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Original Research

Macrophage Migration Inhibitory Factor Promotes Expression of Matrix Metalloproteinases 1 and 3 in Spinal Cord Astrocytes following Gecko Tail Amputation

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Abstract

Background: The matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that play a variety of physiological and pathological roles in development, remodeling of tissues and diseases, mainly through degradation of various components of the extracellular matrix (ECM). Particularly, the MMPs have increasingly been found to mediate neuropathology following spinal cord injury (SCI). Proinflammatory mediators are potent activators of the MMPs. However, how the spinal cord regenerative vertebrates circumvent MMPs-mediated neuropathogenesis following SCI remains unclear. **Methods**: Following the establishment of gecko tail amputation model, the correlation of MMP-1 (gMMP-1) and MMP-3 (gMMP-3) expression with that of macrophage migration inhibitory factor in gecko (gMIF) was assayed by RT-PCR, Western blot and immunohistochemistry. Transcriptome sequencing of primary astrocytes was performed to analyze the intracellular signal transduction of macrophage migration inhibitory factor (MIF). The effects of MMP-1 and MMP-3 induced by MIF on astrocyte migration were assessed by transwell migration assay. **Results**: The expression of gMIF significantly increased at lesion site of the injured cord, in parallel with those of gMMP-1 and gMMP-3 in the gecko astrocytes (gAS). Transcriptome sequencing and *in vitro* cell model revealed that gMIF efficiently promoted the expression of gMMP-1 and gMMP-3 in gAS, which in turn contributed to the migration of gAS. Inhibition of gMIF activity following gecko SCI remarkably attenuated astrocytic expression of the two MMPs, and further influenced gecko tail regeneration. **Conclusions**: Gecko SCI following tail amputation promoted production of gMIF, which induced the expression of gMMP-1 and gMMP-3 in gAS. The gMIF-mediated gMMP-1 and gMMP-3 expression was involved in gAS migration and successful tail regeneration.

Keywords: MIF; astrocytes; MMP-1; MMP-3; spinal cord; reptile; regeneration

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in proteolytic degradation of extracellular matrix (ECM) including collagen, fibronectin and proteoglycan. Also, MMPs can liberate biologically active proteins such as cytokines, growth factors, chemokines and several proproteinases from their inert proforms [1,2]. These significant properties of MMPs endow them with a variety of regulatory roles in physiological and pathological processes, such as in tissue remodeling and organ development, in regulation of wound healing, tissue regeneration, angiogenesis, inflammation, as well as tumor progression [3–7]. The activity of MMPs is closely regulated by transcription level, pro-MMP activation, and inhibition by endogenous inhibitors [8]. Imbalance of the homeostatic regulation of MMPs will results in uncontrolled ECM related diseases, such as tissue ulceration, pulmonary disease, nephritis, arthritis, cardiovascular disease, cancer and neurological disorders [9–12]. To date, at least 28 different types of MMPs have been identified and categorized into four classes according to their substrate specificity, the collagenases, the gelatinases, the stromelysins and a heterogeneous group [13]. All of them consist of a pro-peptide, a catalytic domain, and a hemopexin domain that is linked to the catalytic domain *via* a flexible hinge region [14]. Only after proteolytic removal of the pro-domain or chemical modification of the cysteine residue in the pro-peptide, the MMPs become proteolytically active and mediates turnover of ECM [2].

MMP-1 mainly degrades types I, II and III collagens, but also participates in hydrolyzing other ECM molecules and soluble proteins [15,16]. Like other MMPs, MMP-1 plays an important role in development, tissue morphogenesis and wound repair [17,18]. Generally, MMP-1 maintains at a low level in physiological conditions, and is dramatically induced in response to pathological conditions such as oxidative stress, inflammatory stimulation and tissue remodeling [19]. The abnormal activity of MMP-1 is closely associated with the occurrence of tumors, arthritis, emphy-

sema and fibrosis [20,21]. In the central nervous system (CNS), MMP-1 is involved in neurodegenerative diseases, such as Alzheimer's disease (AD), and Amyotrophic Lateral Sclerosis (ALS) [22]. Inflammatory cytokines have been shown to induce up-regulation of MMP-1 in astrocytes, contributing to neuronal apoptosis and changes in functional circuits [23,24]. It is interesting to note that MMP-1 favors spinal cord regeneration of amphibians by promoting the migration of ependymal cells [18]. Therefore, MMP-1-mediated pathological function in CNS is species-specific across phylogeny. Another family member MMP-3 belongs to the stromelysin-subgroup that can activate various growth factors, cell adhesion molecules, cytokines, chemokines and receptors other than the ECM [6]. In addition, MMP-3 exhibits proteolytic activity towards other subfamily members including pro-MMP-1, -3, -7, -8, -9 and -13, as well as its upstream activators, such as fibrinogen and urokinase plasminogen activator [25,26]. Similarly, MMP-3 not only participates in body development and tissue repair, but also plays multiple pathophysiological roles including tumorigenesis, osteoarthritis, rheumatoid arthritis and other diseases [10,27]. After CNS insults, MMP-3 is inducibly expressed by damaged neurons, astrocytes, oligodendrocytes, microglia/macrophages, and vascular endothelial cells [28]. The dysregulated MMP-3 aggravates a variety of neurodegenerative diseases by disrupting the blood-brain barrier (BBB), promoting apoptosis of neurons and oligodendrocytes, or stimulating excessive inflammatory responses [29,30]. Conversely, MMP-3 promotes neuronal migration, neurite outgrowth and myelinogenesis in the developing CNS [25].

Proinflammatory cytokines including TNF-α, IL-1β and IL-6 can induce the production of MMPs, which in turn activate tissue inflammation [25–27,31]. Such positive feedback loop of inflammatory signaling makes the MMPs become the hub regulators in many inflammation-related diseases [31,32]. Injury to the CNS elicits a severe inflammation, resulting in activation of MMPs [33]. As a critical proinflammatory cytokines of injured spinal cord, macrophage migration inhibitory factor (MIF) is inducibly expressed within neurons, microglia, astrocytes, ependymal cells, and epithelial cells of the choroid plexus [34–36]. The upregulation of MIF at lesion site of cord has been shown to mediate neuronal apoptosis and promote the production of inflammatory mediators from astrocytes, suggesting a key role of MIF in the neuropathogenesis of the injured spinal cord [37,38]. Whether MIF can regulate the astrocytic production of MMP-1 and MMP-3, thereby mediates neuropathology remains unclear.

Unlike mammals, several vertebrates including fish, amphibian and lizards can regenerate spinal cord following injury [39]. The regenerative process is driven under restricted inflammation and astrocyte reactivity [40–42]. Axonal elongation is observed along a "glial bridge" built by a population of glial cells at the lesion site [43,44]. The oriented migration of astrocytes, however, plays important role in supporting the regeneration of the spinal cord. MIF is also upregulated by the cord injury, but it cannot evoke the inflammation during the spontaneous spinal cord regeneration [45]. In the meantime, the MMPs, including MMP-1 and MMP-3, are found to be involved in mediating migration and differentiation of ependymal cells and blastemal cells [17,46]. These lines of evidence suggest that increased activity of MMP-1 and MMP-3 is necessary for either spinal cord or appendage regeneration [18]. Given that MIF has been shown to closely associate with the malignant progression of a variety of human carcinomas by regulation of several MMPs, it is therefore assumed that MIF may promote expression of MMP-1 and MMP-3 to mediate astrocytic migration during the spontaneous spinal cord regeneration [47]. In the present study, *Gekko japonicus* was used as the experimental SCI model to investigate the regulatory function of MIF on MMP-1 and MMP-3 in astrocytes. Our results demonstrated that MIF efficiently induced the expression of MMP-1 and MMP-3 in astrocytes and promoted cell migration after gecko SCI. MIF inhibitor 4-IPP significantly inhibited regeneration of amputated tail, which might provide an important clue for understanding the distinct function of MMP-1 and MMP-3 in tissue repair among different species.

2. Materials and Methods

2.1 Animals

Adult *Gekko japonicus* was used as experimental models as described by Dong *et al.* [48]. Geckos were housed in the room with humidity- and temperature-controlled and fed with water and mealworms (22–25 °C). To mimic the autotomy in the natural environment, gecko tail amputation was performed at the sixth caudal vertebra according to the unique body structure [49]. The animals were anesthetized by freezing on ice before sacrifice. The number of gecko subjected to amputation was calculated by six per experimental group in triplicate. All experimental protocols were approved by the Animal Ethics Committee of Nantong University.

2.2 Drug Treatments

Injections of 4 μL of 40 nM MIF inhibitor 4-IPP (Sigma-Aldrich, St. Louis, MO, USA) or 0.1% DMSO (Sigma-Aldrich) were performed using a Hamilton syringe (Hamilton Co., Reno, NV, USA) at the injury sites following gecko tail amputation. The regenerating tails were photographed at 3 and 14 d, respectively.

2.3 Sequence Analysis of gecko MMP-1 and MMP-3

The amino acid sequences of gecko MMP-1 (gMMP-1) and MMP-3 (gMMP-3) were obtained from the National Center for Biotechnology Information [50]. MegAlign program with the Clustal X 2.0 (http://www.clustal.org/clustal2/) method was used for alignment of multiple protein sequences [51].

2.4 Cell Culture

Astrocytes (gAS) were isolated and cultured from the spinal cord of adult gecko according to previously described methods [52]. Briefly, the cells were dissociated using 0.25% trypsin (Gibco-BRL, Grand Island, NY, USA) for 20 min at 30 °C, and the suspension was then centrifuged at 1000 rpm for 5 min, before cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY, USA), 1% penicillin/strepto-mycin at 30 °C in 5 % CO_2. A monolayer of astrocytes was obtained nearly 12–14 days after plating in the culture flasks. Third or fourth passage cells were prepared for the experiments. Astrocytes were identified by the staining of the astrocytic marker glial fibrillary acid protein (GFAP, Abcam, Cambridge, MA, USA).

2.5 Sequencing of mRNA

Total RNA of gAS stimulated with 2.5 μg/mL recombinant gMIF for 0 h and 12 h, was extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The library for RNA-seq analysis was constructed by using the Illumina TruSeq RNA sample Prep Kit v2 and sequenced by the Illumina HiSeq-2000. High-quality reads that passed the Illumina quality filters were kept for the sequence analysis. Using the SOAP program (BGIShenzhen, Shenzhen, China), clean reads were mapped to the reference genomes (Assembly Gekko_japonicus_V1.1 and RGSC Genome Assembly v6.0) and gene sequences. An average read depth of 30$\times$ per sample was achieved, and all samples were analyzed in triplicate.

2.6 Bioinformatics Analysis

Differentially expressed mRNA was designated in criteria of greater or less than twofold changes in comparison with control. Function of genes was annotated by Blastx against the NCBI database or the AGRIS database (http://arabidopsis.med.ohio-state.edu/downloads.html) with E-value threshold of 10^{-5}. Gene ontology (GO) classification was obtained by WEGO (http://wego.genomics.org.cn/cgibin/wego/inde-x.pl) *via* GO id annotated by Perl and R program. For all heatmaps, genes were clustered by Jensen–Shannon Divergence. The reconstructed gene networks were created using IPA on the basis of differentially expressed genes in gMIF-stimulated gAS.

2.7 Western Blot

Proteins were extracted from 0.5 cm gecko cord segments above the amputation site or from cultured cells with RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration of each sample was detected by BCA kit (Beyotime). The extracts were heat-denatured at 95 °C for 5 min, electrophoretically resolved by 10% SDS-PAGE, and transferred to PVDF membranes. The membranes were incubated with primary antibodies in TBS buffer at 4 °C for 18 h, followed with a HRP-conjugated secondary antibody (1:1000, Proteintech Group, Inc., Chicago, IL, USA) at room temperature for 2 h. The signal of HRP activity was detected using an ECL detection kit (Vazyme, Nanjing, Jiangsu, China). Antibodies used in Western blot were: β-actin (1:5000, Proteintech), Erk1/2 (Cell Signaling Technology, Danvers, MA, USA), p-Erk1/2 (Cell Signaling Technology), SAPK/JNK (Cell Signaling Technology), p-SAPK/JNK (Cell Signaling Techno-logy), P38 (Cell Signaling Technology), and p-P38 (Cell Signaling Technology). The image was scanned with a Tanon-5200 Chemiluminescent Imaging System (Tanon, Shanghai, China), and the data were analyzed using PDQuest 7.2.0 software (Bio-Rad, Hercules, CA, USA).

2.8 Real-Time Quantitative PCR

Total RNA was prepared with Trizol (Thermo Fisher Scientific, Waltham, MA, USA) from gecko cord segments or cultured cells as mentioned above. The first-strand cDNA was synthesized using HisScript II Q Select RT SuperMix for qPCR (R223-01, Vazyme, Nanjing, China) in a 20-μL reaction system that contained 2 μg total RNA. The cDNA was diluted 1:6 before use in the quantitative PCR (qPCR) assays. Two pairs of primers were designed based on the genome sequences with sense primer 5'- CAA GCC TGC CAT CTG GAA TAG-3' and anti-sense primer 5'- TAG CCA CGT ACA ATG TCA TAG C -3' for gMMP-1, sense primer 5'- TCTGACACTTGGGGGTCTCT -3' and anti-sense primer 5'- TTTCTCCTCGGAAAGTGGCG -3' for gMMP-3. Reactions were performed in a final volume of 20 μL according to protocol of ChamQ^{TM} SYBR qPCR Master Mix kit (Q711-02, Vazyme, Nanjing, China). The LightCycler96 software (Roche, Basel, Switzerland) was used for real-time PCR analysis. At the end of each PCR run, data were automatically analyzed by the system.

2.9 Immunohistochemistry

The transverse sections from the spinal cord segments or the cultured cells were incubated with S100β antibody (1:500 dilution, Sigma-Aldrich, St. Louis, MO, USA), MMP-1 antibody (1:200 dilution, Abcam, Cambridge, MA, USA), MMP-3 antibody (1:200 dilution, Abcam), CD74 antibody (1:200 dilution, Bioss, Woburn, MA, USA), or GFAP antibody (1:200 dilution, Abcam) at 4 °C for 24 h. Then, the samples were further incubated with Cy3-labeled goat anti-rabbit or anti-mouse IgG (1:400 dilution, Abcam, ab6939, ab97035), or FITC-labeled donkey anti-mouse or anti-rabbit IgG (1:400 dilution, Abcam, ab150105, ab150073) at 4 °C for 18 h. The images were obtained by using Leica TCS SP5 confocal laser scanning microscope system (Leica, Heidelberg, Germany).

2.10 Transwell Migration Assay

The migration of gAS was assayed in triplicates using 24-well transwell chambers with 8 μm pores (3422, Corn-

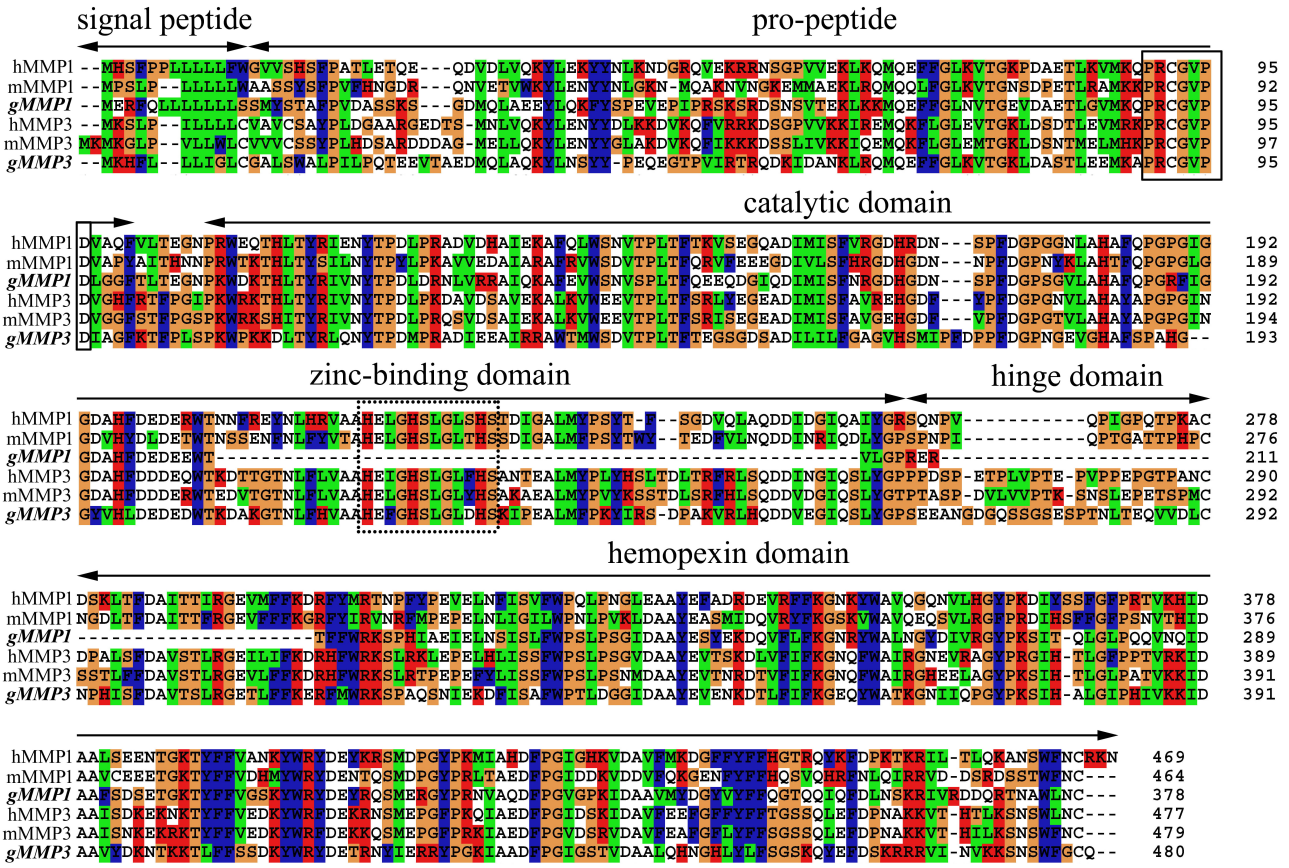

Fig. 1. Sequence analysis of gMMP-1 and gMMP-3. Multiple alignment of amino acid sequences of gMMP-1 and gMMP-3 with the sequences of other representative vertebrates. Gaps introduced into sequences to optimize alignment are represented by dashes. Signal peptide, Pro-peptide, Catalytic domain, Cysteine Switch motif, Zinc-binding domain, Hinge domain and Hemopexin domain are indicated. Sequences obtained from GenBank: gecko-MMP-1 (XP_015277097), mouse-MMP-1 (NP_032633.1), human-MMP-1 (AIC54763.1), gecko-MMP-3 (XP_015277100), mouse-MMP-3 (NP_034939.1) and human-MMP-3 (NP_002413.1).

ing, NY, USA) as previously described [48]. A total of 100 μL of gAS (3×10^{-5} cells/mL) with or without siRNA knockdown for 48 h was transferred to the upper chambers at 30 °C in 5 % CO_2. Furthermore, 1.5 μg/mL recombinant gMIF was added into the lower chambers for 24 h. Cells adhering to the bottom surface of the membranes were fixed and stained with 0.1% crystal violet. Each chamber was imaged under a DMR inverted microscope (Leica Microsystems, Leica, Heidelberg, Germany).

2.11 Statistical Analysis

Differences between groups were analyzed by one-way analysis of variance with SPSS 23 software (SPSS, Chicago, IL, USA). Normality and homoscedasticity of the data were verified using Levene's test before statistical analysis. $p < 0.05$ was considered statistically significant.

3. Result

3.1 Characteristic Analysis of gMMP-1 and gMMP-3 Sequence

To understand the physiological functions of MMP-1 and MMP-3 in gecko after tail amputation, we analyzed the structural characteristics of gMMP-1 (GenBank: XP_015277097) and gMMP-3 (GenBank: XP_015277100) amino acids sequence. The gMMP-1 is composed of 378, while gMMP-3 480 amino acids (Fig. 1). Both matrix metalloproteinases contain a signal peptide, a pro-peptide with a cysteine switch, a catalytic domain and a hemopexin domain, suggesting the conserved primary structure of MMPs in the vertebrates (Fig. 1).

3.2 Dynamic Changes of gMMP-1 and gMMP-3 Expression in the Injured Spinal Cord following Gecko Tail Amputation

To clarify the potential roles of gMMP-1 and gMMP-3 in the injured spinal cord, a 0.5-cm cord segment at lesion site was collected at 0 d, 1 d, 3 d and 7 d after gecko tail amputation. The expression of *gMMP-1* and *gMMP-3*

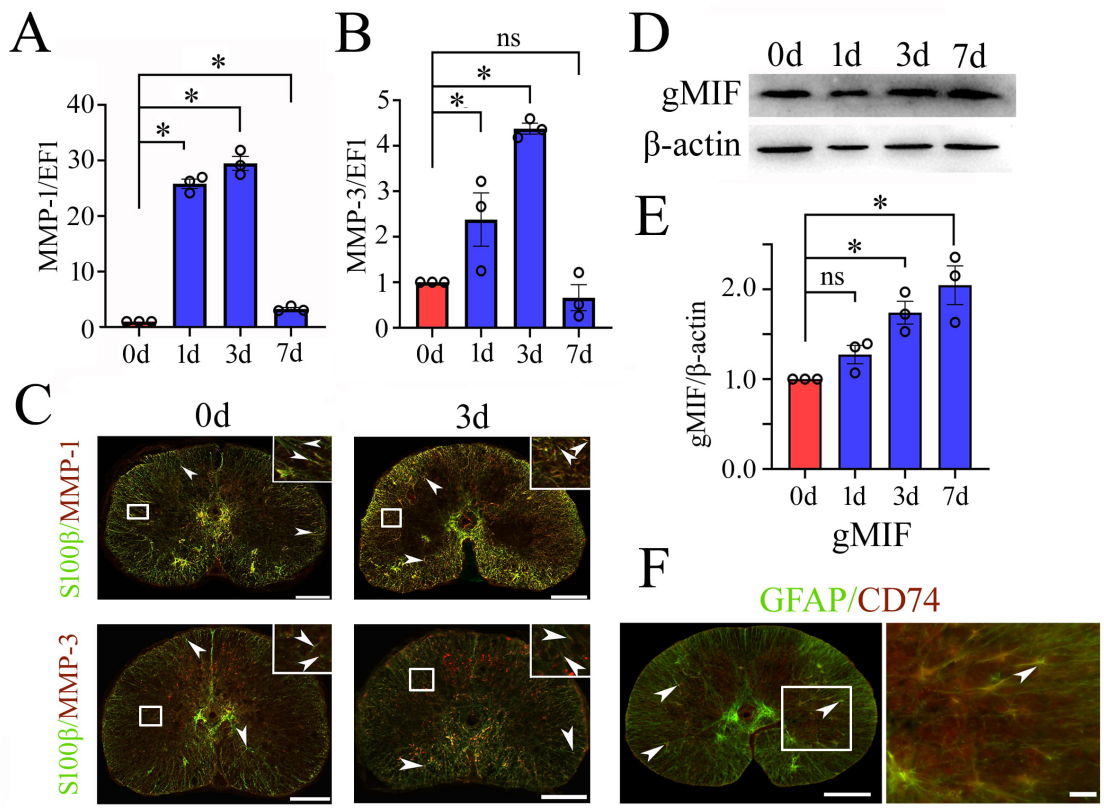

Fig. 2. Determination of gMMP-1 and gMMP-3 in the injured spinal cord of gecko. (A,B) RT-PCR analysis of *gMMP-1* and *gMMP-3* in the 0.5 cm segments of the injured cord following gecko tail amputation (n = 6) at 0 d, 1 d, 3 d and 7 d, respectively. (C) Immunofluorescence showed the colocalization of gMMP-1 (red) with S100β-positive astrocytes in the injured spinal cord following gecko tail amputation at 0 d and 3 d (n = 5). (D) Western blot analysis for gMIF in the 0.5 cm segments of the injured cord following gecko tail amputation (n = 6) at 0 d, 1 d, 3 d and 7 d respectively. (E) Statistical analysis of (D). (F) The colocalization of CD74 (red) with GFAP-positive astrocytes detected by immunofluorescence in the gecko spinal cord. The rectangle indicates the region magnified. Arrowheads indicate the colocalized signals. Experiments were performed in triplicate. Data are expressed as mean $\pm$ SEM; *$p < 0.05$. Scale bar, 100 μm in (C) and (F), 20 μm in the magnification (F).

was detected by RT-PCR, showing that both MMPs transcription dramatically increased from 1 d with a peak at 3 d, and returned to the control level at 7 d (Fig. 2A,B). Immunostaining was then carried out to observe whether gAS was involved in the production of gMMP-1 and gMMP-3 in response to SCI. Results indicated that gecko SCI significantly induced the astrocytic expression of gMMP-1 and gMMP-3, as were analyzed by the colocalization of S100β-positive astrocytes with anti-gMMP-1 or anti-gMMP-3 antibody at 0 d and 3 d following tail amputation (Fig. 2C).

MMPs can be induced by various inflammatory cytokines including TNF-α and IL-1β in multiple cell types [53–57], which however were not inflamed by the gecko SCI [42]. We thus turned to the link between proinflammatory gMIF, a cytokine involved in the neuropathology, with the activation of gMMP-1 and gMMP-3 in astrocytes. Western blot analysis showed that the protein levels of gMIF were temporally increased at lesion site of the cord (Fig. 2D). As MIF initiates intracellular signal transduction through interaction with its cell membrane receptor CD74 [58], immunostaining was performed to examine the expression of CD74 in the astrocytes of the spinal cord, Results revealed that CD74 constitutively colocalized with GFAP-positive astrocytes (Fig. 2F). The data indicate that gMIF may be implicated in the inducing astrocytic expression of gMMP-1 and gMMP-3 following gecko SCI.

3.3 The gMIF is Efficient in Promoting Expression of gMMP-1 and gMMP-3 in Primary gAS

To validate the regulatory roles of gMIF on the astrocytic activation of gMMP-1 and gMMP-3, the primary gAS was cultured with purity over 95% (Fig. 3A,B). Transcriptome sequencing (RNA-Seq) was then performed following gAS stimulation with 2.5 μg/mL gMIF for 12 h. GO analysis of the differentially expressed genes (DEGs) demonstrated gMIF-mediated biological process of gAS was relevant to regulation of cell migration and chemotaxis (Fig. 3C). These DEGs displayed dynamic alteration as shown by Heatmap and cluster dendrogram, among which gMMP-1 and gMMP-3 were found to be significantly in-

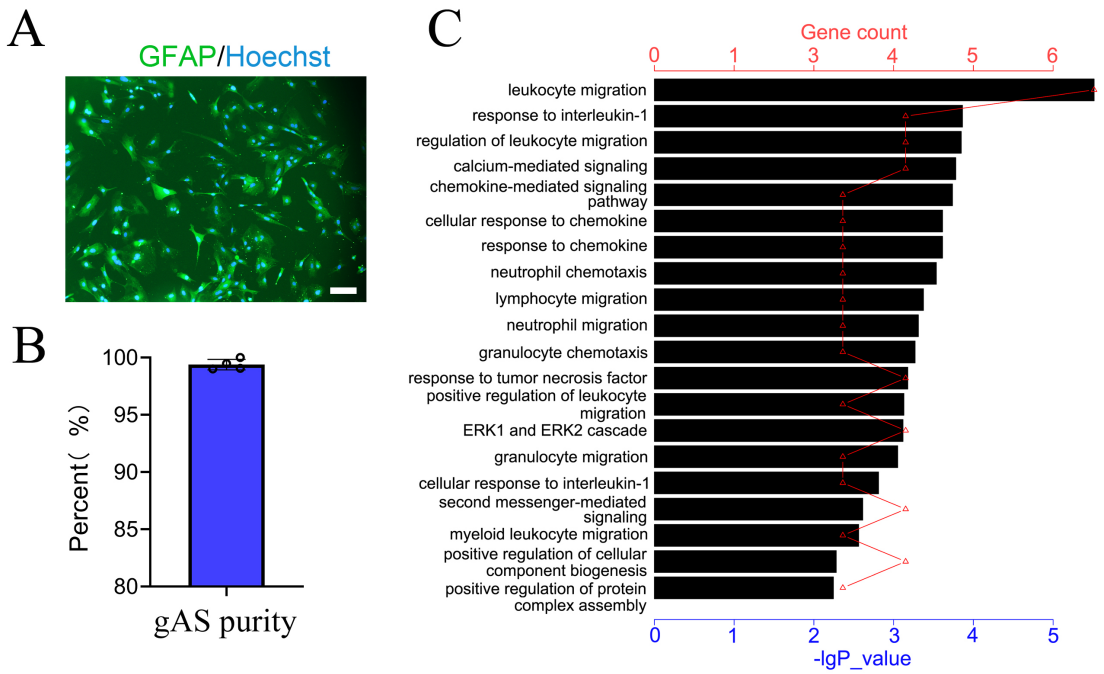

Fig. 3. Transcriptome sequencing analysis of gAS following stimulation with 2.5 μg/mL gMIF for 12 h. (A) Primary cultured gecko astrocytes stained with GFAP and Hoechst 33342. (B) Statistical analysis of primary astrocyte purity. (C) GO terms of DEGs in gAS following stimulation with or without 2.5 μg/mL gMIF for 12 h.

duced by gMIF (Fig. 4A). A gene network was constructed by the Ingenuity Pathway Analysis (IPA) using the DEGs, showing that gMMP-1 and gMMP-3 were driven by gMIF (Fig. 4B).

To confirm the inference from RNA-Seq, the expression changes of gMMP-1 and gMMP-3 in gAS were determined by RT-PCR following cell exposure to 0–2.5 μg/mL recombinant gMIF for 24 h. The primary gAS was examined to constitutively express CD74, gMMP-1 and gMMP-3 (Fig. 5A–C). Once the cells were challenged by gMIF, the transcripts of gMMP-1 and gMMP-3 remarkably increased in concentration-dependent manner (Fig. 5D,E). However, addition of 40 nM 4-IPP, the inhibitor of MIF, obviously attenuated such effects (Fig. 5F,G). These data suggest that gMIF significantly promotes the expression of gMMP-1 and gMMP-3 in gAS.

3.4 Interference of gMMP-1 and gMMP-3 Inhibits the Migration of gAS

To explore the roles of gMIF-mediated gMMP-1 and gMMP-3 expression on the cell event of gAS, the gMMP-1 or gMMP-3 was knocked down by siRNA in the presence of gMIF. The siRNA oligonucleotides gMMP-1-s3 and gMMP-3-s2 with the highest efficiency for interference were accordingly selected (Fig. 6A,B). The cells were transfected with gMMP-1-s3 or gMMP-3-s2 oligonucleotides for 48 h, followed by stimulation with 1.5 μg/mL recombinant gMIF for 24 h. Results demonstrated that gMMP-1-s3 or gMMP-3-s2 was efficient in decreasing gMIF-induced gMMP-1 or gMMP-3 expression (Fig. 6C,D). Subsequently, transwell assays were performed to examine the influence of gMMP-1 and gMMP-3 on the migration of gAS. The results showed that knockdown of gMMP-1 and gMMP-3 significantly attenuated the migratory effects of gMIF (Fig. 6E,F). The data indicate that gMIF-mediated expression of gMMP-1 and gMMP-3 is involved in the migration of gAS.

3.5 The gMIF is Inefficient in Activation of Intracellular MAPKs in gAS

MIF influences a variety of cell behaviors through activation of intracellular MAPKs, such as inflammatory response, cellular survival, and proliferation [59]. To unveil the potential regulatory mechanism of gMIF on the expression of gMMP-1 and gMMP-3, 2.5 μg/mL recombinant gMIF protein was used to stimulate the gAS for 24 h. Results revealed that neither gMIF nor its inhibitor 4-IPP could make any effects on the activation of ERK, P38 and JNK. The data indicate that gMIF-mediated expression of gMMP-1 and gMMP-3 is not governed by MAPKs signaling (Fig. 7A–D).

3.6 Inhibitor of gMIF Attenuates Gecko Tail Regeneration by Decreasing Expression of gMMP-1 and gMMP-3

To clarify the physiological significance of gMIF-regulated expression of gMMP-1 and gMMP-3, a total of 4 μL of 40 nM 4-IPP or 0.1% DMSO was injected into the amputated caudal vertebrae. Immunofluorescence showed

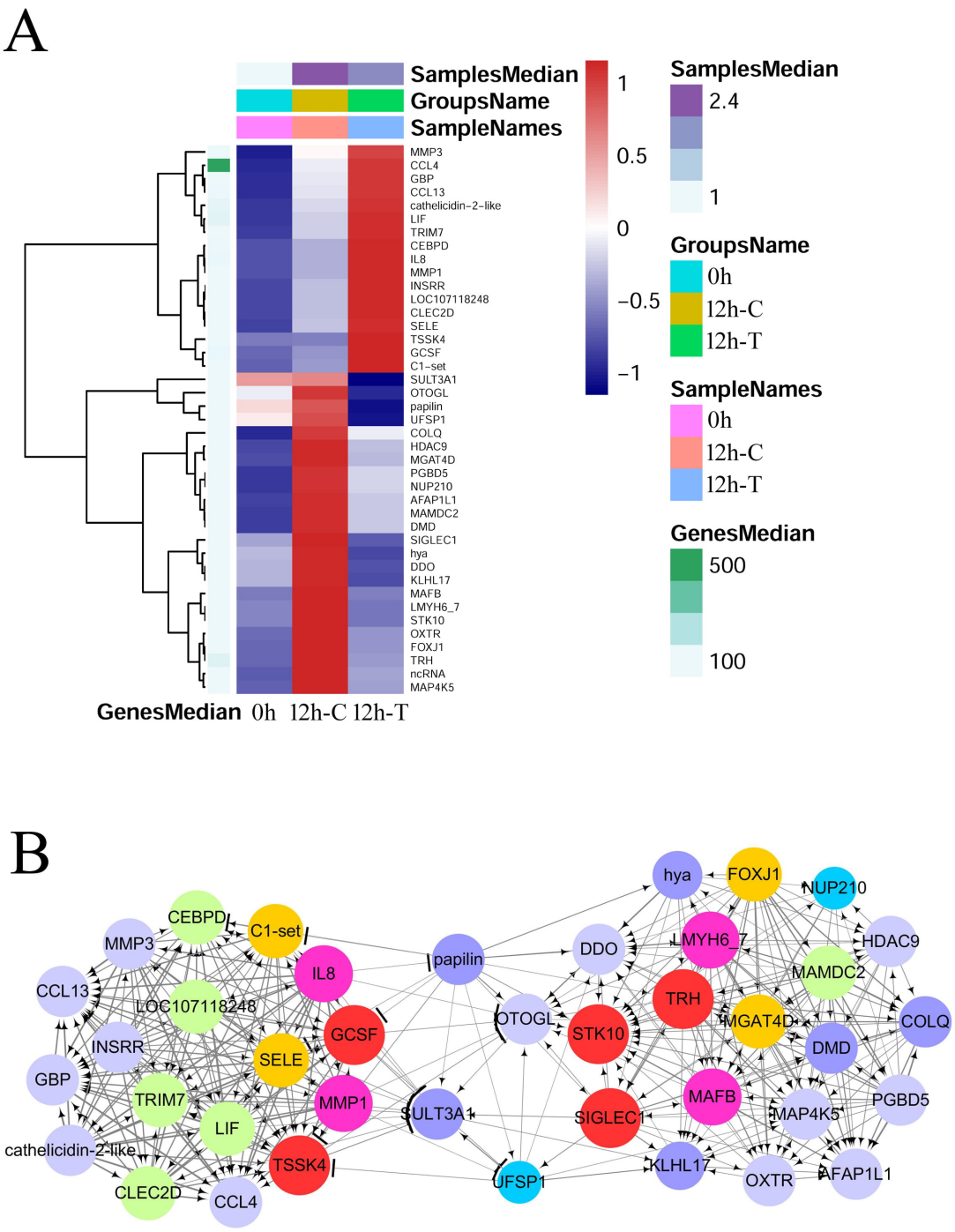

Fig. 4. Heatmap and the inferred gene network of integrated DEGs. (A) Heatmap of integrated DEGs in response to stimulation of 2.5 μg/mL gMIF for 12 h. The color scale shown at the right illustrates the relative expression level of the indicated mRNA across all samples: red denotes expression >0 and blue denotes expression <0. (B) A reconstructed gene network was created using IPA on the basis of integrated DEGs.

that the treatment of 4-IPP significantly suppressed the expression of gMMP-1and gMMP-3 in gAS (Fig. 8A). Meanwhile, the tail regeneration of the animals was attenuated by the inhibitor, as was observed at 3 d and 14 d, respectively (Fig. 8B,C). The data indicate that both gMMP-1 and gMMP-3 induced by gMIF are required for gecko tail regeneration.

4. Discussion

SCI often triggers a series of molecular and cellular events, including disruption of blood spinal cord barrier, activation of excessive inflammation, apoptosis of neurons and oligodendrocytes, and formation of a permanent glial scar [60–62]. These neuropathogenesis severely ham-

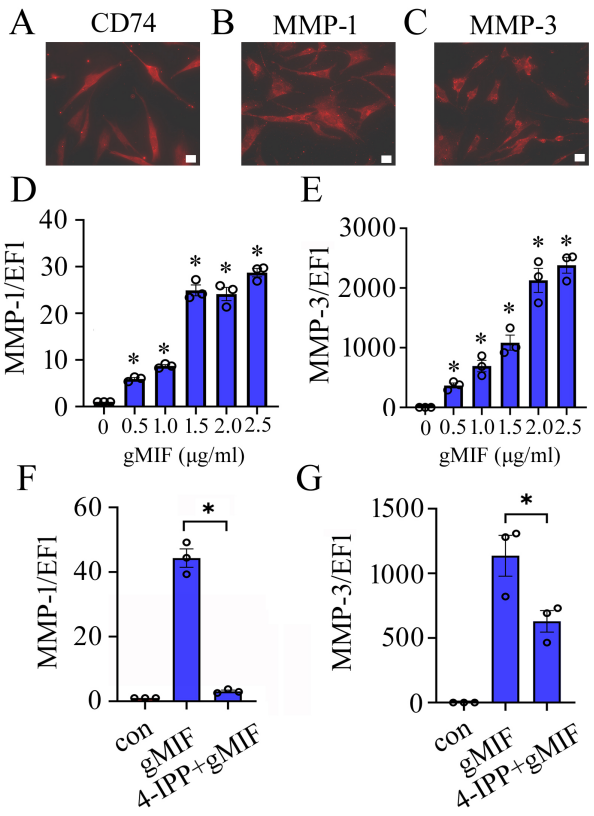

Fig. 5. Examination of gMMP-1 and gMMP-3 expression in astrocytes following treatment with gradient recombinant gMIF. (A–C) Immunostaining showed the expression of CD74, gMMP-1 and gMMP-3 in astrocytes. (D, E) RT-PCR analysis for gMMP-1 and gMMP-3 in astrocytes following stimulation with 0–2.5 μg/mL recombinant gMIF for 24 h. (F, G) RT-PCR shows the expression of gMMP-1 and gMMP-3 following astrocyte treatment with 1.5 μg/mL gMIF in the presence of 100 μM 4-IPP. Experiments were performed in triplicate. Data are expressed as mean ± SEM; *$p < 0.05$. Scale bars, 20 μm in (A–C).

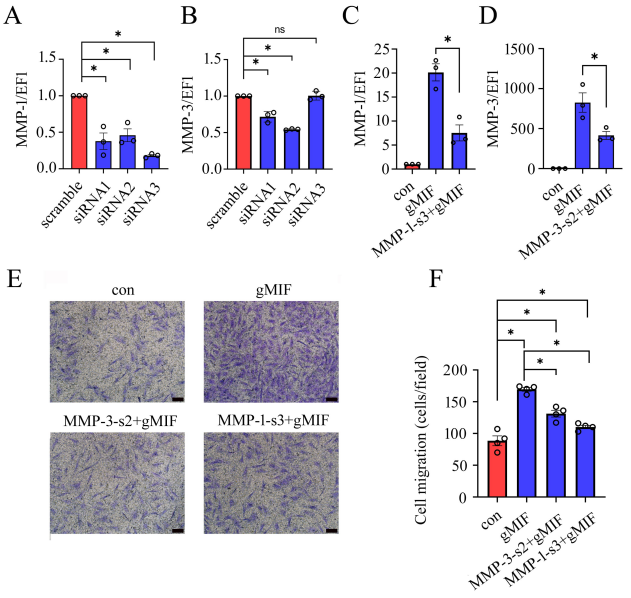

Fig. 6. Effects of gMIF-mediated gMMP-1 and gMMP-3 expression on gAS migration. (A, B) Interference efficiency of three siRNA oligonucleotides for gMMP-1 and gMMP-3 was measured by RT-PCR, respectively. gMMP-1-siRNA3 and gMMP-3-siRNA2 were selected for the knockdown experiments. (C, D) The expression of gMMP-1 and gMMP-3 after siRNA knockdown for 48 h, followed by stimulation with 1.5 μg/mL gMIF for 24 h. (E) Transwell determination of gAS migration after siRNA knockdown for 48 h, followed by stimulation with 1.5 μg/mL gMIF for 24 h. (F) Quantification data as shown in (E). Experiments were performed in triplicates. Error bars represent the standard deviation ($p < 0.05$). Scale bars, 100 μm.

pers the functional outcomes. Damage-associated molecular patterns (DAMPs) are key mediators to initiate and accelerate the degenerative process of the injured spinal cord [22,63]. Lines of evidence demonstrate that DAMPs elicit excessive inflammation by interacting with the pattern recognition receptors (PRRs) on the surface of microglia and astrocytes, thereby contributing to neurotoxicity [64]. MIF is recognized as an important DAMPs molecule following CNS insults. It is immediately elevated after SCI by inducibly expressed in multiple cells including the neurons, oligodendrocytes, microglia, astrocytes and vascular endothelial cells, in association with neuronal apoptosis and demyelination [35,65]. Also, MIF is able to interact with membrane surface receptor CD74 in microglia and astrocytes to activate inflammatory and chemotactic responses [38,66]. D-Dopachrome tautomerase (D-DT), a homolog of MIF, shares a 35% sequence similarity with MIF in human. The tautomerase enzymatic activity of D-DT is ten times less active than MIF [45,67]. Our previous works have shown that gMIF, rather than D-DT, plays roles in promoting the spinal cord regeneration without evoking the excessive inflammation following gecko tail amputation [45,68]. Such functions are completely opposite to what MIF acts in the mammals. Mechanistically, the gMIF-mediated inflammation is either blocked by VAV1 in astrocytes, or transformed to promoting phagocytose of microglia/macrophages [66,68]. In the present study, we have demonstrated that gMIF promoted astrocytic migration by up-regulating the expression of MMP-1 and MMP-3 in astrocytes, suggesting the diverse roles of MIF in promoting the spontaneous spinal cord regeneration.

Astrocytes in response to SCI often become "reactive", undergoing dramatic shifts from morphology to functions, including hypertrophy of soma, reduction of processes, susceptive to inflammatory stimuli and formation of a scar [69]. Originally, a population of reactive astrocytes with a barrier-like structure is mobilized to spatially isolate the diffusion of inflammatory factors and other toxic substances and protect injured nerves [70]. Afterwards, the

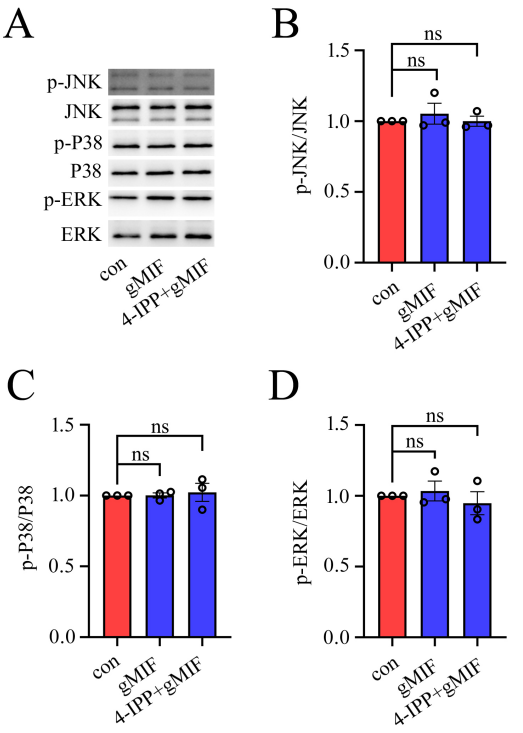

Fig. 7. Effects of gMIF on activation of intracellular MAPKs in gAS. (A) Western blot analysis of p-JNK, p-P38 and p-ERK protein levels following astrocytes stimulation with 2.5 μg/mL recombinant gMIF with or without 4-IPP (40 nM) for 24 h. (B–D) Statistical analysis of (A). Experiments were performed in triplicates. Error bars represent the standard deviation (*$p < 0.05$).

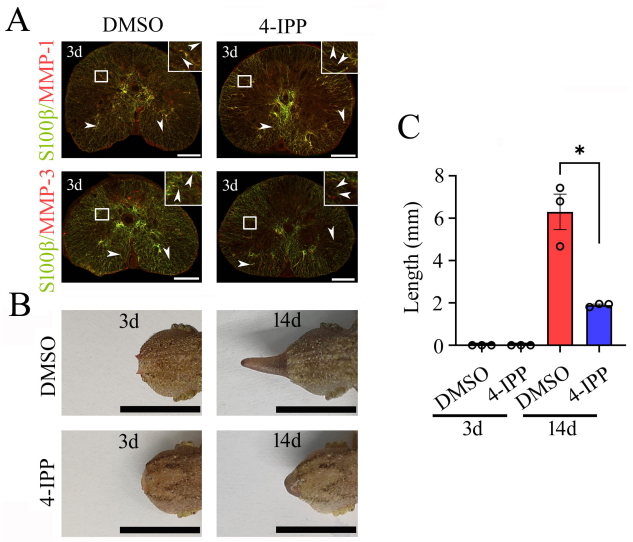

Fig. 8. Inhibitor of gMIF attenuates gecko tail regeneration. **(A)** Immunofluorescence of gMMP-1 and gMMP-3 expression in gAS after cord treatment with 4-IPP or vehicle following gecko tail amputation (n = 6) at 3 d. The rectangle indicates the region magnified. Arrowheads indicate the colocalized signals. (B) Observation of gecko tail regeneration following injection of 4 μL of 40 nM 4-IPP at lesion site at 3 d and 14 d (n = 6). (C) Statistical analysis of (B). Experiments were performed in triplicate. Data are expressed as mean ± SEM; *$p < 0.05$. Scale bars, 100 μm in (A) and 10 mm in (B).

reactive astrocytes form a glial scar that limits the plasticity of axons [71]. In stark contrast, certain vertebrate species, including fish and salamanders, can lead to less astrocyte reaction after SCI, although it is still controversial whether these two species have "true astrocytes" [39]. Glial cells have been found to be arranged along the injured site, creating a "glial bridge" upon which axons can grow across [39]. As a consequence, the oriented migration of astrocytes is essential for the axonal growth and successful spinal cord regeneration. SCI of gecko is similar to those of fish and amphibian in limited inflammation and absence of glial scar [42,52]. In the present study, we showed that gMIF promoted astrocytic migration through regulation of gMMP-1 and gMMP-3, suggesting a beneficial role of astrocytes during the spontaneous spinal cord regeneration.

As important endopeptidases, MMPs mainly degrade the structural components of ECM to participate in many physiological and pathological processes, such as inflammation, wound healing, angiogenesis, cell apoptosis, and tumor invasion [3,72]. Increasing evidence reveals that intracellular MMPs have also several alternative substrates, and play novel biological roles independent of its action on the extracellular matrix. For instance, MMP-2 located in cardiac myocytes is involved in the degradation of troponin to mediate ischemia-reperfusion injury [73]. MMP1, however, has been found to facilitate cell invasion in glioma by intracellular regulation of the MAPK pathway [74]. Similarly, MMP-3 is found in the cytoplasm and nucleus of dopamine neurons and myofibroblasts. Although the indeed substrate is unclear, MMP-3 performs in tumor progression and angiogenesis by inducing the transcription of CTGF gene [75]. In this study, we found that gMIF induced the expression of gMMP-1 and gMMP-3 in astrocytes after gecko SCI. Whether gMMP-1 and gMMP-3 can mediate cell events of astrocytes through intracellular substrates remains to be further studied.

5. Conclusions

Gecko SCI increased the protein levels of gMIF at lesion site, which significantly induced the expression of gMMP-1 and gMMP-3 in the astrocytes. The gMIF-mediated gMMP-1 and gMMP-3 activity contributed to the migration of astrocytes, which in turn were involved in the successful tail regeneration of gecko.

Abbreviations

ANOVA, analysis of variance; MIF, macrophage migration inhibitory factor; DAMPs, damage associated molecular pattern molecules; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate buffered saline; MAPK, mitogen-activated protein kinase; SCI, spinal cord

injury; GFAP, glial fibrillary acid protein; 4-IPP, 4-iodo-6-phenylpyrimidine; IPA, Ingenuity Pathway Analysis Software; Q-PCR, Quantitative real-time polymerase chain reaction.

Author Contributions

YonW and YinW designed this work. HZ and CS performed the experiments. HZ, CS, BH, XZ, HH, YH, AL, YonW and YinW analyzed the data. HZ and YonW joined discussions. YinW wrote the paper. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All animal experiments were approved by the Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee (S20190420-405).

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Conflict of Interest

The authors declare no conflict of interest.

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