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Silencing of *PHLPP1* promotes neuronal apoptosis and inhibits functional recovery after spinal cord injury in mice

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ABSTRACT

Aim: Spinal cord injury (SCI) causes increased apoptosis of neurons, leading to irreversible dysfunction of the spinal cord. In this study, we investigated the effects of the progression of SCI and potential regulation of apoptosis after the Pleckstrin homology (PH) domain and leucine rich repeat protein phosphatase 1 (*PHLPP1*) gene was silenced.

Main methods: Spinal cord injection, and neuronal transfection with a recombinant adenovirus vector encoding small interfering RNA (siRNA) against PHLPP1 (AdsiPHLPP1) successfully silenced *PHLPP1*. These created *in vivo* and *in vitro* PHLPP1-silenced models, respectively, resulting in stable expression of the transgene in neurons.

Key findings: The results showed that silencing of *PHLPP1* evidently reduced levels of the nuclear factor erythroid 2-related factor 2 (Nrf2) after SCI. Western blot analysis revealed that the mice injected with AdsiPHLPP1 showed increased the expression of pro-apoptotic factors (Bax and cleaved-caspase 3), and reduced levels of neurotrophic (BDNF) and anti-apoptotic (Bcl-2) factors, both *in vivo* and *in vitro*. The motor function of AdsiPHLPP1-injected mice was restored more slowly than that of wild type (WT) mice. In addition, the number of motor neurons surviving in the anterior horn of the spinal cord was also reduced after SCI.

Significance: Our results confirm that silencing of *PHLPP1* promotes neuronal apoptosis and inhibits functional recovery after injury *in vivo* and *in vitro*. Consequently, PHLPP1 represents a potential therapeutic target gene for the clinical treatment of SCI.

1. Introduction

Spinal cord injury (SCI) is one of the most difficult conditions to diagnose. It can lead to severe dysfunction of the lower limbs, high levels of disability, and mortality. It commonly includes both primary injury and secondary injury. Primary injury is caused by external trauma acting directly on the spinal cord, thereby causing irreversible repair. Secondary injury is the result of a series of biochemical reactions caused by a primary injury, including oxidative stress, an inflammatory response, or an increase in neuronal apoptosis [1–3]. Currently there is no effective clinical treatment for primary injury of the spinal cord. Furthermore, we know very little about the molecular mechanisms underlying secondary injury. However, the development of techniques that could reduce the number and extent of biochemical reactions that occur in secondary injury would represent a very useful step forward in the treatment of SCI.

The Pleckstrin homology (PH) domain and leucine rich repeat protein phosphatase (PHLPP) belongs to the family of Ser/Thr protein phosphatases and consists of an N-terminal portion of the PH domain structure, a leucine-rich repeat (LRR) structure domain, a PP2C phosphatase domain, and a C-terminal PDZ ligand [4,5]. There are two known sub-types, PHLPP1 and PHLPP2, expressed in hippocampal neurons [6,7]. Furthermore, PHLPP1 can promote the apoptosis of tumor cells [8,9] and inhibit the invasive ability of tumor cells, thus suppressing tumorigenesis. A recent report shows that the expression of PHLPP1 is increased in a model of myocardial ischemia-reperfusion injury [10]. In contrast, selectively inhibited PHLPP1 in primary hippocampal neurons reportedly increases the levels of pro-apoptotic factors levels [11]. Therefore, PHLPP1 may play different roles in different cells.

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Protein kinase B (AKT) survival signaling provides a major substrate for PHLPP1. High levels of PHLPP1 leads to the dephosphorylate of pAkt473, which promotes apoptosis [12,13]. Reduced expression of PHLPP1 is highly correlated with a shorter survival in patients with pancreatic ductal adenocarcinoma (PDAC), because a reduction in PHLPP1 levels leads to pAkt473 activation, and mediates the development of PDAC [14]. Furthermore, *in vitro* reports on cardiomyocytes show that knockdown of pAkt473 inhibits Nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear translocation and induces apoptosis [15]. As a transcription factor, Nrf2 binds to the Kelch-like ECH-associated protein (Keap1) in the cytoplasm in an inactive state [16]. When tissue has been injured, Nrf2 is released from the cytoplasm to the nucleus to assume an active state, simultaneously activates downstream target heme oxygenase-1 (HO-1) [17,18], and has a protective effect on damaged neuronal cells [19]. As far as we know, pAkt473/Nrf2 can regulate apoptosis in diseased states, but the regulatory mechanisms associated with the action of PHLPP1 on pAkt473/Nrf2 in the spinal cord remains unclear.

Therefore, in this study, we used AdsiPHLPP1 to silence *PHLPP1*, and thereby create *PHLPP1*-silenced models *in vivo* and *in vitro*. We then investigated the effects of *PHLPP1* silencing and its role in the regulation of pAkt473/Nrf2 and neuronal apoptosis after SCI. Our findings maybe provide evidence of the potential of *PHLPP1* as a therapeutic target gene for effective treatment following SCI.

2. Materials and methods

2.1. Animals and the establishment of an animal model for SCI

Adult female mice (weight: 18–23 g; age: 6–7 weeks) were purchased from, and fed in the specific pathogen-free Experiment Animal Center of Jinzhou Medical University. All experimental procedures were approved by the Ethics Committee of Jinzhou Medical University (Permit Number: SCXK (Liao) 2014-0004). A total of 60 adult mice were randomly assigned to four groups, five mice per group within three repeated experiments. The four groups were treated as follows: Sham (T9–10 laminectomy only); SCI; *PHLPP1* silencing (spinal cord injection AdsiPHLPP1 for 3 days); and *PHLPP1* silencing + SCI. Wild type (WT) mice were those that had not been injected with AdsiPHLPP1. A mouse model of SCI was established using Allen's method [20]. Mice were anesthetized by an injection of 10% chloral hydrate (3.0 mL/kg, i.p.). The skin and muscles were then incised along the midline, in order to perform a laminectomy at the T9–10 level. A 1 mm diameter, 3 g impactor was then dropped from a height of 50 mm onto the surface of the spinal cord at T9–10, leading to a spinal cord contusion injury. The muscles and skin were then sutured in layers. Bladders were manually expressed twice a day until normal function was recovered.

2.2. Cell culture

A six-well plate was pretreated with poly-D-lysine (Sigma-Aldrich, USA) one night before seeding. Pregnant mice were euthanized and a cesarean section was performed 13–15 days into pregnancy. The spinal cords of fetal mice were removed, minced well, and digested in papain (Solarbio, China). Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Sera Pro, Germany) and 0.5% penicillin/streptomycin (Gibco, USA) for 8 h. The culture medium was then changed to a neurobasal medium (Gibco) composed of 2% B27 (Gibco), 0.25% GlutaMAX (Gibco), and 0.5% penicillin/streptomycin (Gibco, USA), and maintained in an incubator at 37 °C, with 5% CO_2 for 7–10 days. The purity of cultured neurons was determined by immunofluorescence. Cells were treated in groups, based on the treatment groups of the mice.

2.3. Adenovirus-mediated gene silencing

2.3.1. Production of recombinant adenovirus

We used the AdMax™ system to generate the recombinant adenovirus as follows: three small interfering RNAs (siRNAs) bearing different *PHLPP1* cDNAs and an adenoviral vector bearing green fluorescent protein (GFP) were cotransfected into human embryonic kidney 293 (HEK293) cells. Recombinant adenovirus (AdsiPHLPP1) was obtained *via* the action of the Cre/loxP recombinase system. After AdsiPHLPP1 was transfected into HEK293 cells, viral plaques were allowed to develop for 15 days, after which the viral supernatant was collected. Crude viral lysates were used for plaque purification. The virus was propagated in HEK293 cells, purified by cesium chloride density gradient centrifugation, and stored at −80 °C. The resultant titer of AdsiPHLPP1 was 1×10^{12} infectious units/mL (IFU/ml).

2.3.2. Animals

Laminectomy at the level of T9–10 was carried out on mice, as described above. A 30-gauge needle was then fitted to a Hamilton syringe and used to inject 10 μL AdsiPHLPP1 or AdsiGFP (designed by Sangon Biotech, China) at rate of 2 μL per min into the exposed area of the spinal cord. After injection, the needle was slowly withdrawn and the wound was sutured, as described above. After three days, mice were euthanized and the spinal cord was resected for western blot analysis, to determine whether the AdsiPHLPP1 injection had the desired silencing effect [21]. Thus, an animal model of gene silencing was established, which had been created for study of the SCI model, as described above. All *in vivo* transduction studies used a viral vector titer of 6.0×10^{11} IFU/mL. The sequences of AdsiPHLPP1 were as follows:

AdsiPHLPP1-1: F: ccggcgcagccctgtctgtaaataactcgagttatttacagacagggctgcgttttttg,

R: aattcaaaaaacgcagccctgtctgtaaataactcgagttatttacagacagggctgcg.

AdsiPHLPP1-2: F: ccggggctgtctcattcgattctatctcgagagaatcgaatgagacagccaatttttg,

R: aattcaaaaaattggctgtctcattcgattctctcgagatagaatcgaatgagacagcc.

AdsiPHLPP1-3: F: ccgggctgagtgaaatcacgttaccctcgagtaacgtgatttcactcagctctttttg,

R: aattcaaaaaagagctgagtgaaatcacgttactcgagggtaacgtgatttcactcaga.

2.3.3. Primary neurons

Primary neuronal culture was carried out as described above. On day 7, cells were transfected with AdsiPHLPP1 in serum-free medium diluted to a multiplicity of infection (MOI) of 40 for 8 h [22–24]. Fresh medium was then added and cells were left to incubate for an additional 3 days [25]. Cells were collected for protein analysis or observation of the effects of transfection under a fluorescence microscope.

2.4. Cell viability assay

Cultured cells were seeded in a 96-well plate at a density of 1×10^3–1×10^4 cells/mL. Cells were then treated with 25, 50, 100, and 200 μmol/L H_2O_2, applied for 4, 8, 12, 24, and 36 h, respectively. The MTT (5 mg/mL) (Sigma-Aldrich, USA) solution was prepared in phosphate buffer saline (PBS), filtered, and added to each well, after which the plates were incubated for 4 h at 37 °C. Subsequently, purple formazan crystals were dissolved in 150 μL of dimethyl sulfoxide (DMSO). The absorbance was then measured at a wavelength of 470 nm. Data were calculated as the proportion of surviving cells using the following formula:

$$\text{Survival} = A_t/A_c \times 100\%$$

where A_t and A_c are the absorbance of the treatment and control groups, respectively.