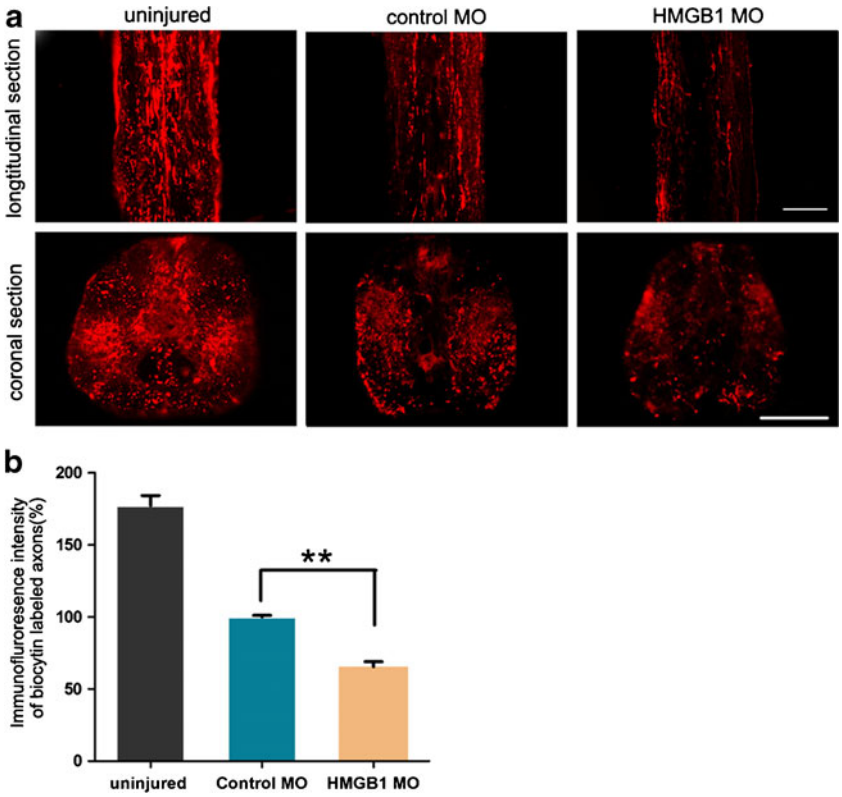

Fig. 6 Inhibition of HMGB1 protein expression after SCI impairs regrowth of axons. **a** Coronal and longitudinal sections show fluorescence intensity of axons beyond the lesion site at 6 weeks after injury. Zebrafish treated with HMGB1 MO showed significantly less axonal regeneration than fish treated with control MO. The sham injury group was used as a control. Regenerating axons (*red*). *Scale bars* 200 μm. **b** Biocytin staining analysis of axonal regeneration in SCI zebrafish treated with HMGB1 MO or standard MO (**$P<0.01$, one-way ANOVA with Tukey's post-hoc test; $n=4$ fish/group). Values represent means±SEM

released into the blood between 6 and 24 h after ischemia [13, 38]. The rapid and persistent elevation of HMGB1 seen in higher vertebrates contrasts the rapid decrease in HMGB1 levels following SCI observed here. Because HMGB1 is a highly pro-inflammatory factor, it is possible that lower levels of HMGB1 after SCI in zebrafish may ameliorate persistent inflammation, resulting in greater cell survival and less tissue damage during a period of recovery, enabling regeneration to proceed as signaled by the later return of HMGB1 to basal levels.

It is noteworthy in this context that acute inflammation in zebrafish after central nervous system injury has led to improved recovery. Cerebroventricular injection of immunogenic zymosan A into adult zebrafish induces acute neuroinflammation and results in increased numbers of newly born neurons compared to animals injected with vehicle only [39]. In zebrafish with traumatic brain injury, injection of the anti-inflammatory agent Pranlukast before the injury inhibits leukotriene signaling in activated radial glial cells and decreases proliferation of neurons when compared to vehicle-only injected animals [39]. Given that acute inflammation has been suggested to stimulate regeneration [39], our demonstration that an increase in HMGB1 mRNA levels occurs at 4 h after SCI in zebrafish suggests that acute inflammation occurs at very early stages after injury and would suggest a model in which an initial, transient inflammatory HMGB1 release occurs immediately after SCI to stimulate neurogenesis and/or promote cell survival, followed by a decrease in HMGB1 levels to prevent persistent inflammation that would be detrimental to neurogenesis.

The increase in HMGB1 mRNA, observed at 4 h after SCI, occurs in the absence of changes in protein level. One possible explanation is that the short duration of increased mRNA expression of HMGB1 may not be sufficient to accumulate protein. Alternatively, the increase in HMGB1 mRNA occurs to counter mechanisms responsible for the rapid decrease in HMGB1 expression, observed at 12 h, in order to maintain sufficient HMGB1 levels to stimulate neurogenesis or promote cell survival, consistent with our model. The detailed mechanism remains to be studied.

HMGB1 is Expressed in Both Motoneurons and Blood Vessels

HMGB1 is expressed in tyrosine hydroxylase-expressing neurons, as well as in microglia and astrocytes in the central nervous system of adult vertebrates [7, 8, 22]. In the present study, we further demonstrate that HMGB1 is expressed in motoneurons and blood vessels. Interestingly, at 4 h after SCI, localization of HMGB1 in motoneurons is mainly cytoplasmic, possibly making HMGB1 accessible to release or more accessible to the cellular protein degradation machinery, while at 12 h after SCI, HMGB1 protein remains cytoplasmic, but at reduced levels in motoneurons through 11 days after SCI, after which HMGB1 increases. It is thus not unlikely that HMGB1 translocates from the nucleus into the cytoplasm during the acute stage after SCI. By 21 days after SCI, most HMGB1-positive motoneurons revert to the pattern of the sham-injured controls, with most of the signal localized in the nuclei. At this time, HMGB1 expression is at similar levels as the sham injury group and parallels the improvement in locomotor function, suggesting that the presence of HMGB1 in motoneuron nuclei facilitates functional restoration during the first 3 weeks after SCI.

Expression of HMGB1 in blood vessels after SCI suggests a role for HMGB1 in neurovascular protection. Previous studies show that transgenic mice overexpressing HMGB1 show smaller myocardial infarction volumes than wild-type mice. These mice also show higher capillary and arteriole densities at 4 weeks after myocardial infarction than wild-type mice [40]. After intracerebral hemorrhage-induced brain injury in rats, inhibition of HMGB1 decreases levels of vascular endothelial growth factor and retards functional recovery [41], suggesting that HMGB1 enhances angiogenic regeneration. In the present study, HMGB1 expression was remarkably elevated in the neural vasculature after SCI. In addition, the number of motoneuron cell bodies touching blood vessels is increased, accompanied by sprouting of vessels apparently induced by HMGB1. This suggests that HMGB1 promotes nervous system regeneration at least partly through facilitating angiogenesis after SCI.

Taken together, our results indicate that HMGB1 enhances functional recovery in adult zebrafish by stimulating neurogenesis and increasing angiogenesis after severe complete spinal cord transection. Studying the HMGB1 signaling pathway in neurons and blood vessels in zebrafish after SCI and overexpression of this molecule in endothelial cells can be expected to delineate the protective function of HMGB1 in the lesioned adult central nervous system not only of fish, but also of mammals, with the hope to provide new approaches for therapy.

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Conflicts of Interest The authors declare that there are no conflicts of interest.

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