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Major vault protein promotes locomotor recovery and regeneration after spinal cord injury in adult zebrafish

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Abstract

In contrast to mammals, adult zebrafish recover locomotor functions after spinal cord injury (SCI), in part due to axonal regrowth and regeneration permissivity of the central nervous system. Upregulation of major vault protein (MVP) expression after spinal cord injury in the brainstem of the adult zebrafish prompted us to probe for its contribution to recovery after SCI. MVP is a multifunctional protein expressed not only in many types of tumours but also in the nervous system, where its importance for regeneration is, however, unclear. Using an established zebrafish SCI model, we found that MVP mRNA and protein expression levels were increased in ependymal cells in the spinal cord caudal to the lesion site at 6 and 11 days after SCI. Double immunolabelling showed that MVP was co-localised with Islet-1 or tyrosine hydroxylase around the central canal of the spinal cord in sham-injured control fish and injured fish 11 days after surgery. MVP co-localised with the neural stem cell marker nestin in ependymal cells after injury. By using an *in vivo* morpholino-based knock-down approach, we found that the distance moved by MVP morpholino-treated fish was reduced at 4, 5 and 6 weeks after SCI when compared to fish treated with standard control morpholino. Knock-down of MVP resulted in reduced regrowth of axons from brainstem neurons into the spinal cord caudal to the lesion site. These results indicate that MVP supports locomotor recovery and axonal regrowth after SCI in adult zebrafish.

Introduction

Spinal cord injury (SCI) is a central nervous system (CNS) lesion that leads to severe and often permanent disability in mammals (Karnezis *et al.*, 2004; Silver & Miller, 2004; Harel & Strittmatter, 2006; Schwab *et al.*, 2006). In contrast to mammals, adult zebrafish have a remarkable capacity for functional recovery after injury to parts of the nervous system such as spinal cord, brain and optic nerve (Bernhardt *et al.*, 1996; Becker *et al.*, 1997, 2004; Guo *et al.*, 2011; Kroehne *et al.*, 2011; Baumgart *et al.*, 2012). The ability to move partly depends on networks of rhythmically active neurons in the spinal cord, and their activation relies on descending inputs from the brain or on sensory inputs (McLean & Fetcho, 2009; Kyriakatos *et al.*, 2011). After SCI, neurons in adult zebrafish can regenerate axons which reconnect to their appropriate targets, and the injured fish regain swimming function several weeks later (Becker *et al.*, 1997, 1998, 2004; Bhatt *et al.*, 2004; Becker & Becker, 2008). In the complex regeneration process, intrinsic molecules operant in the brainstem and molecules in the microenvironment of the de-afferented spinal cord play important roles in recovery (Becker *et al.*, 2004; Abdesselem *et al.*, 2009; Yu *et al.*, 2011a,b). The zebrafish has, thus, become an important model for exploring the molecular mechanisms underlying functional recovery after SCI.

To search for molecules involved in successful regeneration we have analysed, by expression profiling, the mRNA levels in the nucleus of the medial longitudinal fascicle (NMLF) as an indicator for nuclei with regenerative capacities after SCI (Ma *et al.*, 2012). One of the molecules found to be upregulated in its expression was the 100-kDa major vault protein (MVP), the predominant component of vaults which contain a small untranslated vault RNA, telomerase-associated protein-1 and vault poly (ADP-ribose) polymerase. MVP accounts for up to 75% of the total complex and is highly conserved from mammals to fungi (Kedersha *et al.*, 1986, 1990; Vasu & Rome, 1995; Herrmann *et al.*, 1996, 1998; Kickhoefer *et al.*, 1998; Yoshinari *et al.*, 2009). MVP is identical to the human lung resistance protein, and its expression is increased in tumours and multiple drug resistance models (Scheffer *et al.*, 1995, 2000; Mossink *et al.*, 2003; Lloret *et al.*, 2008). Interestingly, MVP is also widely expressed in the nervous system of different species (Herrmann *et al.*, 1996; Aronica *et al.*, 2003; Komori *et al.*, 2007; Paspalas *et al.*, 2009; Liu *et al.*, 2011). The wide expression of MVP in various cell types and considerable phylogenetic conservation point to its important cellular functions (Kedersha *et al.*, 1990).

In this report, we use a zebrafish SCI model and show that mRNA and protein levels of MVP are upregulated at 6 and 11 days after SCI. Morpholino (MO)-mediated knock-down of MVP blocked

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re-acquisition of swimming motility after SCI and was associated with reduced axonal regrowth. Our data indicate that MVP is beneficial for functional recovery and axonal regrowth after SCI in adult zebrafish.

Materials and methods

Animals

Adult zebrafish (*Danio rerio*, 6 months old) were purchased from Huiyuan Aquatic Animals Company (Shantou, Guangdong, China). Fish 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 (Becker *et al.*, 2004; Guo *et al.*, 2011; Yu *et al.*, 2011a). Briefly, fish were anesthetised by immersion in 0.033% aminobenzoic acid ethylmethylester (MS222; Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS), pH 7.4, 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, ~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-injured control had the incision but without spinal cord transection. All surgical procedures were performed on ice under a microscope.

Real-time quantitative RT-PCR (qPCR)

To study the expression of MVP mRNA in the spinal cord caudal to the lesion site, total RNA was extracted from the 4-mm pieces of spinal cord directly caudal to the lesion site at different time points after SCI. First-strand cDNA was generated using random primers and ReverTraAce^{R} qPCR RT Kit (Toyobo, Osaka, Japan). qPCR was performed with SYBR^{R} Green Real Time PCR Master Mix (Toyobo) as described (Goff *et al.*, 2004). Primers for qPCR were designed using Primer Express 5.0 software (Applied Biosystems, Foster City, CA, USA). All assays were performed in duplicate samples, and assay products were validated using melting curves to confirm the presence of single PCR products. GAPDH served as the internal control. The following primer sequences were used: zebrafish MVP forward, GGAAAGTCGGGCTAAGAA; reverse, ATCACAGCACGCACCTTT. GAPDH forward, GTGTAGGCGT GGACTGTGGT; reverse, TGGGAGTCAACCAGGACAAATA (Pei *et al.*, 2007).

In situ hybridization

In situ hybridization probes (sense and antisense) for MVP mRNA (NM_201325) 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)-labelled sense and antisense 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 adult zebrafish CNS was performed as described (Bernhardt *et al.*, 1996). The 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, prehybridised for 2 h at 55 °C and hybridised with the DIG-labelled probes at 55 °C overnight. After extensive washings 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. For negative control, sense probes were developed in parallel under the same conditions as the antisense probes. The sense probe showed no reactivity (data not shown). Sections from sham-injured control and injured fish were analysed on the same slides. Sections were viewed and photographed using an epifluorescence microscope (Axio Imager Z1; Zeiss, Oberkochen, Germany).

Immunohistochemistry

All tissues were processed for immunofluorescence after fixation in 4% formaldehyde in PBS at 4 °C overnight. Serial sections (16 μm thick) from spinal cord (longitudinal, 0–4 mm caudal to lesion site) were used. Sections were prepared as described above and processed for immunostaining (Guo *et al.*, 2011). The primary antibodies were rabbit anti-MVP (1 : 200; Bioss, Beijing, China), mouse anti-Islet-1 (1 : 100; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), mouse anti-tyrosine hydroxylase (TH+; 1 : 400; Millipore), Rabbit anti-nestin (1 : 200; Boster, Wuhan, China) and mouse anti-synaptic vesicle protein 2 (SV2; 1 : 200; Developmental Studies Hybridoma Bank). Secondary antibodies were Alexa Fluor CY3 goat antirabbit IgG (1 : 800; Jackson ImmunoResearch) and FITC goat antimouse IgG (1 : 800; Jackson ImmunoResearch). Fluorescence images were collected using an epifluorescence microscope (Axio Imager Z1; Zeiss).

Western blot analysis

Total protein was extracted by homogenizing the spinal cord tissue (0–4 mm caudal to lesion site) in RIPA buffer (Soliba, Beijing, China). After centrifugation of the homogenate at 14 000 ***g*** and 4 °C for 5 min, the protein concentration in the supernatant was determined with a BCA kit (Pierce, Rockford, IL, USA). The supernatant was denatured by boiling for 5 min in SDS sample buffer. Fifty micrograms of total protein were then subjected to SDS-PAGE on 8% gels. Proteins were transferred to a polyvinylidene difluoride membrane and probed with the following antibodies: rabbit anti-MVP (1 : 200; Bioss) and mouse anti-GAPDH (1 : 1000; Beyotime, Haimen, China). Goat antirabbit IgG and goat antimouse IgG (1 : 1000; Jackson ImmunoResearch) conjugated to horseradish peroxidase were used as secondary antibodies. The grey value of each band was measured and normalised to that of the GAPDH b and by IMAGEJ software (NIH).

MO treatment

MOs are synthetic molecules that are the product of a redesign of natural nucleic acid structures. MOs targeting the start codon region can interfere with the progression of the ribosomal initiation complex from the 5′ cap to the start codon and prevent translation of the coding region of the targeted transcript, thus decreasing the protein expression level (Summerton & Weller, 1997). Previous data from our lab showed that the fluorescein-tagged MOs are still detectable 6 weeks after application, indicating that MOs can reduce protein levels for as long as 6 weeks after SCI (Becker *et al.*, 2004).

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MVP antisense MO 5′-GATCAGCGTCCATGTTTCTTCCTAC-3′ and standard control MO 5′-CCTCTTACCTCAGTTACAATTTA-TA-3′ vivo-porter coupled (Gene Tools, Philomath, OR, USA) were dissolved in Danieau solution (Nasevicius & Ekker, 2000) and soaked onto small pieces of Gelfoam (Upjohn, Kalamazoo, MI, USA). The pieces were divided into smaller pieces to yield 600 ng of MO, ~0.22 μL per piece, and allowed to dry. One piece was applied to the transection site immediately after spinal cord transection.

Swim tracking

Swimming capabilities of injured fish were assessed each week after MO application for 6 weeks in two trials of 5 min each (trial interval, 4 h). In each trial, a zebrafish was placed in a brightly illuminated (100 lux) tank (42 × 30 × 30 cm) filled with aquarium water (5 cm deep) at 25 °C. A video camera recorded the trials from 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 graphical presentation and statistical analysis. The experimenter was blinded to the treatment of the animals.

Retrograde tracing

Retrograde axonal tracing was performed by application of tracer biocytin (Sigma) 4 mm caudal to the spinal lesion site 6 weeks after transection (see Fig. 5 for illustration; Becker *et al.*, 1997, 2004; Yu *et al.*, 2011a). In brief, Gelfoam pieces soaked in a saturated solution of biocytin or rhodamine dextran amine were left at the lesion site for the tracer to be retrogradely transported. Twenty-four hours after application, brains were dissected and fixed in 4% formaldehyde in PBS overnight and embedded in 15% sucrose at 4 °C. Serial coronal brain sections (20 μm thick) were obtained using a cryostat. Biocytin labelling in the sections was detected with Streptavidin-Cy3 (1 : 200; Bioss). Labelled neurons in the whole NMLF were counted in brainstem sections using an epifluorescence microscope (Axio Imager Z1; Zeiss) by an investigator blind to treatment. Three animals were in each group (treated with control MO and MVP MO).

Anterograde tracing

To study presynaptic endings from regenerating axons past the lesion site, biocytin was applied at the brainstem–spinal cord junction through a second surgery 6 weeks after SCI (see Fig. 6 for illustration; Becker *et al.*, 1997, 2004; Yu *et al.*, 2011a). After 24 h, spinal cords were removed, and tissue 0–4 mm in length caudal to the original spinal lesion site was fixed in 4% formaldehyde in PBS at 4 °C overnight, embedded in 15% sucrose, and sectioned (20-μm-thick sections) coronally on a cryostat. Biocytin labelling was detected with Streptavidin-Cy3. Fluorescence intensity is presented as the mean intensity evaluated by IMAGEJ software (NIH) from 12 randomly counterpart selected sections of spinal cords 0–4 mm caudal to the lesion site from each group. Three animals were in each group (treated with control MO or MVP MO).

Statistical analysis

Using SPSS 13.0 software, Student's *t*-test was used for comparing two treatment groups, and one-way ANOVA was used for multiple group comparisons. All data are presented as means ± SEM. $P < 0.05$ is set as indicating significance ($^*P < 0.05$, $^{**}P < 0.01$). All experiments were performed three times independently.

Results

MVP mRNA expression levels were increased caudal to the lesion site

To measure the levels of MVP mRNA after SCI, we collected spinal cord tissue extending 4 mm caudally from the lesion site at 4, 12 h, 6 and 11 days after SCI. The 4- and 12-h time points represent the acute response phase while those of 6 and 11 days after SCI represent the chronic response phase (see, for instance, Guo *et al.*, 2011). At 4 and 12 h after SCI, MVP mRNA expression levels (with 1.16 ± 0.14 and 1.38 ± 0.18-fold increases, respectively; one-way ANOVA with Tukey's *post hoc* test, $P > 0.05$, $n = 6$) were not different from those of sham-injured controls, while significant upregulation was observed at 6 days, with a 1.74 ± 0.098-fold increase (one-way ANOVA with Tukey's *post hoc* test, $^{**}P < 0.01$, $n = 6$), and at 11 days, with a 1.82 ± 0.226-fold increase (one-way ANOVA with Tukey's *post hoc* test, $^*P < 0.05$, $n = 6$) after SCI (Fig. 1A).

To analyse the cells that express MVP in the spinal cord caudal to the lesion site, we performed *in situ* hybridization (Fig. 1B). MVP mRNA-positive signals were limited to the ependymal cells lining the central canal and the area surrounding the central canal corresponding to the gray matter (Fig. 1B). More cells expressed MVP at 6 and 11 days after SCI than at 4 and 12 h after SCI or in the sham-injured control groups. Uninjured fish were not different from the sham-injured control fish (data not shown). The MVP sense probe showed no reactivity (data not shown). These observations are in agreement with the levels of mRNA expression seen by qPCR, suggesting that MVP mRNA levels are not changed in the acute phase after SCI but are increased in the chronic phase.

MVP protein expression levels were increased caudal to the lesion site

To investigate whether the increase in MVP mRNA levels corresponds to an increase in protein expression, we performed immunoblotting of the 100-kDa MVP with spinal cord tissue harvested at 4 and 12 h and at 6 and 11 days after SCI (Fig. 2A). No significant differences in MVP expression levels were observed at 4 or 12 h after SCI (Fig. 2B) between lesioned fish and sham-injured controls. Uninjured fish showed expression levels not different from those of sham-injured fish (not shown). Consistent with the mRNA data, levels of MVP protein were increased in the spinal cord caudal to the lesion site at 6 and 11 days after SCI (Fig. 2A and B). Densitometric analysis showed an increase in MVP protein levels at 6 days (1.602 ± 0.072, one-way ANOVA with Tukey's *post hoc* test, $^{**}P < 0.01$; $n = 4$) and 11 days (1.798 ± 0.136, one-way ANOVA with Tukey's *post hoc* test, $^{**}P < 0.01$; $n = 4$) after SCI compared to sham-injured controls (Fig. 2B).

To further study MVP protein expression and distribution, we performed immunofluorescence staining on spinal cord sections. MVP-positive cells were located predominantly in the area surrounding the central canal at 11 days after SCI (Fig. 2C), and MVP protein was mainly detectable in the cytoplasm. MVP protein expression levels in injured fish were increased at 6 and 11 days after SCI in cells surrounding the central canal when compared to the sham-injured controls (Fig. 2D, m–x), but were similar in sham-injured controls and injured fish at earlier time points (Fig. 2D, a–i). Uninjured fish were not different from the sham-injured control fish (data not shown, but see Supplemental Fig. 1 in EJN blog for the time point of 4 hours).

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MVP co-localised with Islet-1, tyrosine hydroxylase (TH) and nestin caudal to the lesion site

To examine which type of neurons expresses MVP we performed double immunofluorescence labelling for MVP and the motor neuronal marker Islet-1 or the noradrenergic and dopaminergic neuronal marker TH. The results showed that MVP is expressed in motor neurons and noradrenergic and dopaminergic neurons at 11 days after SCI (Fig. 3A and B). MVP-immunopositive cells were limited to the area surrounding the central canal, which prompted us to identify whether MVP is expressed in nestin-positive stem cells. Double immunostaining for MVP and nestin showed that MVP and nestin are coexpressed in ependymal cells lining the central canal and in some cells distributed in the spinal cord parenchyma (Fig. 3C).

MVP promoted locomotor recovery after SCI

To investigate whether application of an antisense MVP MO to the freshly injured spinal cord affects MVP protein expression *in vivo*, Western blot analysis was performed 11 days after SCI. MVP protein levels were reduced by 75% in the MVP MO-treated group at this time point after SCI compared to standard control MO (Fig. 4A and B).

To examine the swimming ability of adult zebrafish treated with MVP MO at the lesion site and at the time of SCI, we analysed locomotor recovery weekly by measuring the distance swum in 5 min. No differences were observed between the groups treated with MVP MO and standard control MO up to 3 weeks after SCI (Fig. 4C). However, reduced locomotor recovery of MVP MO vs. standard control MO-treated fish was found at 4, 5 and 6 weeks (Fig. 4C). At 4 weeks after SCI, the total distance moved by fish treated with MVP MO (1376.1 ± 214.4 cm, $n = 11$, one-way ANOVA with *post hoc* Student–Newman–Keuls test, $*P < 0.05$) was reduced to 67.7% of that of fish treated with standard control MO (2032.7 ± 201.9 cm, $n = 11$). Similarly, the total distance moved by fish treated with MVP MO (5 weeks, 1323.6 ± 149.6 cm, $n = 11$, one-way ANOVA with *post hoc* Student–Newman–Keuls test, $*P < 0.05$; 6 weeks, 1557.5 ± 205.2 cm, $n = 11$, one-way ANOVA with *post hoc* Student–Newman–Keuls test, $*P < 0.05$) was decreased to 66.2 and 71.7% of that from the control group (5 weeks, 1998.7 ± 140.8 cm, $n = 11$; 6 weeks, 2171.4 ± 183.4 cm, $n = 11$) at 5 and 6 weeks, respectively. Standard control MO did not affect locomotor recovery as swim distance between spinal cord-injured fish and spinal cord-injured fish treated with standard control MO were not different at 6 weeks after SCI, as observed previously (Becker *et al.*, 2004). Thus, MVP MO treatment significantly impaired locomotor recovery, indicating a requirement for MVP in spinal cord regeneration.

MVP MO application reduced the number of NMLF neurons with regenerated axons

NMLF neurons play a critical role in swimming activity and can regrow axons to the ipsilateral spinal cord after SCI (Uematsu & Todo, 1997; Takeda *et al.*, 2007). To investigate whether MVP MO treatment affects axon regrowth after SCI, we performed retrograde tracing by applying biocytin 6 weeks after the lesion, 4 mm caudal to the lesion site in the same fish tested for locomotor function

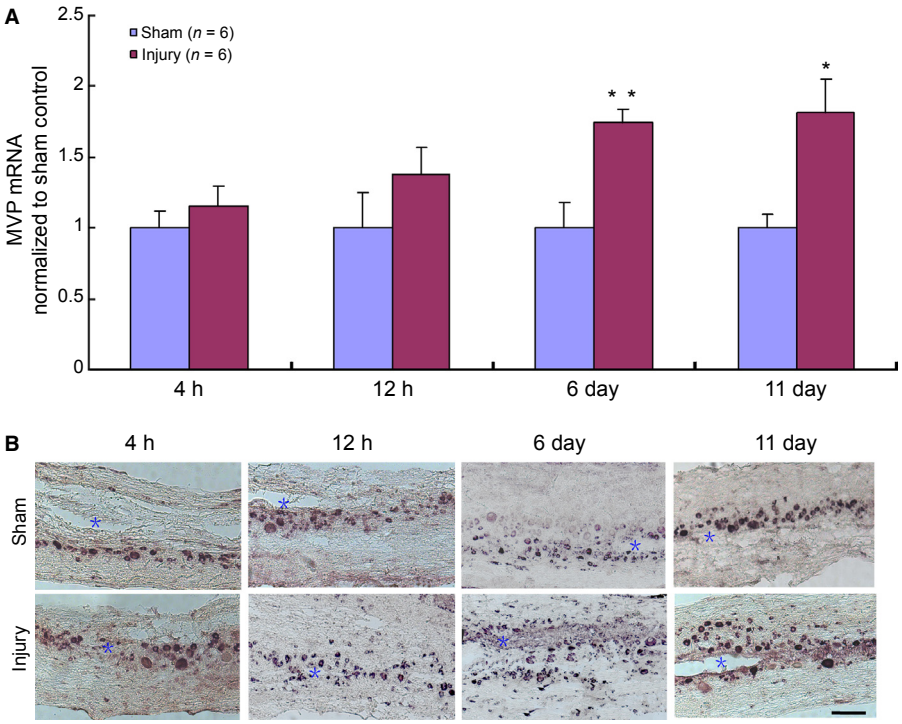

FIG. 1. Time course of MVP mRNA expression after SCI. (A) MVP mRNA expression levels were determined by qPCR in the spinal cord caudal to the lesion site at four time points after SCI. Significant upregulation was observed at 6 days ($**P < 0.01$, one-way ANOVA with Tukey's *post hoc* test; $n = 6$ animals per group) and 11 days, ($*P < 0.05$, one-way ANOVA with Tukey's *post hoc* test; $n = 6$ animals per group) after SCI when compared to sham-injured controls. GAPDH was used as an internal control. Values are means ± SEM. (B) *In situ* hybridization shows expression of MVP mRNA in representative longitudinal sections of the spinal cord 3 caudal mm to the lesion site at 4 and 12 h, and at 6 and 11 days after SCI compared to sham-injured controls ($n = 3$ animals per group); *Central canal. Scale bar, 100 μm.

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(Fig. 5A). Brain sections from fish treated with MVP and control MO were probed for the biocytin tracer using streptavidin-Cy3. Fish treated with MVP MO showed a reduced number of biocytin-labelled NMLF neurons (Fig. 5B and C), indicating impaired re-growth of the axons from NMLF neurons following MVP knock-down. The number of retrogradely labelled neurons was significantly reduced, by 60.3% in MVP MO-treated fish (15 ± 0.58 cells, $n = 3$) relative to the number in control MO-treated fish (25 ± 0.89 cells, $n = 3$; t-test, $^*P < 0.05$; Fig. 5D).

MVP MO treatment reduced the number of synapses caudal to the lesion site

The reformation of functional synapses is essential for locomotor recovery after SCI (Becker *et al.*, 2004). To label synapses formed by regrown axons of supraspinal origin caudal to the injury site, biocytin was applied at the brainstem–spinal cord transition after MO treatment, and co-localization of the tracing signals with the synaptic marker SV2 was analysed 6 weeks after SCI (Fig. 6A). The extent of anterograde tracing (red) was considerably higher in sections of control MO-treated fish (Fig. 6B), with a 2.17-fold increase (t-test, $^*P < 0.05$, $n = 3$) compared to MVP MO-treated fish (Fig. 6C and D), indicating that knock-down of MVP reduces numbers of synapses caudal to the injury site. Few co-localization signals were observed in MVP MO-treated fish when compared to control MO-treated fish (Fig. 6E), confirming that knock-down of MVP prevented regrowth of axons in parallel with a reduction in number of new synapses caudal to the lesion site.

Discussion

Zebrafish have the remarkable ability to regenerate injured tissue compared to many higher vertebrates (Becker *et al.*, 1997; Raya *et al.*, 2003; Reimer *et al.*, 2008; Stewart *et al.*, 2009; Guo *et al.*, 2011; Kroehne *et al.*, 2011). Using an adult zebrafish spinal cord injury model, we demonstrate for the first time that both MVP

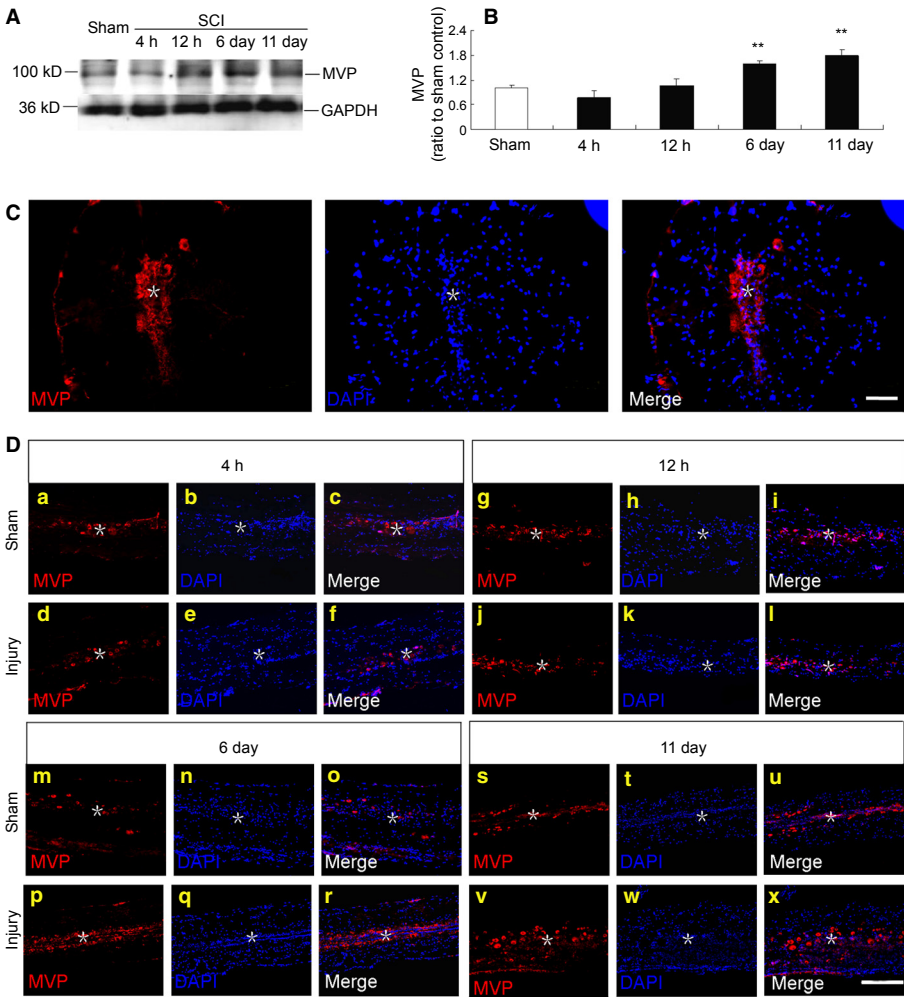

FIG. 2. Time course of MVP protein expression after SCI. (A) MVP protein expression in the spinal cord caudal to the lesion site as detected by immunoblotting at different time points after SCI. MVP corresponds to the band with 100-kDa apparent molecular weight. GAPDH was used as loading control. (B) Change in MVP levels was quantified by IMAGEJ software. The lesion-induced increase in MVP expression achieved significance at 6 days ($^{**}P < 0.01$, one-way ANOVA with Tukey's *post hoc* test; $n = 4$ animals per group) and 11 days ($^{**}P < 0.01$, one-way ANOVA with Tukey's *post hoc* test; $n = 4$ animals per group) after SCI. Values are means ± SEM. (C) MVP-immunopositive cells and DAPI-stained nuclei are shown in a coronal section of the spinal cord 3 mm below the injury site at 11 days after SCI. MVP expression was mainly detected in the cytoplasm; *Central canal. (D) MVP expression was examined by immunohistology at 4 and 12 h, and at 6 and 11 days after SCI. MVP expression was increased surrounding the central canal at 6 and 11 days after SCI when compared to the sham-injured control. MVP (red), DAPI (blue); *Central canal. Scale bars, 50 μm (C), 200 μm (D). [Color version of figure available online]

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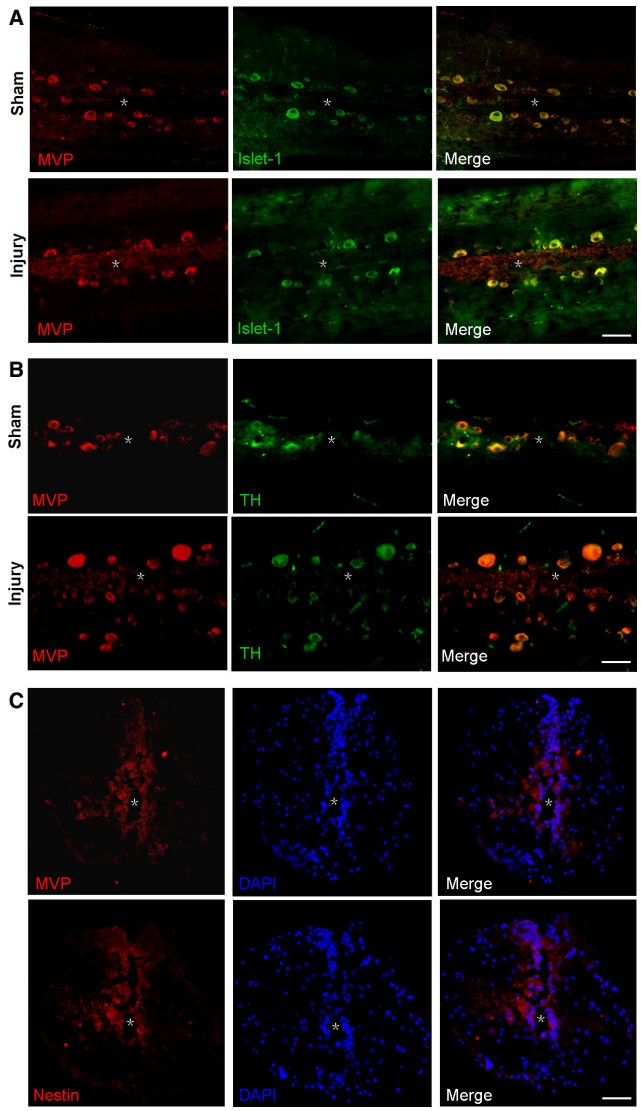

FIG. 3. Double immunolabelling for MVP with markers for two neuronal cell types 11 days after SCI. (A) Double labelling for MVP and Islet-1 (marker for motor neurons) in longitudinal sections of spinal cord 3 mm caudal to the lesion site after SCI and sham injury. (B) MVP and TH (a marker for noradrenergic and dopaminergic neurons) in longitudinal sections of the spinal cord after SCI and sham-injury. (C) MVP and nestin in longitudinal sections of spinal cord after SCI and sham-injury (adjacent sections had to be stained due to the identical species origin of the primary antibodies); *Central canal. All scale bars, 50 μm.

mRNA and protein expression levels are increased at 6 and 11 days, but not at 4 and 12 h, after SCI, using qPCR and Western blot analysis, suggesting that MVP plays a critical role in spinal cord regeneration in the chronic phase but not in the early phase of recovery after SCI.

Double immunostaining of MVP with Islet-1 or TH at 11 days after SCI supports the view that subpopulations of neurons express MVP. These neurons have been shown previously to be newly formed neurons around the injury site 10 days after SCI in adult zebrafish (Hui *et al.*, 2010). Expression of MVP in different types of neurons is in agreement with reports showing MVP expression in the nervous system of humans, non-human primates, rodents and electric fish (Herrmann *et al.*, 1996; Aronica *et al.*, 2003; van Vliet *et al.*, 2004; Paspalas *et al.*, 2009; Liu *et al.*, 2011). In addition to being expressed by newly formed neurons in the vicinity of the central canal, MVP is also expressed by nestin-immunoreactive neural stem cells that comprise the radial glia-type progenitor cells lining the central canal as ependymal cells. These ependymoradial glial cells are progenitor cells for motor neurons after SCI (Reimer *et al.*, 2008). Radial glial cells in the ependymal layer begin to migrate away from the lesion site at 3 days after injury, and accumulation in the parenchyma around the injury site continues till 7 days (Hui *et al.*, 2010). Extending these time points we found that, at 11 days after SCI, MVP and nestin double-immunopositive cells were still observed. Indeed, a recent study on brain stab lesions in adult zebrafish shows that, during the recovery phase, ventricular radial glial progenitor cells that had proliferated and migrated to the lesion site contributed to the population of newly generated neurons which could survive for > 3 months (Kroehne *et al.*, 2011). Spinal cord motor neurons in zebrafish derive from the progenitor domain and differentiate into motor neurons by mechanisms that appear to be highly conserved between mammals and zebrafish (Kimmel *et al.*, 1994; William *et al.*, 2003; Cheesman *et al.*, 2004; Park *et al.*, 2004; Reimer *et al.*, 2008). Similarly, we found that MVP expression is upregulated in motor neurons identified with the marker Islet-1 after SCI.

MVP is expressed not only in motor neurons but also in TH-immunoreactive noradrenergic and dopaminergic neurons around the central canal after SCI. Dopaminergic innervation of the spinal cord is important for locomotion (Sallinen *et al.*, 2009). For example, MPTP, a neurotoxin ablating TH+ neurons in mammals, destroys ~50% TH+ neurons in larval fish, leading to impairment of their swimming capacities at 5, 6 and 7 days post-fertilization (Sallinen *et al.*, 2009). Thus, reinnervation of the caudal spinal cord by axons regrown from intraspinal TH+ neurons may be important for recovery after SCI, with uninjured spinal intrinsic neurons compensating for lost synaptic contacts resulting from plasticity of the intraspinal circuitry caudal to the lesion site.

Upregulation of MVP levels has been reported in the nervous system for different injury models and species. For instance, MVP was found to be upregulated at 7 days after spinal nerve ligation injury in rats (Komori *et al.*, 2007). In the electric ray, MVP expression is also increased in neurons following injury (Li *et al.*, 1999). Expression of MVP is not limited to neurons in the CNS; it is also present in reactive astrocytes and microglia, which may provide a supportive and/or hostile microenvironment for regeneration (Prewitt *et al.*, 1997; Berger *et al.*, 2001; Aronica *et al.*, 2003; Faulkner *et al.*, 2004; van Vliet *et al.*, 2004).

MVP protein expression was decreased by MO application, leading to impaired functional recovery as measured by distance swum at 4–6 weeks after SCI, being most likely at least in part based on reduced axonal regrowth of injured axons and formation of functional synapses by the regrown axons (Zottoli *et al.*, 1994; Becker *et al.*, 1997). The axonal contribution to regeneration and locomotor recovery is associated with an upregulation of MVP expression in the brain stem comprising the NMLF as shown by microarray analysis (Ma *et al.*, 2012). MVP MO is indeed taken up by axons of NMLF neurons that are severed by the transection injury and are therefore considered to be directly affected by the MO treatment. Thus, the tracing studies reveal the effects of the MO treatment on both CNS regions in combination – the NMLF neurons into which the MO is retrogradely transported via the severed axons and the spinal cord where MOs are taken up by severed and non-severed cells. Anterograde tracing experiments showed that in the caudal part of the spinal cord axonal regrowth and number of synapses was reduced when MVP expression in the spinal cord was reduced.

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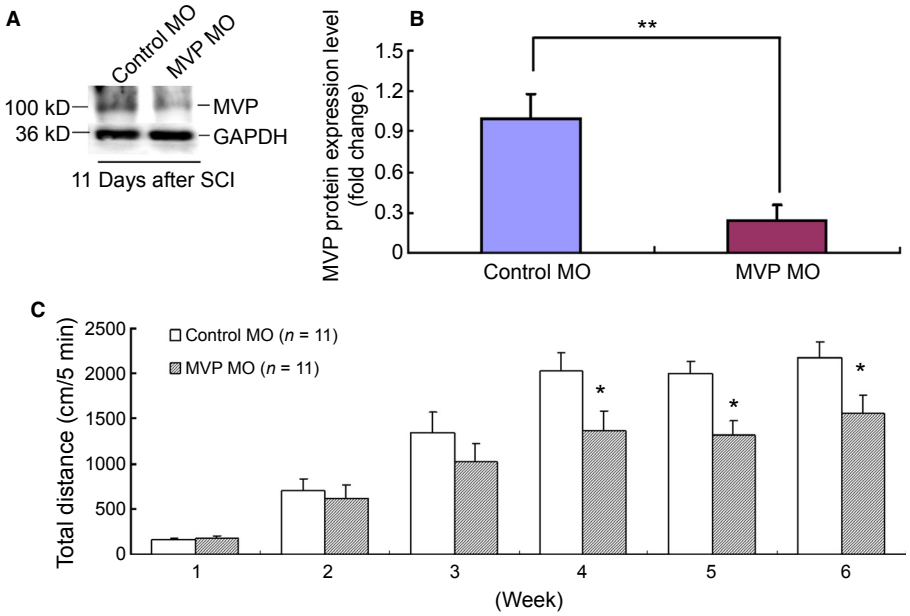

FIG. 4. Inhibition of MVP protein expression reduced locomotor recovery after SCI. (A) MVP protein expression level was reduced 11 days after SCI after application of MVP antisense MO. GAPDH was used as a loading control. (B) Change in MVP protein levels after application of MVP MO was quantified by IMAGEJ software. MVP protein levels were reduced by 75% after application of MVP MO compared to standard control MO (**$P < 0.01$, *t*-test; $n = 3$ animals per group). Values are means ± SEM. (C) Total distance moved by zebrafish treated with MVP MO or standard control MO was studied every week after SCI. At 4, 5 and 6 weeks after SCI the total distance moved by fish was reduced in the MVP MO-treated group (*$P < 0.05$, one-way ANOVA with *post hoc* Student–Newman–Keuls test. Tukey's *post hoc* test did not yield significant differences; $n = 11$ animals per group). Values are means ± SEM.

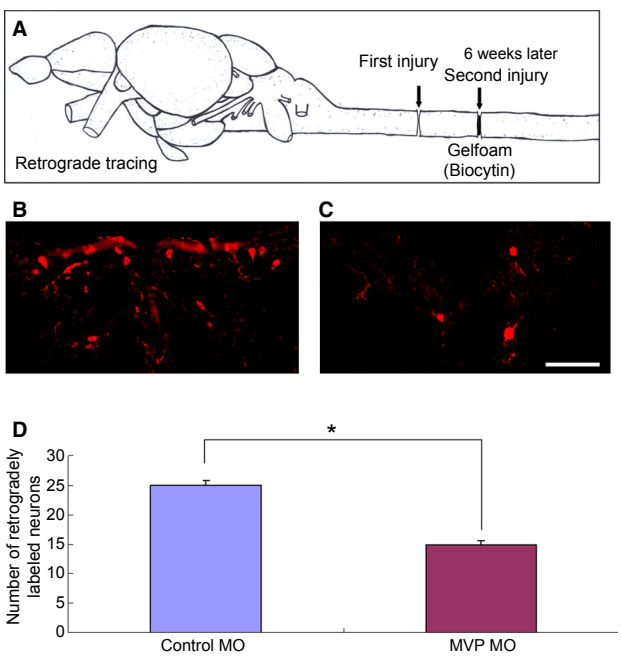

FIG. 5. MVP MO reduced the numbers of NMLF neurons retrogradely labelled 6 weeks after SCI. (A) Schematic illustration of the experiment. Biocytin was applied for retrograde tracing at the site indicated as the second injury at 6 weeks after SCI (first injury). (B) Biocytin-labelled neurons in the NMLF of fish treated with standard control MO. (C) Biocytin-labelled neurons in the NMLF of fish treated with MVP MO. (D) Reduction in numbers of retrogradely labelled neurons in the NMLF after application of MVP MO (*$P < 0.05$, *t*-test; $n = 3$ animals per group). In (B, C) the NMLF area in a coronal section is shown. Scale bar, 50 μm (B, C).

Retrograde tracing experiments showed that the number of NMLF neurons projecting beyond the injury site was reduced when MVP expression in the spinal cord and in the NMLF was reduced. These combined results support the view that expression of MVP both in the spinal cord and in the NMLF is beneficial to axon regrowth. Interestingly, MVP is found not only in axons but also in dendrites (Paspalas *et al.*, 2009). Thus, not only descending and ascending axons regrown by several weeks after SCI, but also reformation and/or reshuffling of dendrites in the spinal cord caudal to the lesion site may contribute to functional recovery (Becker *et al.*, 1997, 1998, 2004; Hanna *et al.*, 1998; Becker & Becker, 2001; Schweitzer *et al.*, 2003, 2007). Neurons can axonally transport MVP to accumulate in nerve terminals presynaptically (Herrmann *et al.*, 1996; Li *et al.*, 1999; Paspalas *et al.*, 2009). Whether MVP also plays a significant role in the elaboration of new dendrites and/or reshuffling of existing ones to new targets after SCI in zebrafish remains to be seen. The fact that MVP is upregulated in the second response phase after injury and remains upregulated until 3 weeks after injury (data not shown, please see Supplemental Fig. 2 in EJN blog) would favour the interpretation that its major function is not in acute neuroprotection after injury by counteracting apoptotic and/or necrotic cell death, but by sustained neuroprotection and by allowing supraspinal axons to regrow and form synapses, while also allowing neurons to be generated from ependymoradial glial cells, with their neurites elaborating to form new synaptic contacts. It is noteworthy in this context that MOs remain detectable until at least 6 weeks after application (Becker *et al.*, 2004).

MVP is also considered to be important for cell survival (Schroeijers *et al.*, 2002; Kolli *et al.*, 2004). For example, serum deprivation of MVP-deficient mouse embryonic fibroblasts (MEFs) leads to significantly increased cell death compared to normal MEFs (Kolli *et al.*, 2004). MVP enhances expression of the anti-apoptotic protein bcl-2 in senescent human fibroblasts (Ryu *et al.*, 2008). Recently, several

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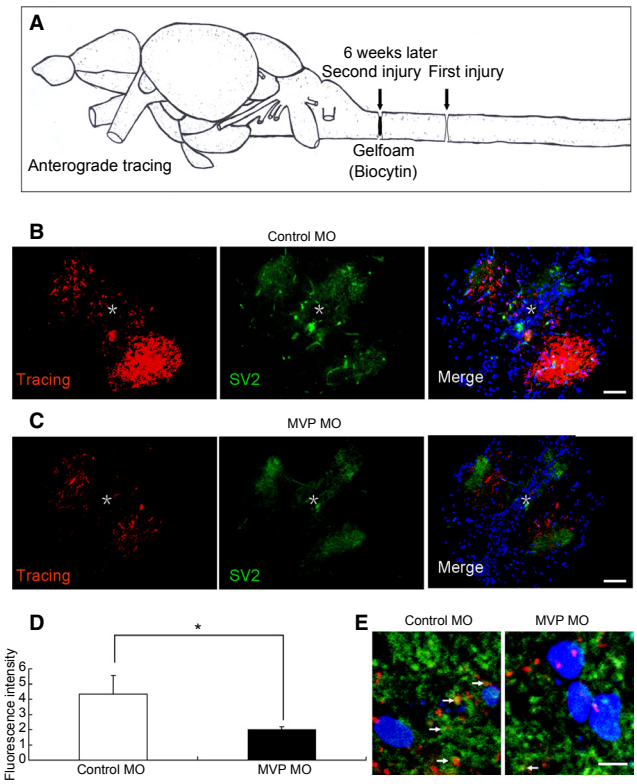

FIG. 6. MVP MO reduced the numbers of regrown descending axons and synapses at 6 weeks after SCI. (A) Schematic illustration of the experiment. Biocytin was applied for anterograde tracing at the site indicated as the second injury at 6 weeks after SCI (first injury). (B, C) Structures, anterogradely labelled with biocytin, in the spinal cord of fish treated with (B) standard control MO and (C) MVP MO. (D) Relative fluorescence intensity was analysed with IMAGEJ software. A 2.17-fold higher intensity was observed with standard control MO application than with MVP MO application (*$P < 0.05$, *t*-test; $n = 3$ animals per group). (E) Images of coronal sections of spinal cord co-labelled for SV2 and anterogradely labelled for biocytin tracing. Arrows indicate co-labelled signals for SV2 (green) and biocytin (red). DAPI (blue) indicates nuclear staining; *Central canal. Scale bar, 50 μm (B, C), 5 μm (E). [Color version of figure available online]

studies have shown that MVP modulates cellular signalling pathways which are related to cell survival and proliferation, pathways such as the Ras–ERK, PI3K–Akt and JAK–STAT signalling pathways (Kolli *et al.*, 2004; Minaguchi *et al.*, 2006; Steiner *et al.*, 2006; Berger *et al.*, 2009). It thus conceivable that MVP is involved not only in functional recovery after injury but also in maintenance of the circuitry governing swim behaviour in uninjured fish. The contribution of MVP-triggered signalling pathways to functional recovery after SCI in adult zebrafish remains to be studied. Future investigations on the functions of MVP after SCI in fish and mammals are expected to yield insights into potential strategies for SCI therapy in humans.

Conflict of interests

The authors declare no conflict of interests.

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Abbreviations

CNS, central nervous system; MO, morpholino; MVP, major vault protein; NMLF, nucleus of the medial longitudinal fascicle; PBS, phosphate-buffered saline; qPCR, quantitative RT-PCR; SCI, spinal cord injury; SV2, Synaptic vesicle glycoprotein 2; TH, tyrosine hydroxylase.

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