FGF-18 protects the injured spinal cord in mice by suppressing pyroptosis and promoting autophagy via AKT-mTOR-TRPML1 axis.

Feida Li  
Wenzhou Medical University

Tingwen Cai  
Wenzhou Medical University

Letian Yu  
Wenzhou Medical University

Haojie Zhang  
Wenzhou Medical University

Yibo Geng  
Wenzhou Medical University

Jiaxuan Kuang  
Wenzhou Medical University

Yongli Wang  
Wenzhou Medical University

Yuepiao Cai  
Wenzhou Medical University

Xiangyang Wang  
Wenzhou Medical University

Hui Xu  
Wenzhou Medical University

Wenfei Ni  
Wenzhou Medical University

Kailiang Zhou  (zhoukailiang@wmu.edu.cn)  
Wenzhou Medical University  https://orcid.org/0000-0001-7795-576X

Research Article

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Abstract

Spinal cord injury (SCI) refers to a severe medical condition with lasting effects. The efficacy of numerous clinical treatments is hampered by its intricate pathophysiological mechanism. FGF-18 has been found to exert neuroprotective effects after brain ischaemia, while its effect after SCI has not been well explored. In the present study, we used a mice model of SCI and found that FGF-18 may significantly affect functional recovery. The present findings demonstrated that FGF-18 directly promoted functional recovery by increasing autophagy and decreasing pyroptosis. In addition, FGF-18 upregulated autophagy, and the well-known autophagy inhibitor, 3-methyladenine (3MA), reversed the therapeutic benefits of FGF-18 on SCI, suggesting that autophagy mediates the therapeutic benefits of FGF-18 on SCI. After stimulation of the AKT-TRPML1-calcineurin signalling pathway, mechanistic analysis revealed that the FGF-18-induced increase in autophagy was mediated by the dephosphorylation and nuclear translocation of TFE3. Together, these findings indicated that FGF-18 is a robust autophagy modulator capable of accelerating functional recovery after SCI, suggesting that it may be a promising treatment for application in the clinic.

Introduction

Spinal cord injury (SCI) commonly leads to critical motor and sensory dysfunctions [1]. SCI not only results in health problems and disabilities for the victims and their families but also places a strain on the health care system and the economy due to lost productivity and excessive medical expenses [2]. Currently, aggressive intensive care approaches, early surgical decompression, corticosteroids, other medications, and physiotherapy rehabilitation are used in clinical treatments to treat SCI [3, 4]. SCI occurs in two stages as follows: 1) the first stage involves destroying the neural parenchyma, disrupting the axonal network, haemorrhaging, and disrupting the glial membrane [5]; and 2) the second stage involves further mechanical and chemical damage to spinal tissues, leading to increases in reactive oxygen species (ROS), inflammation, apoptosis, and programmed cell death (PCD) [5–7]. These modifications cause worse functional recovery after SCI. Inhibiting this damage by concentrating on the mechanisms of PCD, such as pyroptosis and autophagy, may allow effective treatment of SCI [8, 9].

Plasma membrane integrity weakens during pyroptosis, which is a type of cell death resulting from activated inflammasome sensors [10]. Pyroptosis negatively impacts various neurological diseases [11]. ROS accumulation is affected by pathological alterations, including bleeding, hypoxia, and oedema, following spinal cord injury. These ROS function as secondary messengers to trigger NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome-mediated pyroptosis [12, 13]. The NLRP3 inflammasome is a multiprotein complex that causes the activation of Caspase-1, which in turn promotes the maturation and release of IL-1 and IL-18. The NLRP3 inflammasome consists of NLRP3, apoptosis-associated speck-like protein with a caspase recruitment domain (ASC), and Caspase-1 [14]. Thus, inhibition of pyroptosis is a potential direction for SCI treatment.
Every eukaryotic organism conserves the catabolic process of autophagy [15]. Autophagy is also an important effector mechanism of neurological diseases by regulating various types of neuronal cells [16]. It has been demonstrated that after central nervous system (CNS) trauma in various animal models, autophagic flux is blocked [17], which contributes to the accumulation of autophagosomes after reduced lysosomal function in neurons [18]. Neuronal death and damage increase due to autophagy flux blockage, which also disturbs the intracellular environment and triggers an inflammatory response [17–19]. Autophagy prevents NLRP3 inflammasome activation and suppresses IL-1b and IL-18 expression, which prevents pyroptosis [20, 21]. Autophagy helps to inhibit pyroptosis and promote functional recovery after SCI. Fibroblast growth factor 18 (FGF-18) is a member of the fibroblast growth factor (FGF) family [22]. FGF-18 is essential for the bone development, bone growth, blood vessel formation, tumour growth, and other cell development processes [22–24]. According to recent studies, FGF-18 functions as a neuroglial cell stimulator and a brain tissue protector [25, 26]. In addition, previous studies have demonstrated that FGFs partially pass through the blood–brain barrier [27, 28]. FGF-18 has been reported to regulate cell proliferation, reduce apoptosis, and promote autophagy [29, 30]. FGF-18 has been demonstrated to reduce ROS production [31, 32], and ROS generation is often associated with macrophage pyroptosis [33]. Thus, we hypothesized that FGF-18 may potentially inhibit pyroptosis. The potential implications of FGF-18 in the management of SCI have not been previously reported. Furthermore, it is unknown whether FGF-18 blocks pyroptosis by triggering autophagy. Researchers have investigated the impact of FGF-18 on motor function and nerve cells in SCI, and they have suggested that in SCI, FGF-18 may promote the repair of SCI by enhancing autophagy.

Materials And Methods

Animals and Ethics statement

Healthy C57BL/6 mice (female, 6–8 weeks old, and average weight of 20–30 g) were obtained from the Experimental Animal Centre of Wenzhou Medical University (Zhejiang Province, China). All mice were kept in standard housing (21–25°C, 12-hour light/dark cycle, and 50–60% humidity), and they had free access to food and water. The Animal Care and Use Committee of Wenzhou Medical University approved the Guide for the Care and Use of Laboratory Animals of the China National Institutes of Health as the standard for the care and use of experimental animals.

Antibodies and reagents.

The present study used the following reagents: FGF-18 (ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel; CYT-064); pentobarbital sodium, a Masson staining instrument, and a HE staining tool (Solarbio Science & Technology, Beijing, the People’s Republic of China); 3-methyladenine (3MA; Sigma–Aldrich St. Louis, MO, the United States of America; Cat. No. M9281); dorsomorphin (purity of 98.0%; MedChemExpress Monmouth Junction, NJ, the United States of America; Cat. No. HY-18749); AAV-TFE3 shRNA (serotype 9, without fluorescent reporter gene; Shanghai Genechem Co., Ltd.); NE-PER™ Nuclear and Cytoplasmic Extraction Reagent and BCA instruments (Thermo Fisher Scientific; Rockford, IL, United
States of America); Beclin1, NLRP3, AKT, p-AKT, p-mTOR, and mTOR primary antibodies (Cell Signaling Technology; (Beverly, MA, United States of America; Cat. Nos. 3738, 15101, 9272, 4058, 2983, and 5536, respectively); VPS34, CTSD, CASP1, histone H3, and GAPDH antibodies (Proteintech; Chicago, IL, United States of America; Cat. Nos. 12452-1, 21327-1, 22915-1, and 10494-1, respectively); ASC, IL-18, calcineurin, synaptophysin (SYN), microtubule (Alexa Fluor 594), Anti-Mouse Goat IgG H&L (Alexa Fluor 488), Alexa Fluor 488 Goat Anti-Rabbit IgG H&L Goat Anti-Rabbit IgG H&L (Alexa Fluor 594) antibodies (Abcam, Cambridge, UK; Cat. Nos. ab207323, ab52761, ab52636, ab183830, ab104224, ab272608, ab56416, ab150114, ab150113, ab150077, and ab150080, respectively); TFE3 antibody (Sigma--Aldrich Chemical Company; Milwaukee, WI, United States of America; Cat No. HPA023881); LC3B antibody (Novus Biologicals; Cat. No. NB600-1384); IL-1 antibody (ABclonal Technology; Cambridge, MA, United States of America; Cat. No. A1112); MLKL, GSDMD, and NLRP1 antibodies (Affinity Biosciences; OH, United States of America; Cat. Nos. DF7412, AF4013, and DF13187, respectively); HRP-conjugated IgG secondary antibody (Santa Cruz Biotechnology (Dallas, TX, the United States of America); FITC-conjugated IgG secondary antibody (Boyun Biotechnology ;Nanjing, the People's Republic of China); and 4',6-diamidino-2-phenylindole (DAPI) solution (Beyotime Biotechnology ;Jiangsu, the People's Republic of China).

**Animal Model Of Sci**

Animals were given anaesthesia by intraperitoneal injection of 50 mg/kg 1% (w/v) pentobarbital sodium. To reveal a dura circle, a conventional laminectomy was then performed in the T9-T10 vertebrae. A weight drop injury model for causing SCI damage was then generated as previously described [34]. A 5 g weight was released from a height of 30 mm to hit the exposed spinal cord to induce moderate spinal cord injury according to the manufacturer's instructions (W.M. KECK+, Model + III, USA) [35]. After SCI, 4 − 0 nonabsorbable silk sutures were used to suture the muscle, fascia, and layers of the skin. The mice in the sham group underwent the same treatment, but there was no injury caused by the contusion. After the surgery, the mice received three artificial urinations each day.

**Adeno-associated Virus (Aav) Vector Packaging**

Shanghai Genechem Company constructed and packaged AAV-TFE3 shRNA. To create pAV-U6-shRNA (TFE3)-CMV-EGFP, the TFE3-facilitated protein kinase shRNA sequence was cloned and ligated into the plasmid. The AAV Rep/Cap expression plasmid, Ad helper, and pAV-U6-shRNA (TFE3)-CMV-EGFP were cotransfected into AAV-293 cells to obtain AAV9-U6-shRNA(TFE3)CMV-EGFP. AAV9-U6-shRNA(scramble)-CMV-EGFP was used as the scramble control. The iodixanol gradient technique was utilized to isolate the viral particles. Quantitative PCR was used to determine the AAV9-U6-shRNA(TFE3)-CMV-EGFP and AAV9-U6-shRNA(scramble)-CMV-EGFP viral titres, which were 4.82x10^{12} and 6.56x10^{12} genomic copies/ml, respectively.
Drug And Aav Vector Administration

In total, 100 mice were randomly divided into the following 8 cohorts: sham (n = 15); SCI (n = 15); SCI + FGF-18 (n = 15); SCI + FGF-18 + 3MA (n = 15); SCI + FGF-18 + scrambled shRNA control (n = 15); SCI + FGF-18 + TFE3 shRNA (n = 15); SCI + SC79 (n = 5) and SCI + FGF-18 + SC79 (n = 5). The SCI + FGF-18 cohort received intraperitoneal injection of FGF-18 (90 µg/kg/day, once a day) after SCI for three days. The FGF-18 dose was based on previous data with some modification [25, 36–38]. The sham and SCI cohorts received the same treatment but were injected with saline instead of FGF-18. Daily intraperitoneal injections of SC79 (10 mg/kg) and 3-methyladenine (15 mg/kg) were given 30 minutes before rFGF-18 was administered. Fourteen days prior to SCI, 100 µl of intravenous viral vectors in PBS with 1x10^10 packaged genomic particles were administered to the SCI + FGF-18 + Scramble and SCI + FGF-18 + TFE3 shRNA cohorts. The SCI + FGF-18 + scrambled shRNA control and SCI + FGF-18 + TFE3 shRNA cohorts were treated the same as the SCI + FGF-18 cohort after 14 days. On the third day after pentobarbital sodium overdose, animals were killed, and histology samples were collected.

Functional Behaviour Assessment

To assess functional recovery after SCI, the Basso Mouse Scale (BMS) was used to measure locomotion on Days 0, 1, 7, 14, 21, and 28 [39]. BMS was in the range of 0 to 9 with 0 and 9 representing total paralysis and normal motor function, respectively. At 28 days following the operation, footprints were observed using various coloured dyes to stain the forelimbs (blue) and hind limbs (red) of mice. Toe dragging refers to the proportion of the overall length of the hindlimbs being dragged to the walking distance. Stride length refers to the distance between the footprints of the hind limbs [40]. The hind limb strength was assessed using the inclined plane test, in which a sloped plane with a rubber surface was used. The mouse dropped off the plane as the angle of inclination reached a particular point. The greatest inclined plane that the mouse was capable of maintaining for 5 seconds was noted. Three evaluations were performed on each mouse with a one-minute gap between each evaluation [41]. Measurements were made by two independent testers blinded to the experimental setup.

Preparation Of Tissue Slides For He And Masson Staining

Pentobarbital sodium (2%, w/v) was used to anesthetize mice, which were then perfused with regular saline. The tissues were harvested and cut into 10-mm long segments with the epicentre in the middle, and they were fixed with 4% (w/v) paraformaldehyde for 24 hours. The tissues were then embedded in paraffin and cut into 4-µm sections, which were mounted onto slides precoated with poly-L-lysine for HE staining according to previously reported methods [42]. For Masson's staining, the mordant longitudinal sections were incubated with 10% TCA and with 10% potassium dichromate. Haematoxylin was used for nuclear staining. Slides were then incubated with ethanol and hydrochloric acid, and they were then rendered blue with less ammonia followed by staining with Masson's solution according to a previously described protocol [43]. Finally, images were acquired using an Olympus light microscope (Tokyo, Japan).
Western Blot Analysis

Western blot analysis
On Day 3 after SCI, the mice were anesthetized, and the spinal cords (1.5 cm) that covered the injury site were collected and stored at -80°C until used for Western blot analysis. Some samples were lysed with lysis buffer for protein extraction, and other samples were used to extract cytoplasmic and nuclear proteins using NE-PER™ Nuclear and Cytoplasmic Extraction Reagent. BCA assays were used for protein quantification. Equal amounts of protein samples (60µg) were electrophoresed on 12% SDS-PAGE gels (Roche Applied Science) followed by transfer onto polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked with 5% (w/v) skimmed milk and then incubated overnight at 4°C with the following primary antibodies: ASC (1:1,000), CASP1 (1:1,000), GSDMD-N (1:1,000), IL-18 (1:1,000), NLRP1 (1:1,000), IL-1β (1:1,000), SQSTM1/p62 (1:1,000), Beclin1 (1:1,000), VPS34 (1:1,000), CTSD (1:1,000), p-AKT (1:1,000), AKT (1:1,000), p-mTOR (1:1,000), mTOR (1:1,000), GAPDH (1:1,000), TRPML1 (1:1,000), calcineurin (1:1,000), TFE3 (1:1000), and histone (1:1000). The membranes were then incubated with HRP-conjugated IgG secondary antibodies for 2 hours at room temperature. A ChemiDoc™ XRS+ Imaging System (Bio-Rad) and ECL immunodetection equipment were utilized to detect and examine the band signals.

Immunofluorescence Staining

Spinal cord samples were collected on the third day following SCI for immunofluorescence staining of 1-mm long rostral spinal cord (4 mm from epicentre) sections according to a previously reported protocol [44]. After deparaffinization, rehydration, and washing, the sections were incubated in 10.2 mM sodium citrate buffer for 20 minutes at 95°C. The sections were permeablized for with 0.1% (v/v) PBS-Triton X-100 for 10 min and then blocked with 10% (v/v) BSA in PBS for 1 h. The sections were then incubated overnight at 4°C with the following primary antibodies: MAP2 (1:200), SYN (1:200)/NeuN (1:400), Caspase-1 (1:200)/NeuN (1:400), GSDMD (1:150)/NeuN (1:400), p62 (1:200)/NeuN (1:400), LC3 (1:200)/NeuN (1:400), and TFE3 (1:200)/NeuN (1:400). The sections were then washed three times (10 min each) at room temperature followed by incubation with a secondary antibody conjugated to FITC at room temperature. An Olympus fluorescence microscope (Tokyo, Japan) was used to acquire images and for analysis of six random fields of view in three randomly selected areas of each sample.

Statistical analysis

SPSS software (version 19) was used for all statistical analyses with a double-blind analytic strategy. Values are presented as the mean ± standard error of the mean (SEM). In the present study, data normalization served to eliminate undesirable sources of variation. ANOVA combined with Tukey’s test (equal variances assumed) or Dunnett’s T3 (equal variances based on no assumption) was used to evaluate the significant differences between two cohorts or among three or four cohorts, respectively. An
Results

1. FGF-18 ameliorates functional recovery after SCI

HE staining, Masson staining, Immunofluorescence (IF) staining, Basso Mouse Scale (BMS) scores, inclined plane test, and footprint analysis were used to evaluate mouse motor function after SCI. The ISC lesion area was examined with HE and Masson staining, which demonstrated that SCI mice had significantly wider glial scars than sham mice. Compared to the sham control, IF staining in the SCI group indicated reduced MAP2 expression as well as downregulated SYN-positive synapses on ventral motor neurons (VMNs). In FGF-18-treated mice, glial scars were fewer, neuronal MAP2 expression was higher, and VMNs had more SYN-positive synapses compared to the untreated SCI group (Figs. 1A–1F). Considering the motor functional recovery on Day 28 post SCI, footprint analysis and the inclined plane test indicated that the SCI + FGF-18 group presented a better performance (less toe dragging and long stride length) than the SCI group without treatment (Figs. 1G–1J). The sham group had significantly higher BMS scores than the SCI group at Days 1, 3, 7, 14, 21, and 28 postinjury. Similarly, the SCI + FGF-18 group possessed higher BMS scores than the SCI group on Days 7, 14, 21, and 28 postinjury (Fig. 1K). Overall, these results indicated that FGF-18 ameliorates functional recovery after SCI.

2. Fgf-18 Weakens Pyroptosis After Sci

We next evaluated the expression of ASC, Caspase-1, GSDMD, NLRP3, IL-1, and IL-18 in the spinal cord following SCI to evaluate pyroptosis following injury. IF staining revealed significantly higher Caspase-1 and GSDMD staining intensity in VMNs in the SCI group, and the staining intensity was decreased after FGF-18 therapy (Fig. 2A-D). Figure 2E shows the WB analysis of ASC, Caspase-1, GSDMD, NLRP3, IL-1β, and IL-18 levels. Compared to the sham group, the SCI group had higher expression of proteins related to pyroptosis, including ASC, Caspase-1, GSDMD, NLRP3, IL-1, and IL-18. Compared to the SCI group, FGF-18 therapy reduced the expression of proteins related to pyroptosis (Fig. 2F). Together, these findings indicated that FGF-18 decreases markers related to pyroptosis and may help to suppress SCI-induced pyroptosis.

3. Fgf-18 Enhances Autophagy After Sci

We next evaluated the levels of LC3II, Beclin1, Vps34, CTSD, and p62 in the spinal cord after SCI to assess autophagic activity after injury. IF staining indicated that the p62 staining intensity in VMNs was significantly increased after SCI (Fig. 3A), but the p62 staining intensity was lower in the SCI + FGF-18 group compared to the SCI group (Fig. 3B). In the SCI + FGF-18 group, the spinal cord had more LC3II puncta in neurons compared to the sham group and SCI group, which indicated that FGF-18 therapy
significantly increased the number of LC3II puncta in neurons (Fig. 3C and 3D). According to WB analysis, the SCI group had higher expression levels of VPS34, Beclin1, LC3II, and p62 but lower expression levels of CTSD compared to the sham group. Compared to the SCI group, the SCI + FGF-18 group had higher expression levels of VPS34, Beclin1, LC3II, and CTSD but lower expression levels of p62 (Fig. 3E and 3F).

4. Autophagy Suppression Reverses The Influence Of Fgf-18 On Pyroptosis After Sci

To further explore whether FGF-18 has a favourable effect on functional recovery augmentation after SCI caused by autophagy activation, we coadministered 3MA, which inhibits autophagy, with FGF-18. Immunofluorescence analysis demonstrated higher p62 staining intensity in the SCI + FGF-18 + 3MA group compared to the SCI + FGF-18 group (Fig. 4A and 4B). Compared to the SCI + FGF-18 group, the SCI + FGF-18 + 3MA group presented fewer LC3II puncta in neurons (Fig. 4A and 4C). Immunofluorescence analysis revealed increased Caspase-1 and GSDMD staining intensity in the SCI + FGF-18 + 3MA group compared to the SCI + FGF-18 group (Fig. 4D-4F). Western blot analysis revealed alterations in LC3II, Beclin1 and Vps34, CTSD and p62 levels (Fig. 4H). Compared to the SCI + FGF-18 group, the LC3II, Beclin1, Vps34, and CTSD expression levels were significantly decreased in the FGF-18 + 3MA group, but the expression levels of p62 were significantly increased in the FGF-18 + 3MA group (Fig. 4I). Moreover, the FGF-18 + 3MA group had significantly higher expression levels of ASC, Caspase-1, GSDMD, NLRP3, IL-1, and IL-18 than the SCI + FGF-18 group (Fig. 4J). Thus, these findings revealed that coadministration of 3MA with FGF-18 reduces the impact of FGF-18 on reducing pyroptosis, implying that the autophagy-enhancing activities of FGF-18 may support the mechanism through which it suppresses pyroptosis.

5. The Effect Of Fgf-18 On Functional Recovery Following Sci Is Weakened By Autophagy Inhibition

HE and Masson staining demonstrated an expanded lesion area in the spinal cord in the SCI + FGF-18 + 3MA group compared to the SCI + FGF-18 group, which indicated a reversed effect on functional recovery in the SCI + FGF-18 + 3MA group (Fig. 5A and B). We also employed IF staining, which showed decreased MAP2 levels and fewer SYN contacts with motoneurons in the SCI + FGF-18 + 3MA group compared to the SCI + FGF-18 group, which indicated a reversed effect on functional recovery in the SCI + FGF-18 + 3MA group (Fig. 5C-F). On Day 28 after SCI, as assessed by footprint analysis and the inclined plane test, the SCI + FGF-18 + 3MA group exhibited worse functional performance compared to the SCI + FGF-18 group (Fig. 5G-J). The BMS scores of the SCI + FGF-18 + 3MA group 14 days postinjury were significantly worse than those of the group that received FGF-18 treatment alone (Fig. 5K). Therefore, these findings suggested that the strengthened autophagy activities induced by FGF-18 following SCI account for the ameliorated functional recovery with FGF-18 treatment following SCI.
6. Fgf-18 Enhances Autophagy By Upregulating Tfe3 Activity And Inhibits Pyroptosis After Sci

The above findings shed light on the importance of autophagy and attenuated pyroptosis in the effects of FGF-18, but the mechanism of FGF-18 regulation of pyroptosis and autophagy remained unknown. We have previously reported that TFE3 is essential for controlling autophagic flux in SCI (39). Therefore, we examined TFE3 expression in the cytoplasm and nucleus to confirm whether FGF-18 regulates TFE3 in the present study. As shown in Fig. 6A, FGF-18 treatment significantly promoted TFE3 translocation into the spinal cord nucleus (Fig. 6C), which suggested that the activity of TFE3 may have been upregulated by FGF-18. To study the mechanism of TFE3 activation in enhancing autophagy and restricting pyroptosis by FGF-18, TFE3 expression and activity were downregulated by shRNA. The following three groups were compared: FGF-18 only, FGF-18 + scramble, and FGF-18 + TFE3 shRNA. The SCI + FGF-18 + TFE3 shRNA group exhibited significantly lower TFE3 expression in the cytoplasm and nucleus than the SCI + FGF-18 + scramble group, whereas the expression of TFE3 was not significantly different between the SCI + FGF-18 and SCI + FGF-18 + scramble groups (Figs. 6F and 6G), which demonstrated that TFE3 expression was efficiently suppressed by transfection of TFE3 shRNA.

We next investigated whether autophagy and pyroptosis are regulated by FGF-18-induced nuclear translocation of TFE3. According to the IF results, the number of LC3II puncta in neurons in the SCI + FGF-18 and FGF-18 + scramble groups were not significantly different; however, the proportion of LC3II puncta was significantly decreased in the SCI + FGF-18 + TFE3 shRNA group (Figs. 6B and 6E). Similarly, WB analysis demonstrated no appreciable difference in terms of LC3II, Beclin1, Vps34, CTSD, and p62 expression between the FGF-18 and FGF-18 + scramble groups. Compared to the FGF-18 + scramble group, the SCI + FGF-18 + TFE3 shRNA group had significantly reduced expression levels of LC3II, Beclin1, Vps34, and CTSD but significantly increased expression levels of p62 (Figs. 6K and 6M). The expression levels of proteins related to pyroptosis, including ASC, Caspase-1, GSDMD, NLRP3, IL-1β, and IL-18, were increased after transfection of TFE3 shRNA (Figs. 6B, 6J and 6L). The results confirmed that TFE3 nuclear translocation induces a stronger effect of FGF-18 as it increases autophagy and inhibits pyroptosis.

7. FGF-18 promotes functional recovery after SCI by enhancing autophagy and inhibiting pyroptosis by upregulating TFE3 activity

Compared to the SCI + FGF-18 group after SCI, the SCI + FGF-18 + TFE3 shRNA group had a larger lesion area (Figs. 7A and 7B), lower MAP2 expression (Figs. 7C and 7E), and fewer SYN-positive synapses (Figs. 7D and 7F) on VMNs. The posterior limb function of the SCI + FGF-18 + TFE3 shRNA mice was substantially affected as evidenced by a discontinuous footprint of the SCI + FGF-18 + TFE3 shRNA group on Day 28 postinjury and a shorter distance between posterior limbs relative to the SCI + FGF-18 group (Fig. 7G-I). The results of the inclined plane test were similar (Fig. 7J). On Days 1, 3, and 7, there was a negligible change in the BMS score among the SCI + FGF-18, SCI + FGF-18 + scramble shRNA, and SCI + FGF-18 + TFE3 shRNA groups. On Days 14, 21, and 28 after SCI, the SCI + FGF-18 + TFE3 shRNA group
had significantly lower BMS scores than the SCI + FGF-18 and SCI + FGF-18 + scramble shRNA groups (Fig. 7K). These results confirmed that FGF-18 promotes functional recovery after SCI by enhancing autophagy and inhibiting pyroptosis via upregulating TFE3 activity.

8. Fgf-18 Facilitates Tfe3 Via The Akt-trpml1-calcineurin Signalling Pathway After Sci

Previous studies have shown that the AMPK-TRPML1-calcineurin signalling cascade controls the function of the MiT/TFE family (40, 41), suggesting that FGF-18 and the calcium signalling system are related. The present study demonstrated that FGF-18 decreased p-AKT and p-mTOR expression but enhanced TFE3 nuclear translocation (Figs. 8A and 8B). Western blot analysis demonstrated that FGF-18 treatment resulted in significantly higher levels of TRPML1 and calcineurin, which are downstream signalling molecules (Figs. 8A and 8B). These findings suggested that FGF-18 activates the AKT-TRPML1-calcineurin pathway.

We next investigated the effects of SC79, a well-known AKT activator, on the AKT-TRPML1-calcineurin signalling pathway to ascertain whether FGF-18-induced TFE3 activation is influenced by this signalling pathway. FGF-18 stimulated the AKT-TRPML1-calcineurin signalling pathway and enhanced TFE3 nuclear translocation, but this effect was abolished by SC79 (Fig. 8A-B). In addition, Western blot analysis indicated that SC79 significantly suppressed the FGF-18-induced enhancement of autophagy and subsequent suppression of pyroptosis in SCI (Fig. 8C-D). Overall, these results demonstrated that FGF-18 activates TFE3 activity in SCI through the AKT-TRPML1-calcineurin signalling pathway.

Discussion

Trauma patients with SCI may experience anything from a small annoyance to devastating paralysis, and this range of injuries is commonly observed after SCI [45]. After SCI, a reduced ability to regenerate new neurons and increase neuronal death may affect the ability of the brain and limbs to continue transmitting signals, thereby obstructing functional recovery [7, 46]. Therefore, promoting neuronal regeneration and inhibiting neuronal death should be given much attention. Autophagy and pyroptosis engage the responses of multiple cell types and regulate recovery following SCI [47–49]. FGF-18 mediates various physiological processes. Previous studies have demonstrated that FGF-18 has a neuroprotective effect on Parkinson's disease and cerebral ischaemic injury [25, 26]. FGF-18 also regulates autophagy in bone growth and chondrocytes [30, 50]. The present study demonstrated that TFE3 activity via the AKT-TRPML1-calcineurin signalling pathway is responsible for the therapeutic impact of FGF-18, which in turn increases autophagy and further decreases pyroptosis.

Autophagy, a self-degradative process, is important for many physiological processes [51]. The study of mammalian physiology and the pathophysiology of human diseases has come into a new era with the identification of the key genes required for autophagy [52]. Disruption of autophagy after an SCI caused by the secondary damage mechanism together with the enhancement or restoration of autophagy flux
may be a possible therapeutic target for treating SCI [17]. A previous study has shown that impaired autophagy after SCI is related to ER stress, which causes neuronal death [18]. The present study assessed the autophagy flux levels in SCI and found higher expression of proteins associated with autophagy in the SCI + FGF-18 group, which demonstrated that FGF-18 facilitated autophagy. Together, these findings suggested that FGF-18 activates autophagy and that this effect is beneficial for SCI recovery.

Activation of Caspase-1 inside the inflammasome complex, Caspase-11 activation (Caspase-4/5 in humans), and signalling pathways under the control of GSDMD are all thought to contribute to pyroptosis, which regulates cell death by promoting inflammation [53, 54]. Pyroptosis is an inflammatory and PCD [53]. The canonical pyroptosis pathway, which involves NLRP3, is the main pathway in SCI [47]. Moreover, numerous other possible pathways, including noncanonical pyroptosis caspases and other NLR inflammasomes, may potentially play a substantial role [13, 55, 56]. In the present study, IF staining was used to evaluate the Caspase-1 and GSDMD staining intensities in SCI neurons to quantify FGF-18 activity in pyroptosis. WB analysis showed that FGF-18 significantly inhibited pyroptosis in the SCI model as manifested by decreased expression levels of IL-1B, IL-1, Caspase-1, GSDMD, NLRP1, NLRP3, and ASC after FGF-18 treatment.

Anomalies in PCD signalling cascades, such as pyroptosis, cell death linked to autophagy, and unprogrammed necrosis, are detected in the aetiology of many neurological diseases and result in an unintended loss of neuronal cells and function [57]. By degrading a variety of proteins, autophagy, a prosurvival process, has been reported to inhibit pyroptosis [20, 58]. According to previous studies, autophagy activation helps to recover neurological function after moderate traumatic brain injury (TBI) or SCI by suppressing pyroptotic neuronal death [21, 59]. Few studies, however, have examined how autophagy affects pyroptosis after SCI. To demonstrate that FGF-18 prevents pyroptosis via increasing autophagy, we used 3MA to suppress autophagy in SCI with FGF-18 therapy. Pyroptosis-associated markers were partially modified when autophagy was suppressed, and FGF-18 therapy reduced functional recovery in SCI. However, further research should be performed to determine the precise relationship and biological mechanism. These findings indicated that FGF-18 reduces pyroptosis by promoting autophagy, which is essential for the therapeutic action of FGF-18 following SCI.

We also investigated the underlying mechanisms of autophagy to further understand the mechanism underpinning the therapeutic effect of FGF-18 on SCI. The basic helix-loop-helix leucine zipper transcription factors that belong to the microphthalmia family regulate autophagy [60]. TFE3 (also a part of the family) activation benefits many neurological and lysosomal disorders, and it regulates the autophagic system [61]. Therefore, we examined the role of TFE3 in the present study. The TFE3-14-3-3 complex is formed by the interaction of phosphorylated TFE3 and the cytosolic chaperone, 14-3-3, in a physiologically normal environment. The cells sense environmental changes caused by starvation, hypoxia, or toxins, inhibiting mTOR activity, which initiates the dephosphorylation of TFE3 [62]. Target genes are activated when dephosphorylated TFE3 enters the nucleus after being released from the TFE3-
14-3-3 complex [60, 63]. Overall, FGF-18 promotes the nuclear translocation of TFE3 to accelerate autophagy and prevent pyroptosis.

Researchers have studied the mechanism of FGF-18 regulation of TFE3 activity to establish a solid foundation for potential clinical applications based on the positive therapeutic effect of TFE3. Previous research has demonstrated that the phosphorylation of AKT at Ser565 regulates TFE3 function, and pharmacological inhibition of PPP3/calcineurin stimulates AKT phosphorylation while preventing autophagic cell death dependent on TFE3 [64]. To enhance cellular anabolism as well as inhibit cellular catabolism pathways (autophagy), we used a regulator that mainly controls cell growth and metabolism (mTOR) to monitor environmental cues [65, 66]. Dephosphorylated AKT also inhibits mTOR action. Additionally, calcineurin is activated by Ca²⁺ being released through TRPML1 channels in the cytoplasm when mTOR is activated [64, 65, 67]. Recent research has shown that the calcium ion-dependent phosphatase, calcineurin, initiates the dephosphorylation of TFE3 and facilitates its nuclear translocation [68]. Accordingly, FGF-18 treatment for SCI enhanced the AKT-TRPML1-calcineurin signalling pathway. Additionally, we prevented the signalling pathway from being activated by FGF-18 by using SC79, a well-known AKT activator. Overall, FGF-18 increased TFE3 nuclear translocation in SCI via the AKT-TRPML1-calcineurin signalling pathway.

Due to several limitations of the present study, additional in-depth analyses are required. A previous study has reported that during early postnatal bone growth, FGF-18 activates FGFR4, activates JNK kinase, phosphorylates BCL2, and activates the VPS34-beclin-1 autophagy complex [50]. It is thoroughly investigated to identify if FGF-18 also affects autophagy after SCI through the FGFR4-JNK-VPS34-beclin-1 signaling pathways. In-depth analyses will be needed to determine the best FGF-18 dosage and regimen in order to increase the therapeutic effect.

The present study also provided novel research directions to further investigate FGF-18. Patients with osteoarthritis potentially undergo long-lasting cartilage structure alteration with FGF-18 treatment [69, 70], but there are no clinical experimental data on FGF-18 in SCI. Therefore, additional investigation into the potential for therapeutic FGF-18 transformation for SCI is needed. Necroptosis, among many other pathways, may result in neuronal death after SCI [71–73]. Necroptosis has been noted in nervous system neoplasms and is closely related to neurological disorders as well as nervous system disease. Thus, it is worthwhile to study how FGF-18 affects necroptosis after SCI. Previous research has developed a drug delivery system using fast-degradable hydrogels (CHPOA/hydrogels) allowing the regulatory release of FGF-18 over a long-term period, providing additional delivery options for the application of FGF-18.

In conclusion, the present study showed that FGF-18 increases autophagy in SCI by activating the AKT-TRPML1-calcineurin signalling pathway. Additionally, after SCI, elevated autophagy inhibits pyroptosis. These FGF-18 effects ultimately lead to better outcomes after SCI. Figure 9 shows a schematic representation of these findings. Overall, these findings provide evidence for the therapeutic value of FGF-18 for SCI. Future studies that focus on translating FGF-18 as a strategy for treating SCI patients in clinical practice are currently highly anticipated.
Declarations

Availability of data and materials

The research data used to support the findings of this study are included within the article.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors’ contributions

Kailiang Zhou, Wenfei Ni and Hui Xu conceived and designed the study. Feida Li, Tingwen Cai, Letian Yu, Haojie Zhang and Yibo Geng performed the experiments. Jiaxuan Kuang, Yongli Wang, Yuepiao Cai and Xiangyang Wang analyzed the data. Feida Li and Letian Yu wrote the manuscript. Kailiang Zhou, Wenfei Ni and Hui Xu reviewed and revised the manuscript and supervised the study. All authors read and approved the manuscript.

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Ethics approval

All institutional and national guidelines for the care and use of laboratory animals were followed. All experimental procedures used in this study were approved by the Chinese Institutional Animal Care Committee of Wenzhou Medical University, China (Approval no. wydw 2017-096).

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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References


Figures
FGF-18 ameliorates Functional Recovery after SCI. (A) Masson staining and HE staining were used to examine longitudinal spinal cord slices from the indicated groups on the 28th day following SCI (scale bar = 1000 µm). (B) Quantitative evaluations of the Masson-positive spinal cord lesions among the relevant groups. (C) Photographs of slices of spinal cord from each group that had been incubated with an anti-MAP2 antibody (scale bar = 50 µm). (D) Photos (150×) of damaged spinal cord slices (T11-T12)
taken on day 28 while being stained with a SYN/NeuN antibody (scale bar = 25 µm). (E) MAP2 optical density within a spinal cord subjected to injury on the 28th day. (F) Quantitative analyses for motor neuron contacting synapse. (G) Images of mouse footprints 28 days post injury. (H), (I) Quantification of stride length and toe dragging in the footprint analysis. Test on an inclined plane in (J) for the specified groups and time points. Basso Mouse Scale (BMS) scores at the designated cohorts and time periods (K). Data are expressed as the mean ± SEM, n=5 per cohort. *P < 0.05, **P < 0.01.
**Figure 2**

**FGF-18 attenuates pyroptosis following spinal cord injury.** (A) Caspase-1 and NeuN colocalized by immunofluorescence staining in the spinal cords of the SCI, SCI+FGF-18, and Sham cohorts (scale bar = 25 µm). (B) Caspase-1’s quantitative mean optical density in spinal cord lesions’ neurons. (C) GSDMD-N and NeuN colocalization were detected using immunofluorescence staining in the spinal cords of the SCI+FGF-18, SCI, and Sham cohorts (scale bar = 25 µm). (D) Quantification graph for optical density of GSDMD-N within neurons. (E) Western blot assay for ASC, Caspase-1, GSDMD-N, IL-18, NLRP3 and IL-1β expression levels in the three groups. Gels were put through the same experimental processes, and cropped blots were shown. (F) ASC, Caspase-1, GSDMD-N, IL-18, NLRP3, and IL-1β optical densities of the expression levels were measured and examined in the corresponding cohorts. Data are expressed as the mean ± SEM, n=5 per cohort. *P < 0.05, **P < 0.01.
Figure 3

FGF-18 enhances autophagy following spinal cord injury. (A) Colocalization of NeuN and p62 via immunofluorescence labeling at the ISC lesion after spinal cord injury (scale bar = 25 µm). (B) Quantification graph for p62 in motor neurons of spinal cord lesions. (C) Colocalization of NeuN and LC3II via immunofluorescence labeling at the spinal cord lesion after spinal cord injury (scale bar = 25 µm). (D) The number of LC3II puncta in each neuron in spinal cord lesions. (E) Western blot assay for
p62, LC3II, Beclin1, Vps34 and CTSD expression levels in the three groups. Gels were put through the same experimental processes, and cropped blots were shown. (F) p62, LC3II, Beclin1, Vps34 and CTSD optical densities of the expression levels were measured and examined in the corresponding cohorts. Data are expressed as the mean ± SEM, n=5 per cohort. *P < 0.05, **P < 0.01.

Figure 4
Suppression of autophagy reverses the influence exerted by FGF-18 on pyroptosis following spinal cord injury. (A) The spinal cord lesion tissue sections were stained with p62/NeuN or LC3II / NeuN immunofluorescence antibodies (scale bar = 25 µm). (B), (C) The mean optical density of p62 and the number of LC3II puncta in each neuron in spinal cord lesions in the respective groups. (D) The spinal cord lesion tissue sections were stained with Caspase-1/NeuN or GSDMD-N / NeuN immunofluorescence antibodies (scale bar = 25 µm). (E), (F) The mean optical density of Caspase-1 and GSDMD-N in the spinal cords of each cohort. (G), (I) Western blot assay for the ASC, Caspase-1, GSDMD-N, IL-18, NLRP3, and IL-1β. The expression levels of ASC, Caspase-1, GSDMD-N, IL-18, NLRP3, and IL-1β were measured and studied using optical densities in the respective groups. (H), (J) Western blot assay for the p62, LC3II, Beclin1, Vps34 and CTSD expression levels in the respective groups. The expression levels of p62, LC3II, Beclin1, Vps34, and CTSD were measured and studied using optical densities in the respective groups. Data are expressed as the mean ± SEM, n=5 per cohort. *P < 0.05, **P < 0.01.
Figure 5

Suppression of autophagy reverses the influence exerted by FGF-18 on functional recovery following spinal cord injury. (A) On the 28th day after SCI, longitudinal spinal cord slices from the indicated groups were analyzed using Masson staining and HE staining. (scale bar = 1000 µm). (B) Quantitative analyses of Masson-positive spinal cord lesions in the respective groups. (C) Images (30×) of spinal cord slices from each group stained with an anti-MAP2 antibody (scale bar = 50 µm). (D) Photos (150×) of damaged
spinal cord slices (T11-T12) taken on day 28 while being stained with a SYN/NeuN antibody (scale bar = 25 µm). (E) MAP2 optical density within a spinal cord subjected to injury on the 28th day. (F) Quantitative analyses for motor neuron contacting synapse. (G) Images of mouse footprints 28 days post injury. (H), (I) Quantification of stride length and toe dragging in the footprint analysis (J) inclined plane test for the indicated groups and time points. (K) Scores on the Basso Mouse Scale (BMS) at the specified cohorts and time points. Data are expressed as the mean ± SEM, n=5 per cohort. *P < 0.05, **P < 0.01.
**Figure 6**

**FGF-18 enhances autophagy by upregulating TFE3 activity and inhibiting pyroptosis.**

(A) The spinal cord lesion tissue sections were stained with TFE3 / NeuN immunofluorescence antibodies (scale bar = 25 µm). (B) The spinal cord lesion tissue sections were stained with LC3II / NeuN or GSDMD / NeuN immunofluorescence antibodies (scale bar = 25 µm). (C) There is a quantification graph for the percentage of translocated TFE3 in motor neurons of spinal cord lesions. (D) Average optical density of GSDMD-N in spinal cord lesions measured quantitatively. (E) Number of LC3II puncta in each neuron in the respective groups. (F) Nuclear and cytoplasmic TFE3 expression was measured using a Western blot. (G) TFE3 expression levels in the cytoplasm and the nucleus are reported using the Western blot test in the SCI+FGF-18, SCI+FGF-18+Scramble and SCI+FGF-18+TFE3 shRNA cohorts. (H) Optical densities for nuclear TFE3 and cytoplasmic TFE3 have been quantified. (I) Quantification outcomes for optical densities of nuclear and cytoplasmic TFE3. (J) Western blot assay for the ASC, Caspase-1, GSDMD-N, IL-18, NLRP3, and IL-1β expression levels in the respective groups. (K) Western blot assay for the p62, LC3II, Beclin1, Vps34 and CTSD expression levels in the SCI+FGF-18, SCI+FGF-18+Scramble and SCI+FGF-18+TFE3 shRNA cohorts. (L) The expression levels of ASC, Caspase-1, GSDMD-N, IL-18, NLRP3, and IL-1β were measured and studied using optical densities in the respective groups. (M) The expression levels of p62, LC3II, Beclin1, Vps34, and CTSD were measured and studied using optical densities in the SCI+FGF-18, SCI+FGF-18+Scramble and SCI+FGF-18+TFE3 shRNA cohorts.
Figure 7

FGF-18 promotes the functional recovery after SCI by enhanced autophagy and inhibited pyroptosis through upregulating TFE3 activity. (A) On the 28th day after SCI, longitudinal spinal cord slices from the indicated groups were analyzed using Masson staining and HE staining. (scale bar = 1000 µm). (B) Images (30×) of spinal cord slices from each group stained with an anti-MAP2 antibody (scale bar = 50 µm) and photos (150×) of damaged spinal cord slices (T11-T12) taken on day 28 while being stained...
with a SYN/NeuN antibody (scale bar = 25 µm). (C) Images of mouse footprints 28 days post injury. (D) Quantitative analyses of Masson-positive spinal cord lesions in the respective groups. (E) MAP2 optical density within a spinal cord subjected to injury on the 28th day. (F) Quantitative analyses for motor neuron contacting synapse. (G) Images of mouse footprints 28 days post injury. (H), (I) Quantification of stride length and toe dragging in the footprint analysis. (J) Inclined plane test for the indicated groups and time points. (K) Scores on the Basso Mouse Scale (BMS) at the specified cohorts and time points.

Figure 8
FGF-18 activates TFE3 through the AKT-TRPML1-calcineurin signalling channel. (A) Western blot assay reporting the cytoplasmic expression levels of p-mTOR, mTOR, p-AKT, AKT, TRPML1 and calcineurin, normalized to GAPDH as an internal control; nuclear expression of TFE3, normalized to histone H3 as an internal control. (B) Optical densities of TRPML1, p-mTOR, mTOR, p-AKT, AKT, calcineurin and nuclear TFE3. (C) Western blot assay reporting the expression levels of p62, LC3II, Caspase-1 and GSDMD-N, normalized to GAPDH as an internal control. (D) Optical densities of p62, LC3II, Caspase-1 and GSDMD-N. Data are expressed as the mean ± SEM, n=5 per cohort. *P < 0.05, **P < 0.01.

Figure 9

A schematic representation of this research. FGF-18 protects the injured spinal cord by suppressing pyroptosis and promoting autophagy via AKT-mTOR-TRPML1 pathway.