PERSPECTIVE

Complementary research in mammals and fish indicates MMP-2 as a pleiotropic contributor to optic nerve regeneration

Matrix metalloproteinases (MMPs) are members of the metzincin superfamily named after the zinc ion and the conserved methionine residue at the active site. In addition to their role in extracellular matrix (ECM) remodeling, these proteinases (in)activate many signaling molecules such as growth factors, adhesion molecules and cytokines; and even exert some intracellular functions. Hence, they are long-proven regulators of neurogenesis, axonal outgrowth, guidance and myelinogenesis during vertebrate central nervous system (CNS) development and considered crucial for maintaining normal functioning of the adult CNS (Verslegers et al., 2013). However, next to their involvement in a wide range of physiological processes, uncontrolled MMP activities have been associated with the onset of various neurological disorders and CNS injuries. Nonetheless, recent research indicates MMPs as benefactors in the repair and regeneration of the adult mammalian CNS. This paper then also reviews the attributed roles of MMPs, with a focus on MMP-2, in vertebrate axonal outgrowth and mammalian axonal regrowth, and renders *in vivo* proof for a regulatory function of MMP-2 in zebrafish optic nerve regeneration. Moreover, this paper provides novel evidence that the use of zebrafish (successful) and mouse (unsuccessful) regeneration models can be applied as a two-pronged approach to examine how manipulation of MMPs, or other potential targets, can be used to promote/inhibit axonal regeneration in the injured adult mammalian CNS. During development of retinofugal projections within the visual system – which is a widely used model in axonal outgrowth studies because of its accessibility and well-known morphology – MMPs, and gelatinases (MMP-2 and -9) in particular, have been implicated as promoters of retinal ganglion cell (RGC) axonal outgrowth and modulators of guidance, both in mammals and anamniotes. Indeed, the first *in vivo* evidence was provided by a study on retinotectal development in *Xenopus* embryos, which showed that administration of broad-spectrum MMP inhibitors or more specific gelatinase inhibitors to the bathing medium of developing embryos, disrupted axonal guidance cues at low concentrations, whereas higher concentrations reduced axonal outgrowth (Hehr et al., 2005). Due to high sequence conservation among gelatinases, and MMPs in general, the production of inhibitors which act specifically and solely on one MMP remains a challenge. However, over time, inhibitors more specific to MMP-2, that show minimal activity to MMP-9, have been generated. Through use of those more potent MMP-2 inhibitors and by application of genetic loss-of-function techniques, our research group provided primary proof for MMP-2 as a main player in RGC axonal outgrowth in developing vertebrates and for regulatory interactions between MT1-MMP and MMP-2 herein (Janssens et al., 2013; Gaublomme et al., 2014). In zebrafish embryos - a powerful model system to study retinotectal development due to its transparency and conservation of retinal anatomy - single knockdown of Mt1-mmp, a membrane-bound proteinase, significantly decreased the RGC axon innervation area in the optic tectum (OT). Intriguingly, additional Mmp-2 knockdown further reduced OT innervation as compared to single Mt1-mmp knockdown, indicating a potential co-involvement for both proteinases in RGC axonal outgrowth (Janssens et al., 2013). Indeed, mammalian MT1-MMP has been repeatedly reported as an efficient MMP-2-activator (Visse and Nagase, 2003). Likewise, Mt1-mmp knockdown also resulted in reduced Mmp-2 activity levels in zebrafish embryos, suggestive of Mt1-mmp being a major *in vivo* activator of Mmp-2 in zebrafish retinotectal development (Janssens et al., 2013). A reduced RGC axonal outgrowth was also observed after application of broad-spectrum and more specific MMP-2 inhibitors to *ex vivo* postnatal mouse retinal explants. Moreover, utilization of an antibody that specifically blocks the MMP-2 activating ability of MT1-MMP reduced axonal outgrowth to the same extent as a general MT1-MMP neutralizing antibody, indicating that MT1-MMP mainly contributes to mouse RGC axonal development through activation of MMP-2, similar as in developing zebrafish. Furthermore, explants of MMP-2 deficient, but not of MMP-9 deficient mice, showed a reduced neurite outgrowth as compared to wild-type explants, thereby confirming a specific role for this gelatinase in RGC axonal growth (Gaublomme et al., 2014). Despite this established role for MMP-2 in axonal outgrowth in the developing CNS, its underlying targets remain largely undefined. In general, MMPs have been implicated in the release of ECM-bound growth factors, like NGF, and modification of adhesion molecules, like NCAM and ICAM5, which are known to stimulate neurite outgrowth (Verslegers et al., 2013). Within the optic system, activated MMP-2 has been suggested to interact with β1-integrin, a transmembrane cell adhesion receptor that affects neurite outgrowth of RGCs (Gaublomme et al., 2014). These data are supported by developmental expression studies, which, besides a macroglial localization, localized MMP-2 in/on outgrowing RGC axons and their growth cones in vertebrates (**Figure 1A**, left panel) (Janssens et al., 2013; Verslegers et al., 2013; Gaublomme et al., 2014). Altogether, these data ascribe an intrinsic function to MMP-2, serving as an activator of axonal growth stimulating factors, which are in close contact with, or directly located inside or on RGC axons or growth cones (**Figure 1A**, left panel).

As the adult mammalian CNS, including its retino-thalamic projections, is characterized by poor axonal regeneration, optic neuropathies like glaucoma frequently result in permanent vision loss. To date, no clinical therapy is available to cure these neuropathies, yet considerable progress in understanding the mechanisms underlying regenerative CNS failure has been made. So far, one of the main causes of limited axonal regeneration is presumed to be injury-induced glial scarring, which forms an impenetrable barrier of inhibitory components, and myelin debris derived from degenerating nerve fibers. In addition, mature CNS neurons are characterized by an insufficient intrinsic growth capacity and a loss of neurotrophic support (**Figure 1B**, left panel) (Verslegers et al., 2013). To date, limited long-distance axonal regeneration in mammals can be obtained through suppressive ECM remodeling, increase of trophic support and by induction of controlled ocular inflammation and glial reactivity (Fischer and Leibinger, 2012). Similar to axonal outgrowth during development, MMPs have also been suggested as promoters of axonal regeneration in the adult mammalian CNS (Verslegers et al., 2013). In adult rodents, in which regeneration was triggered after injury of the spinal cord or optic nerve, gelatinase activity was strongly induced in astrocytes in the scar tissue. There, MMPs seemed necessary to degrade its inhibitory constituents, such as chondroitin sulfate proteoglycans (CSPGs) (Verslegers et al., 2013). Indeed, several independent studies identified MMP-2 as a major potential proteinase able to reduce the glial scar through proteolytic cleavage of CSPGs (**Figure 1B**, right panel) (Verslegers et al., 2013). For example, MMP-2 deficient mice showed an impaired structural and functional recovery after spinal cord injury due

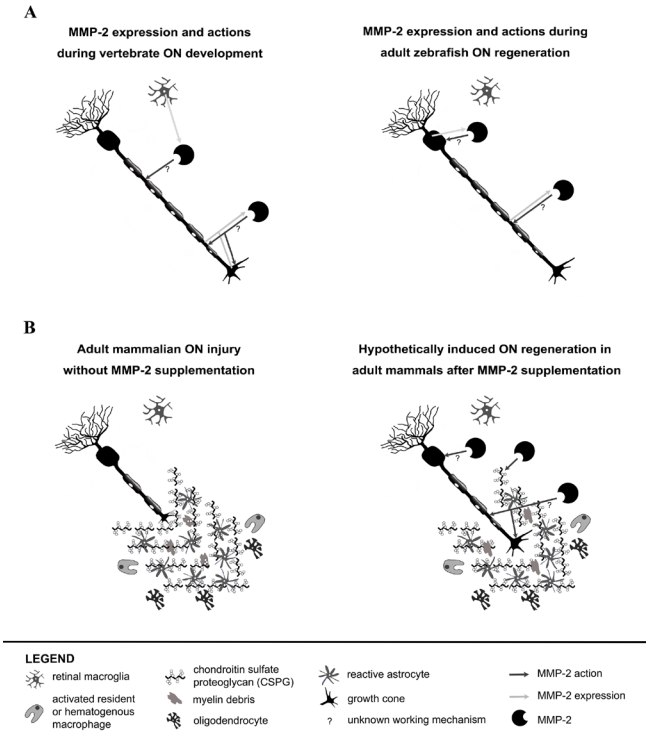

Figure 1 Schematic representation of functional implications for matrix metalloproteinase-2 (MMP-2) in vertebrate optic nerve (ON) development and regeneration.

(A) Left panel: Upregulated MMP-2 has been observed in macroglia, in/on outgrowing retinal ganglion cell (RGC) axons and their growth cones during vertebrate ON development (light-grey arrows). A role for MMP-2 as an activator of axonal-growth stimulating factors was suggested at the level of the outgrowing RGC axon or growth cone (dark-grey arrows). Right panel: A similar neuron-intrinsic function was suggested for MMP-2 during zebrafish ON regeneration (dark-grey arrows), where MMP-2 was expressed in/on RGC somata and their regrowing axons (light-grey arrows). (B) Left panel: Since mature mammalian neurons have a low instrinsic regenerative capacity, receive insufficient trophic support and are exposed to an inhibitory environment, regrowing RGC axons are not able to protrude the glial scar, thereby triggering axonal degeneration and ultimately neuronal cell death. Right panel: However, recent findings indicate that, during induced mammalian ON regeneration, MMP-2 could reduce the glial scar through cleavage of chondroitin sulfate proteoglycans (CSPGs), and therefore support axonal regrowth extrinsically (dark-grey arrow). Furthermore, from research in developing vertebrates and injured adult zebrafish, it is now hypothesized that *in vivo* administration of exogenous MMP-2 after mammalian ON injury, might additionally aide mammalian ON regeneration *via* induction of axonal-growth stimulating factors at the level of the RGC somata, axon or growth cone (dark-grey arrows).

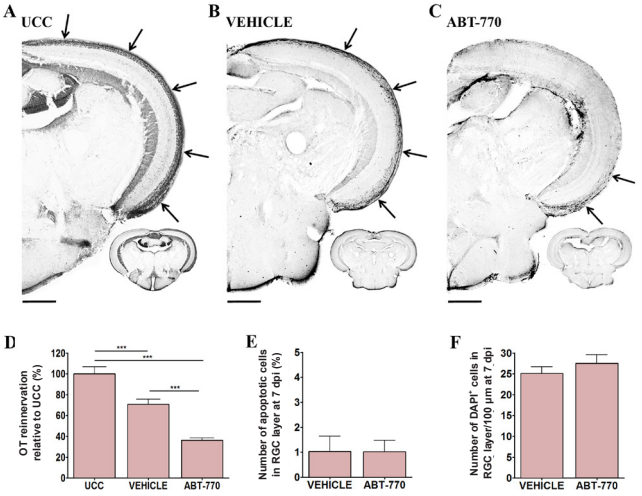

Figure 2 Retinal MMP-2 inhibition after optic nerve crush (ONC) reduces optic tectum (OT) reinnervation, without influencing retinal ganglion cell (RGC) survival.

(A–C) Representative images depicting (re)innervation of the OT (see arrows) by RGC axons in UCC fish (A) and at 7 dpi after repeated vehicle (DMSO) (B) or ABT-770 (C) treatment. Scale bar: 200 µm. (D) Quantification of the area covered by RGC axons in the OT, reveals a clearly diminished reinnervated OT area after Mmp-2 inhibition as opposed to vehicle-injected and UCC fish. Vehicle- and ABT-770-treated fish respectively show ~70% and ~35% reinnervation of the OT relative to the fully innervated OT (100%) in the UCC condition. Data are represented as mean ± SEM, $n = 8$, 10 and 13 animals respectively for the UCC, vehicle and ABT-770 condition over three independent experiments (***$P < 0.001$). (E) Quantitative analysis of activated Caspase-3$^+$ cells in the RGCL reveals no difference between vehicle and ABT-770 treated fish at 7 dpi. (F) Likewise, the number of 4′,6-diamidino-2-phenylindole-positive (DAPI$^+$) cells per 100 µm of RGCL was similar after vehicle and ABT-770 treatment, confirming that no remarkable cell loss is induced after Mmp-2 inhibition. Data are shown as mean ± SEM, $n = 5$ per condition. DMSO: Dimethyl sulfoxide; dpi: days post-injury; RGCL: RGC layer; UCC: uncrushed control.

to increased glial scarring. Furthermore, immature astrocytes, which produce MMP-2, were significantly less able to cross an artificial inhibitory proteoglycan rim when MMP-2 was inhibited (Verslegers et al., 2013). Also olfactory ensheating cell (OECs) grafts, which are known to express very high levels of MMP-2, have been reported to promote adult CNS regeneration in mammals, most likely *via* induction of CSPG degradation. Indeed, CSPG levels present in scar tissue strongly decreased after OEC transplantation in damaged rat spinal cords, suggestive for a role for MMP-2 in CSPG cleavage (Pastrana et al., 2006). Lastly, administration of MMP-2 to dissociated adult rat RGCs promoted axonal regeneration and reduced the amount of CSPGs present in their perineuronal nets. These data then also suggest that degradation of inhibitory CSPGs in a local inhibitory ECM environment is one of the mechanisms through which MMP-2 stimulates axonal outgrowth in adult neurons (Pastrana et al., 2006). Overall, during mammalian CNS repair, gelatinases, and MMP-2 in particular, are predominantly recognized as key players in suppressive environment neutralization, thereby clearing the path for axons to regrow.

Compared to mammals, adult zebrafish can functionally regenerate axons in the injured CNS, due to an increased expression of growth- and pathfinding-associated genes and an environment containing less inhibitory, but more axonal regrowth-promoting molecules. Strikingly, the signaling pathways underlying CNS regeneration in zebrafish and mammals seem conserved (Becker and Becker, 2014). Therefore, zebrafish are frequently used as a model organism to identify pro-regenerative molecules for the injured mammalian CNS. In these fish, RGCs typically survive after optic nerve crush (ONC), regrow long-distance axons and re-establish synaptic contacts with their target neurons in the OT, all within three weeks post-injury (Becker and Becker, 2014). To provide initial insights in molecules involved in zebrafish optic nerve regeneration, RT-PCR and microarray studies were performed on the regenerating zebrafish eye at different time points after ONC. Interestingly, a temporal correlation was shown between the expression of four specific MMPs (*mmp-2*, *-9*, *13a* and *-14*) and different phases of retinotectal regeneration (McCurley and Callard, 2010). Recently, our lab confirmed the dynamic expression pattern of those four specific MMPs in the injured zebrafish retina at protein level, implicating them in axonal regrowth and inner retina remodeling after ONC. Moreover, and identical to their known role in optic system development, MMPs were proven to be *in vivo* regulators of RGC axonal regrowth in adult zebrafish, since

retinal broad-spectrum MMP inhibition after ONC significantly reduced OT reinnervation, without influencing RGC survival (Lemmens et al., 2015). Notably, expression studies revealed significantly upregulated Mmp-2 protein levels in growth-active RGCs and regrowing axons at the level of the retina, but not in retinal or ON macroglia, during zebrafish retinotectal regeneration (**Figure 1A**, right panel) (Lemmens et al., 2015). Since these data suggested Mmp-2 as an important regulator of RGC axonal regrowth, we repeatedly administered ABT-770 - a gelatinase inhibitor reported to be 30-fold more specific for MMP-2 than for MMP-9 (Curtin et al., 2001) - to the zebrafish retina after ONC. Thereto, we either intravitreally injected the potent MMP-2 inhibitor (5 mM ABT-770 (Abbott Laboratories) or its vehicle (5% DMSO) into the zebrafish eye at 1, 3, 4 and 6 days post-injury (dpi). At 7 dpi, axons were anterogradely traced with biocytin and axonal regeneration was quantified at the level of the contralateral OT as previously described (Lemmens et al., 2015). Of note, uncrushed control (UCC) fish, in which OT innervation was analyzed and set as a 100% reference value, were included. Similar to previous observations in our lab, about 70% of the OT of vehicle-injected fish was reinnervated at 7dpi as compared to UCCs, indicating that axonal regeneration was well advanced at one week after ONC (**Figure 2A**, **B** & **D**) (Lemmens et al., 2015). A 50% decrease in tectal reinnervation was observed in fish treated with ABT-770 as compared to vehicle-injected fish (**Figure 2B–D**). Notably, activated Caspase-3 stainings on retinal sections at 7 dpi, did not unveil any difference in the percentage of apoptotic RGCs between vehicle and ABT-770 injected zebrafish. Furthermore, an equal number of cells, visualized by the nuclear marker 4',6-diamidino-2-phenylindole (DAPI), was observed in the RGC layer. This excludes that the diminished OT reinnervation after MMP-2 inhibition is due to apoptotic effects on retinal neurons (**Figure 2E**, **F**). Altogether, these data suggest that lowering Mmp-2 activity in the retina after ONC specifically inhibits RGC axonal regrowth. Importantly, since zebrafish barely have an inhibitory environment after injury, our data suggest a novel, neuron-intrinsic role for MMP-2 in axonal regrowth that is distinct from breaking down environmental barriers, as deducted from various mammalian studies (**Figure 1A**, right panel) (Verslegers et al., 2013; Becker and Becker, 2014). Notably, our observation, which implies a similar role for MMP-2 in zebrafish retinotectal regeneration as during development of retinofugal projections, should not come as a surprise since it is assumed that successful regeneration partly recapitulates molecular mechanisms that are at play during neural development. However, the exact working mechanism of MMP-2 and its underlying targets in zebrafish RGC axonal growth and regeneration remain largely elusive.

Overall, manipulation of MMP signaling pathways holds possible therapeutic potential for mammalian CNS repair. However, as MMPs are widely reported as Yin/Yang players in CNS (patho)physiology, their activity needs to be well controlled. Therefore, a targeted delivery of MMP inhibitors/activators and full understanding of the biological processes in each disease condition seems essential to successfully influence MMP functioning in optic neuropathies (Vandenbroucke and Libert, 2014). Consequently, proteomics approaches to identify MMP underlying pro-regenerative targets crucial for mouse and zebrafish optic nerve regeneration are highly needed and will likely pinpoint important underlying molecules and pathways, which can, after appropriate manipulation, result in successful optic nerve regeneration. We do want to emphasize that, despite an overall conservation of signaling pathways underlying regeneration, glial scar production seems negligible in injured adult zebrafish (Becker and Becker, 2014). As the ultimate goal is to induce optic nerve regeneration and visual repair in mammals, we then also believe in complementary research, combining pro-regenerative targets identified from omics studies in both fish and mammals. In case of MMP-2, and based on the research described above, zebrafish would be useful to identify underlying intrinsic growth-promoting targets, while mammals would primarily serve to characterize its key players in inhibitory environment clearance (**Figure 1A**, **B**, both right panel).

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