

# Low-intensity extracorporeal shock wave therapy promotes recovery of sciatic nerve injury and the role of mechanical sensitive YAP/TAZ signaling pathway for nerve regeneration

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## Abstract

**Background:** Histological and functional recovery after peripheral nerve injury (PNI) is of significant clinical value as delayed surgical repair and longer distances to innervate terminal organs may account for poor outcomes. Low-intensity extracorporeal shock wave therapy (LiESWT) has already been proven to be beneficial for injured tissue recovery on various pathological conditions. The objective of this study was to explore the potential effect and mechanism of LiESWT on PNI recovery.

**Methods:** In this project, we explored LiESWT's role using an animal model of sciatic nerve injury (SNI). Shockwave was delivered to the region of the SNI site with a special probe at 3 Hz, 500 shocks each time, and 3 times a week for 3 weeks. Rat Schwann cells (SCs) and rat perineurial fibroblasts (PNFs) cells, the two main compositional cell types in peripheral nerve tissue, were cultured *in vitro*, and LiESWT was applied through the cultured dish to the adherent cells. Tissues and cell cultures were harvested at corresponding time points for a reverse transcription-polymerase chain reaction, Western blotting, and immunofluorescence staining. Multiple groups were compared by using one-way analysis of variance followed by the Tukey-Kramer test for *post hoc* comparisons.

**Results:** LiESWT treatment promoted the functional recovery of lower extremities with SNI. More nerve fibers and myelin sheath were found after LiESWT treatment associated with local upregulation of mechanical sensitive yes-associated protein (YAP)/transcriptional co-activator with a PDZ-binding domain (TAZ) signaling pathway. *In vitro* results showed that SCs were more sensitive to LiESWT than PNFs. LiESWT promoted SCs activation with more expression of p75 (a SCs dedifferentiation marker) and Ki67 (a SCs proliferation marker). The SCs activation process was dependent on the intact YAP/TAZ signaling pathway as knockdown of TAZ by TAZ small interfering RNA significantly attenuated this process.

**Conclusion:** The LiESWT mechanical signal perception and YAP/TAZ upregulation in SCs might be one of the underlying mechanisms for SCs activation and injured nerve axon regeneration.

**Keywords:** Low-intensity extracorporeal shock wave therapy; Schwann cells; Sciatic nerve injury; YAP/TAZ

## Introduction

Peripheral nerve injury (PNI), a result of damage to the nerves outside of the brain and spinal cord, is an intractable problem in the clinical scenario.<sup>[1,2]</sup> The knowledge of PNI came mainly from experiences on the battlefield historically, but it is not uncommon in modern times in non-combat related trauma cases such as motor vehicle accidents, gunshots, and industrial accidents. These injuries can cause weakness, numbness, and pain which finally lead to significant disabilities and potential lifelong implications.<sup>[1]</sup>

The peripheral nerve trunks can be classified into myelinated or unmyelinated axonal fibers which are mechanically and metabolically supported by myelinated

Schwann cells (SCs) or non-myelinated SCs, respectively.<sup>[3]</sup> These precise structures are further wrapped by three separate layers of connective tissues composed of perineurial fibroblasts (PNFs) which are known as epineurium, perineurium, and endoneurium.<sup>[4]</sup> Axonal injuries usually undergo an anterograde degeneration process distal to the injury location known as Wallerian degeneration that is associated with axonal discontinuity, myelin fragmentation, macrophage scavenging followed by SCs/PNFs activation, and axonal regrowth which can last 4 to 8 weeks long.<sup>[5]</sup> Surgery repair remains the main treatment modality for PNI, but due to the influence of multiple factors (such as surgical repair technique, operation time, and regrowth distance), the consequences of PNI vary from local fibrous scar (failure of axonal regrowth) to full recovery of the end organ (complete axonal regrowth and reinnervation). Axonal regeneration has an estimated rate

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of 1 mm/day and approaches to maintain or accelerate this process will promote histological and functional recovery of end organs, especially in higher degree PNIs.<sup>[6]</sup>

Low-intensity extracorporeal shock wave therapy (LiESWT) is a kind of mechanical therapy that usually involves delivering vibration energy to local tissue through a special probe. LiESWT has been investigated in various clinical settings<sup>[7-9]</sup> and, in our previous research, we have found indirect evidence of LiESWT for promoting injured small nerve fibers recovery.<sup>[10-12]</sup> Meanwhile, research from other investigators also illustrated that low-level mechanical stimuli might activate SCs mitogenic pathways which are independent of the crosstalk between SCs and axons after axonal injury.<sup>[13-15]</sup> The special multilayer structure of peripheral nerves and the physiological property of SCs might make them more sensitive to applied mechanical energy such as LiESWT or other physical stimuli.<sup>[3,16]</sup>

Based on these findings, we hypothesized that SCs could receive mechanical stimuli from LiESWT. The following SCs activation process promotes axonal regeneration which might lead to better histological and function recovery of PNI. The effect of LiESWT was explored using a rat model of sciatic nerve injury (SNI) in this project. Because yes-associated protein (YAP) and transcriptional co-activator with a PDZ-binding domain (TAZ) which are central to the mammalian Hippo pathway have been proven to be the vital mechanosensitive transcription factors that regulate SCs proliferation, differentiation, and myelination during axon-SCs recognition and nerve regeneration,<sup>[17,18]</sup> we focus mainly on the dynamic changes of YAP/TAZ signaling pathway after LiESWT administration. The potential mechanism was further investigated on *in vitro* cultured cells of rat SCs and rat PNFs. Our study would provide preliminary data on how LiESWT was received by local injured nerve tissue and whether LiESWT is a potential candidate in promoting PNI recovery.

## Methods

### Experimental design

All animal experiments were approved by the ethics committee of Peking University First Hospital (Approval No: 201875, comply with the Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA] guidelines). SNI was performed as previously described<sup>[19]</sup> in adult male Sprague-Dawley rats between 7 and 8 weeks of age. In brief, the sciatic nerve was exposed under anesthesia. The nerve was crushed once for 30 s and again for another 30 s at the same site but orthogonal to the initial crush using a pair of delicate forceps (0.4 mm tip angled, Fine Science Instruments, North Vancouver, B.C., Canada). Sham surgery was conducted on the contralateral leg of the same rat and all procedures were the same except no nerve was crushed. All animals undergoing surgery were given appropriate post-operative analgesia and monitored daily. Functional analysis was conducted based on the following grouping method ( $n = 10$  for each group) [Figure 1A and 1B]: Sham surgery and mock

treatment (Sham), SNI and mock treatment (SNI), Sham surgery and LiESWT treatment (Sham + LiESWT), SNI and LiESWT treatment (SNI + LiESWT). After all the LiESWT treatment and functional studies, rats were sacrificed and sciatic nerve tissue (distal to the crush site) was collected for histological and molecular analysis. To further investigate the potential mechanism of LiESWT, *in vitro* cultured rat SCs (RSC96, ATCC CRL-2765, Manassas, VA, USA) and rat PNFs (R1710, ScienCell, Carlsbad, CA, USA) were used in the cell-based experiments. The cells were cultured according to the manufacturers' instructions.

### Low-intensity extracorporeal shock wave therapy

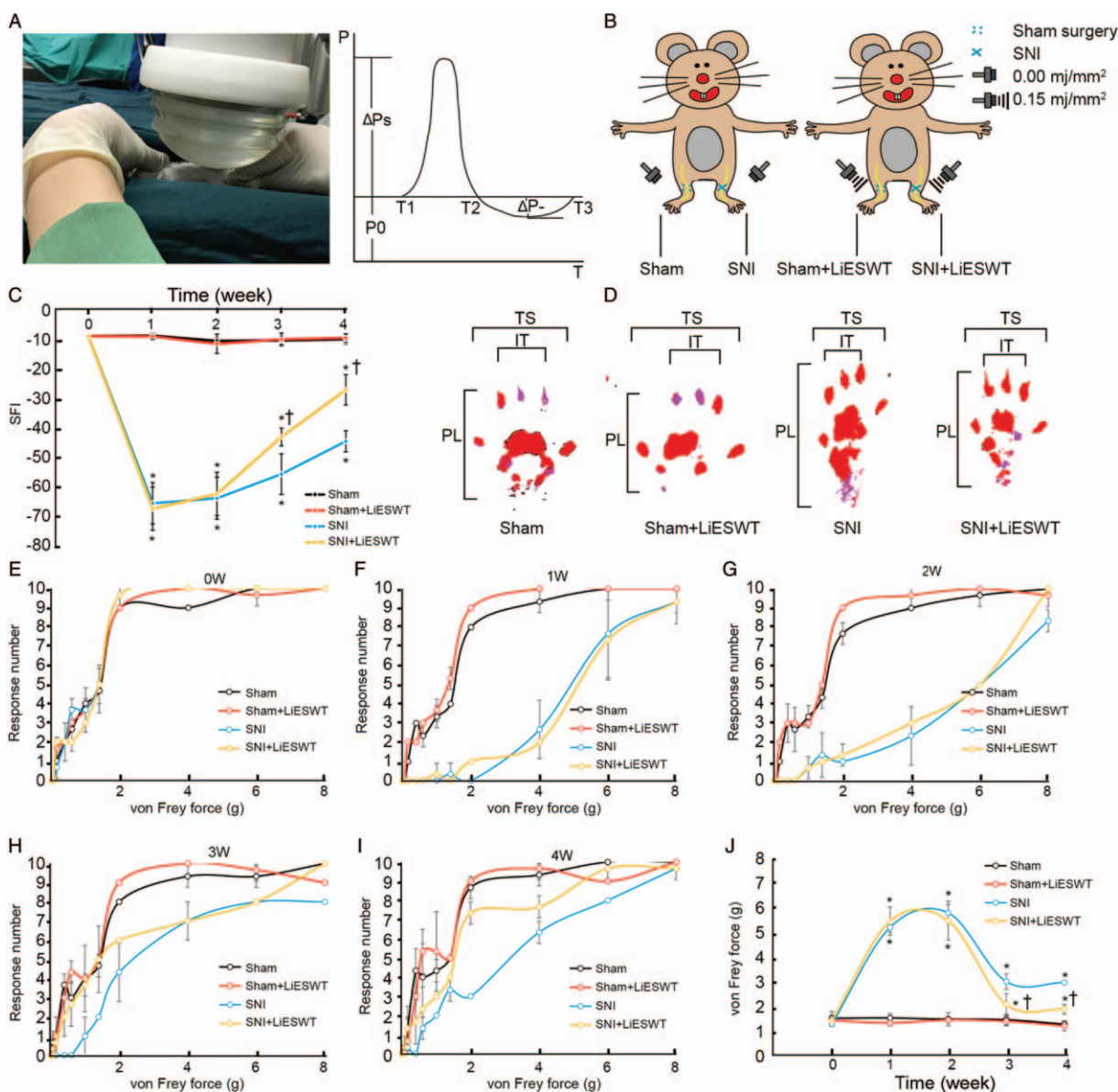
For the animal experiments, the shockwave was delivered to the region of the SNI site with a special probe that was attached to a compact electrohydraulic unit with a focused shockwave source (HaiBing Medical Equipment Limited Corporation, Zhanjiang Economic and Technological Development Zone, Guangdong, China). Under isoflurane anesthesia, each rat was placed in the supine position with its lower extremities shaved and sterilized with 75% alcohol. Standard commercial ultrasound gel (Aquasonic, Parker Laboratories Inc., Fairfield, NJ, USA) was applied between the probe and the skin of the limb region for optimal coupling [Figure 1A]. The treatment was applied at the energy flux density (maximum amount of acoustical energy that is transmitted through an area of 1 mm<sup>2</sup>/pulse) of 0.00 mJ/mm<sup>2</sup> (Sham group and SNI group) or 0.15 mJ/mm<sup>2</sup> (Sham + LiESWT group and SNI + LiESWT group) at 3 Hz (3 shocks/s), 500 shocks each time, and 3 times a week for 3 weeks. The course of treatment was designed based on the clinical application of LiESWT and this was also the course similar to what we used in our previous animal research.<sup>[10,20]</sup> For the *in vitro* experiment, LiESWT treatment was applied to cultured cells. Rat SCs or rat PNFs received one-time mock treatment (0.00 mJ/mm<sup>2</sup>) or LiESWT treatment (0.03–0.12 mJ/mm<sup>2</sup>, 500 pulses at 3 Hz) after reaching 60% to 80% confluence. The probe was handled under the cell culture dish with standard commercial ultrasound gel applied between the dish and probe. The cells were then harvested for the following molecular studies at corresponding time points.

### Small interfering RNA (siRNA) transfection

For siRNA-mediated knockdown, cells were reverse transfected as the cells were plated using Lipofectamine RNAiMAX (Life Technologies, 13778150, Eugene, OR, USA) according to the manufacturer's protocol. The following siRNAs were used: control siRNA (Thermo Scientific, 4390843, South San Francisco, CA, USA) and TAZ siRNA (Thermo Scientific, s148960, 4390771). Three days after transfection, cells were used for the following experiments and analysis.

### Rat functional tests

Functional tests were conducted before the surgical procedure (0 week), after the surgical procedure (1 week), and after each week's treatment procedure (2/3/4 weeks). The rat's sciatic functional index (SFI) measurement was



**Figure 1:** LIESWT administration and functional recovery of SNI. (A) Sketch of LIESWT treatment application and a typical waveform of LIESWT. (B) Grouping method for the *in vivo* experiment. (C) Calculation of SFI among different groups. (D) Typical footprints of different groups. (E–J) Positive response number to stimulation with von Frey filaments of increasing size on a sole region at different time points. (J) Calculation of 50% threshold forces at different time points. \**P* < 0.05 compared with Sham; †*P* < 0.05 compared with SNI. Δ*P*: Pressure change; IT: Intermediary, 2–4 toe spread; LIESWT: Low-intensity extracorporeal shock wave therapy; P0: Initial pressure; PL: Print length; SFI: Sciatic functional index; SNI: Sciatic nerve injury; TS: 1–5 toe spread.

used to assess sensory-motor coordination in rats. Tactile sensitivity tests were used to assess the recovery of sensory function (pressure sensitivity). For SFI measurement, 1 to 5 toe spread (TS), 2 to 4 TS (intermediary [IT]), and print length (PL) were calculated based on footprint analysis. TS and PL are believed to be dependent on both tibial and peroneal divisions of the sciatic nerve, while the IT is believed to be dependent on the tibial division alone. SFI was calculated as the following formula:  $SFI = 38.3 \times (PL_n - PL_0) / PL_0 + 109.5 \times (TS_n - TS_0) / TS_0 + 13.3 \times (IT_n - IT_0) / IT_0 - 8.8$ . Tactile sensitivities of the sole region were measured using a series of 14 von Frey filaments with increasing calibrated forces from 0.008 to 8.0 g (Stoelting, Wood Dale, IL, USA). Beginning with the smallest filament, each filament has applied a total of 5 times for 3 s, with intervals of 8 s between each stimulus. The following

behaviors were considered to be positive responses: (1) retraction of lower limb, (2) instant licking of the sole area, and (3) jumping. The rat will be count as positive if the response positive for >3 times out of 5 times ( $\geq 3/5$ ). The number of rats with positive response was recorded. In addition, the von Frey force which would elicit a positive response in half of the rats in each group (50% threshold) was also calculated and compared among different groups.

### Histological analysis and immunofluorescence staining

Sciatic nerve tissue or cultured cells on coverslips was freshly harvested and fixed and immunofluorescence staining was performed as previously described.<sup>[10]</sup> The primary antibodies included anti-neurofilament (NF, 1:500, ab8135, Abcam, Cambridge, MA, USA), anti-

myelin basic protein (MBP, 1:200, ab40390, Abcam), anti-TAZ (1:300, 4883, Cell Signaling Technology, South San Francisco, CA, USA), and anti-Ki67 (1:500, ab15580, Abcam). Secondary antibodies used included Alexa-594-conjugated antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole. Image analysis was performed by computerized densitometry (K pixel number of integrated optical density) using Image-Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD, USA) for six randomly chosen visual fields.

### Real-time quantitative reverse transcription-polymerase chain reaction (PCR)

Freshly harvested cells were immediately homogenized in TRIzol reagent followed by isolation of total RNA. Complementary DNA (cDNA) was synthesized from the RNA using a SuperScript III cDNA synthesis kit with random hexamer primers (Invitrogen, Carlsbad, CA, USA). Expressions of the following genes were analyzed: TAZ (GTTCCAGCTCGTCAGTTCGTGCGTGACGTG-GATGACT), YAP (CAGGAATTATTTTCGGCAGGA-CATCCTGCTCCAGTGTAGGC), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (AGGTCGGTGT-GAACGGATTTGTGTAGACCATGTAGTTGAGGTCA). Quantitative PCR was performed using the SYBR Green PCR Master kit with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

### Western blotting

Protein isolation and Western blotting were conducted as previously reported.<sup>[21]</sup> A total of 20 mg protein was loaded for each sample. The primary antibodies used in Western blotting were anti-TAZ (1:500, 4883, Cell Signaling Technology), anti-YAP (1:500, 4912, Cell Signaling Technology), anti-Phospho-YAP (Ser127, 1:200, 13008, Cell Signaling Technology), anti-Phospho-TAZ (Ser89, 1:200, 59971, Cell Signaling Technology), anti-p75 (1:300, ab3125, Abcam), anti-Cleaved Caspase-3 (Asp175, 1:800, 9661, Cell Signaling Technology), and anti- $\beta$ -actin (1:1000, ab8227, Abcam). After incubation with the secondary antibody, the resulting images were analyzed with ChemImager 4000 (Alpha Innotech Corp., San Leandro, CA, USA) to determine the integrated density value of each protein band.

### Statistical analysis

Results were analyzed using Prism 5 (GraphPad Software, San Diego, CA, USA) and are expressed as mean  $\pm$  standard deviation. Multiple groups were compared by using one-way analysis of variance followed by the Tukey-Kramer test for *post hoc* comparisons (four or five variables). Statistical significance was set at  $P < 0.05$ .

## Results

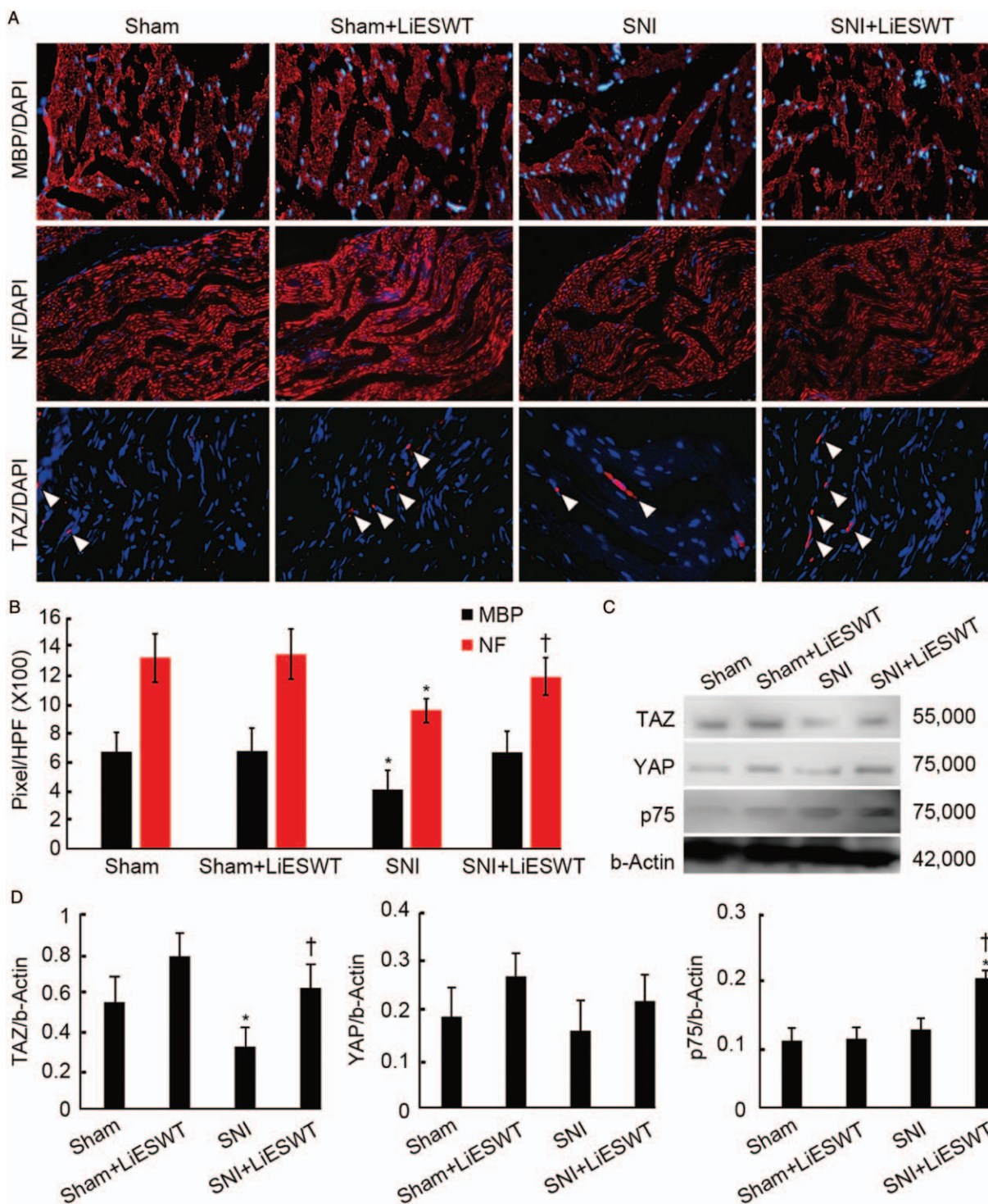
### LiESWT promoted functional recovery of SNI in rats

SFI values were calculated and compared among groups before the surgical procedure (0 week), after the surgical

procedure (1 week), and after each week's LiESWT treatment procedure (2/3/4 weeks) [Figure 1C]. Baseline SFI values were similar among the four groups (0 week). SNI procedure significantly decreased SFI value in groups SNI and SNI + LiESWT (1 week,  $F = 212.791$ ,  $P < 0.05$  compared with Sham), and no fully functional recovery was recorded in both groups until 4 weeks ( $F = 123.722$ ,  $P < 0.05$  compared with Sham). LiESWT treatment, however, partially improved sensory-motor coordination in group SNI + LiESWT and significant changes happened at 3 weeks ( $F = 170.601$ ,  $P < 0.05$  compared with SNI) and 4 weeks ( $F = 123.722$ ,  $P < 0.05$  compared with SNI). According to the footprint analysis, rats had a better ability to abduct their toes and lift the heel after LiESWT treatment reflected as increased TS (TS and IT) and decreased PL [Figure 1D]. Tactile sensitivities of the sole region were further measured using a series of 14 von Frey filaments [Figure 1E-I]. Mechanical stimulation with von Frey filaments of increasing size on the sole region resulted in an increasing percentage of rats with positive responses. This trend was lagged after SNI as a lower level of filaments force induced significantly less positive response in group SNI and SNI + LiESWT. LiESWT treatment partially improved the lagged response, potentially indicating better sensory perception recovery. Calculation of 50% threshold forces (the average force that could induce half of the rats to have a positive response) revealed a significantly higher tactile response threshold in SNI and SNI + LiESWT rats [Figure 1J]. Two to 3 weeks' LiESWT treatment raise the tactile sensitivity, and the 50% threshold forces decreased significantly in SNI + LiESWT group compared with the SNI group ( $F = 17.912$ ,  $P < 0.05$  at 3 weeks;  $F = 85.989$ ,  $P < 0.05$  at 4 weeks).

### Nerve regeneration promoted by LiESWT was accompanied by upregulation of the YAP/TAZ signaling pathway

To examine the histological changes of sciatic nerve fibers after SNI and LiESWT treatment, the amount of myelin sheath and axon were examined using immunofluorescence staining of MBP (a marker of myelin sheath) and NF (a marker of an axon) [Figure 2A]. The result showed that SNI was associated with significantly less axon and myelin sheath distal to the crush site after 3 weeks. LiESWT treatment significantly promoted axonal regrowth and myelination represented as increased expression of MBP and NF 3 weeks after SNI ( $F = 4.259$ ,  $P < 0.05$  and  $F = 7.677$ ,  $P < 0.05$ , respectively) [Figure 2B]. We further examined the expression of YAP and TAZ, the two mechanosensitive transcription factors [Figure 2C and 2D]. LiESWT administration did increase the expression of YAP and TAZ compared with the non-treated groups, and this phenomenon was observed even in the Sham + LiESWT group ( $F = 13.382$ ,  $P < 0.05$  for TAZ expression). The expression of p75, which is a marker for SC dedifferentiation, however, was only significantly increased in the SNI + LiESWT group ( $F = 41.894$ ,  $P < 0.05$  compared with all the other three groups). These indicated that specific cell types in the neural tissue did respond to the LiESWT stimuli. However, the following cellular responses after energy perception might vary according to the surrounding environment (eg, if an injury had happened or if a repair process was needed).

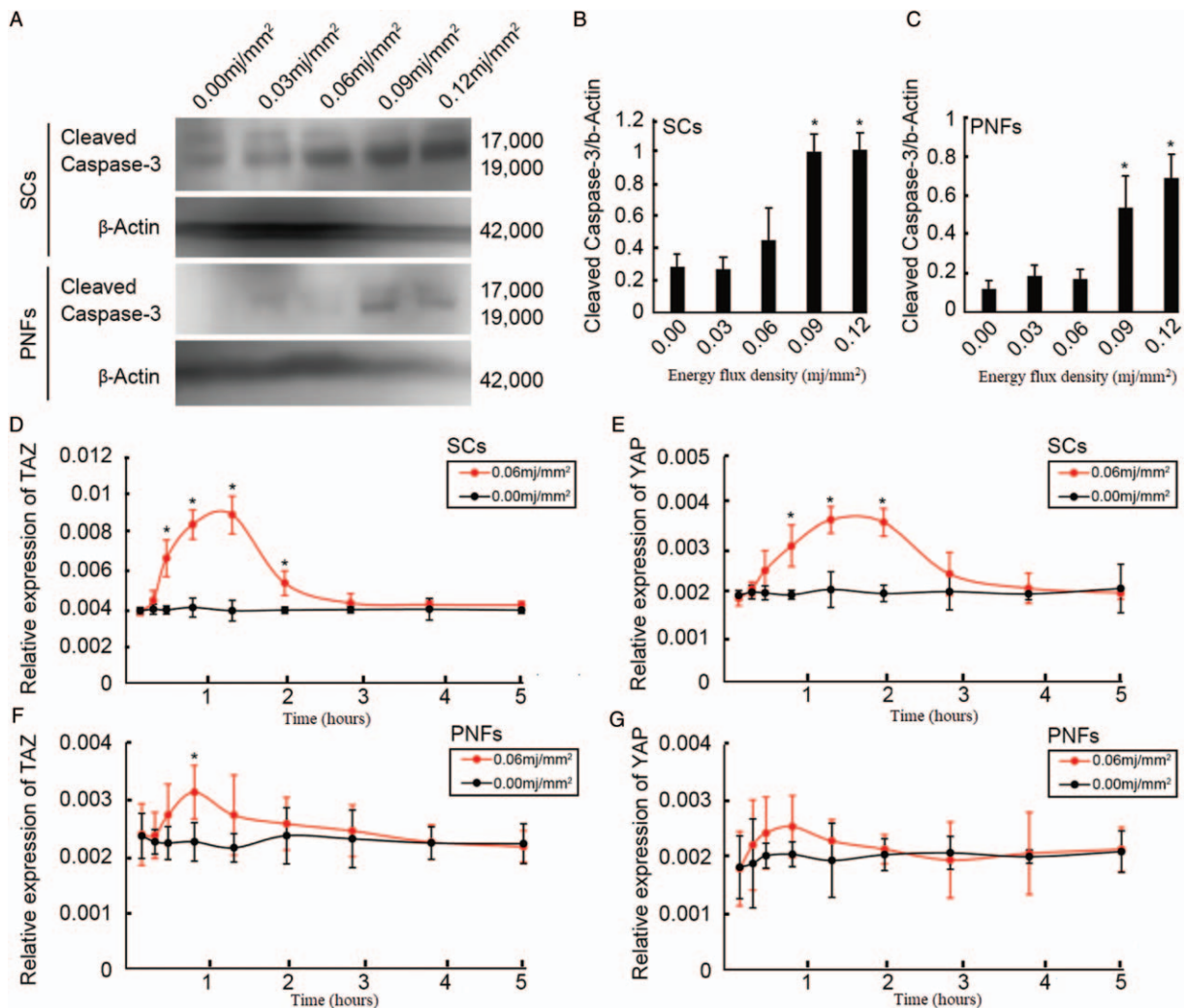


**Figure 2:** Histological recovery of SNI. (A) Representative immunofluorescence staining of MBP (cross-section, original magnification is 200×), NF (longitudinal section, original magnification is 200×), and TAZ (longitudinal section, original magnification is 400×). (B) Calculation of average optical density of MBP and NF. (C) Representative Western blotting examination of protein levels of TAZ, YAP, and p75. (D) Calculation of Western blotting results of TAZ, YAP, and p75 based on bands' grayscale. \**P* < 0.05 compared with Sham; †*P* < 0.05 compared with SNI. DAPI: 4',6-Diamidino-2-phenylindole; HPF: High power field; LiESWT: Low-intensity extracorporeal shock wave therapy; MBP: Myelin basic protein; NF: Neurofilament; SNI: Sciatic nerve injury; TAZ: Transcriptional co-activator with a PDZ-binding domain; YAP: Yes-associated protein.

**SCs responded to a specific dosage of LiESWT in a timely manner**

To examine the specific cell response, *in vitro* studies were carried out using the cell culture of the two main cell types of peripheral neural tissue: rat SCs and rat PNFs. The dose

of LiESWT was adjusted first as *in vitro* cultured cells might be more vulnerable to external stimuli compared with *in vivo* circumstances. The status of apoptosis was examined at various dosages of LiESWT from 0.00 to 0.12 mJ/mm<sup>2</sup> based on the expression of apoptosis marker cleaved Caspase-3 [Figure 3A–C]. Both rat SCs and



**Figure 3:** Dose of LiESWT and checking time points selection for *in vitro* experiments. (A) Representative Western blotting examination of protein levels of cleaved Caspase-3 in SCs and PNFs at a different dosage of LiESWT. (B, C) Calculation of Western blotting results of cleaved Caspase-3 in SCs and PNFs. (D, E) Calculation of RT-PCR results of relative expression of TAZ and YAP in SCs at different time points after LiESWT administration. (F, G) Calculation of RT-PCR results of relative expression of TAZ and YAP in PNFs at different time points after LiESWT administration. \**P* < 0.05 compared with 0.00 mJ/mm<sup>2</sup>. LiESWT: Low-intensity extracorporeal shock wave therapy; PNFs: Perineurial fibroblasts; SCs: Schwann cells; TAZ: Transcriptional co-activator with a PDZ-binding domain; YAP: Yes-associated protein; RT-PCR: Reverse transcription-polymerase chain reaction.

rat PNFs exhibited significantly increased apoptosis at the level of 0.09 and 0.12 mJ/mm<sup>2</sup>. So, the dosage of LiESWT was set at 0.06 mJ/mm<sup>2</sup> for the following cell-based studies. The expression of YAP and TAZ was used as an indicator for cell response to LiESWT stimuli and their expression was examined using real-time reverse transcription PCR at different time points after LiESWT administration. Rat SCs' response was recorded most dramatically at 1 to 2 h post-administration [Figure 3D and 3E]. Meanwhile, rat PNFs did have some response to LiESWT stimuli, but in a much milder way [Figure 3F and 3G]. Rat SCs were believed to be the main cell type of stimuli perception and; therefore, be used as the following cell-based experiments.

**LiESWT promoted the activation of rat SCs through YAP/TAZ pathway**

To examine the role of the YAP/TAZ signaling pathway in the activation process of SCs, TAZ siRNA was used to

knockdown the expression of TAZ. The prior added TAZ siRNA significantly decreased the amount of TAZ mRNA no matter whether LiESWT administration was added or not (1 h after LiESWT treatment, *F* = 55.465, *P* < 0.05) [Figure 4A]. The expression level and distribution of TAZ protein were further examined 4 h after LiESWT administration in different groups [Figure 4B]. Rat SCs were classified as the following rule: negative (N, no TAZ was detected), peripheral (P, TAZ was distributed mainly in the plasma), central+ (C+, TAZ was distributed mainly in the nucleus but covered < 50% of the nucleus area), and central++ (C++, TAZ was distributed mainly in the nucleus, covered > 50% of the nucleus area). Without prior TAZ knockdown, LiESWT increased the percent of C+ and C++ SCs which indicating more TAZ protein was acting as a transcription factor in the nuclei. The expression of TAZ and YAP protein was further confirmed with Western blotting [Figure 4C]. Without prior TAZ knockdown, LiESWT significantly increased the amount of TAZ expression (4 h after LiESWT treatment,



LiESWT treatment under the circumstances of TAZ siRNA knockdown ( $F=4.906$ ,  $P<0.05$ ) indicating the increased degradation process for relative surplus YAP protein. SCs activation is a complex process that is characterized by dedifferentiation, redifferentiation, proliferation, and maturation. Here, we examined the expression of SCs dedifferentiation marker p75 (4 h after LiESWT treatment) and SCs proliferation marker Ki67 (6 h after LiESWT treatment) to further investigate the SCs activation process after perception of LiESWT stimuli [Figure 4C and 4D]. Our results illustrated that both markers are significantly increased after LiESWT treatment only in the presence of adequate TAZ expression (without TAZ knockdown,  $F=25.177$  and  $14.741$ , respectively,  $P<0.05$ ) which further proved the vital role of intact YAP/TAZ signaling pathway for the activation of SCs under LiESWT stimuli.

## Discussion

Physical therapy or physiotherapy is a broad category associated with evidence-based therapeutic forms such as exercise, immobilization, electric current, light/acoustic agents, or mechanical stimuli.<sup>[22,23]</sup> These treatments have been frequently used in the management of physical impairments resulting from trauma or other medical conditions typically of musculoskeletal, cardiovascular, respiratory, and neurological origins.<sup>[24,25]</sup> The mechanisms under these treatment modalities, however, are mostly under-investigated due to the conflicting nature between “invisible energy” of physical therapy and “molecular interaction” of classic modern molecular biology. The same goes for LiESWT, which is a kind of mechanical stimuli that have been investigated in various preclinical and clinical settings, such as myocardial ischemia, musculoskeletal injury, neurodegenerative disease, and erectile dysfunction.<sup>[7-12]</sup> Our present study does show the direct evidence of LiESWT on promoting the functional and histological recovery of SNI on a rat model, and we also find some clues of mechanotransduction related to SC activation which might be the underlying mechanism for LiESWT's beneficial effect on PNI recovery.

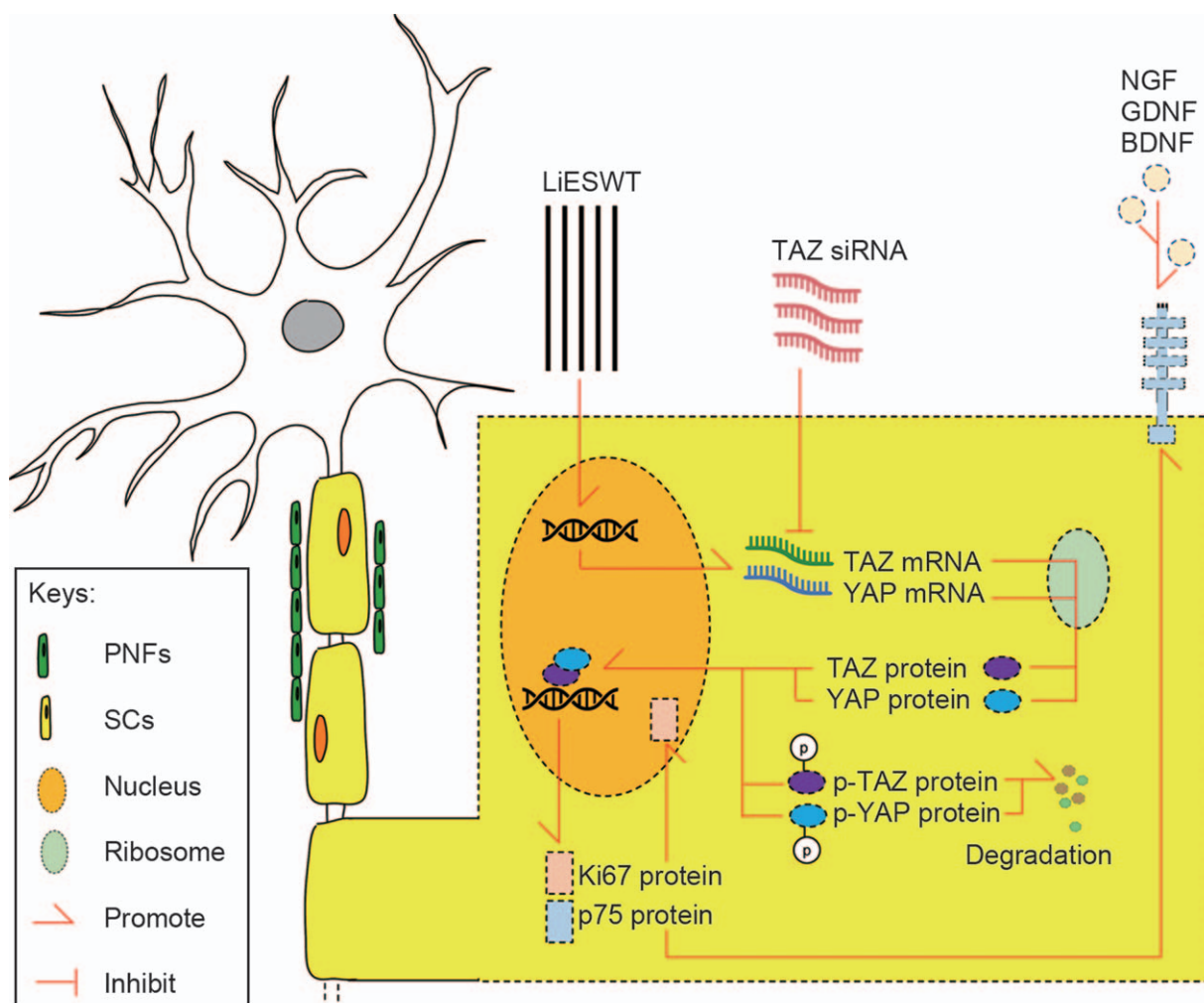
LiESWT is a kind of longitudinal propagating mechanical wave that can penetrate the skin and soft tissue up to 10 cm without significant energy loss and tissue damage.<sup>[26]</sup> During LiESWT administration, the targeted tissues, extracellular matrix (ECM), and cells are experiencing extremely high frequent extrusion force and shear force, and most energy resorption happens at the interface of different “medium” (different inherent properties in wave reflection and refraction), such as the sites of periosteum, perimysium, or perineurium.<sup>[27,28]</sup> The mechanical force (invisible energy) acting on cells eventually results in deformations of cellular structure. But to be recognized by cells as a signal, the deformation must be converted into a biochemical signal (molecular interaction). The majority of cell surface receptors that have been identified to date are characterized by their ability to respond to chemical factors.<sup>[29]</sup> But for some perceptions (such as hearing and touch), they are dependent on mechanical forces. Mechanosensors which are a collection of tethered proteins, enzymes, and ion channels have long been hypothesized to

be able to “feel” the force and translate it into a biochemical signal.<sup>[30,31]</sup> Peripheral nerve tissues which are essentially axonal fibers mechanically and metabolically supported by SCs and wrapped by multiple connective tissues composed by PNFs have long been regarded as mechanical stress-sensitive during development and regeneration.<sup>[32]</sup> Based on previous reports<sup>[33,34]</sup> and our present *in vivo* and *in vitro* results, we believe SCs belong to the ones that respond to a certain dosage of LiESWT stimuli timely in peripheral neural tissue. SCs are surrounded by ECM and the LiESWT signals might transmit through the ECM and the SCs basal side. Certain molecules in ECM (eg, laminins or collagens) and certain proteins on the basal side (eg, integrins or G protein-coupled receptors) might act as mechanosensors for LiESWT in SCs.<sup>[3]</sup>

The molecular aspects of mechanosensors including the mechanosensors in ECM and SCs basal side working as the upstream of mechanotransduction unfortunately remain largely unknown. But the downstream changes in enzymatic activity, gene transcription, and cell behavior could be investigated in a much clearer way. The most notable pathway that responds to actomyosin cytoskeleton transformation and focal adhesion activation in SCs is Hippo signaling pathway that coordinates SCs proliferation, apoptosis, and differentiation.<sup>[17]</sup> In fact, the Hippo signaling pathway has emerged as a major regulator of many tissue development/regeneration processes and central to the mammalian Hippo pathway is the action of the transcriptional regulators' YAP and TAZ, which are controlled by a kinase cascade that is very sensitive to mechanosensory and cell polarity cues.<sup>[35]</sup> Grove *et al*<sup>[18,36]</sup> have found out that YAP/TAZ promotes differentiation of immature SCs and YAP/TAZ dramatically disappears from SCs of adult mice concurrent with axon injury. But they reappear in SCs only if axons regenerate. Work from Sophie *et al*<sup>[37]</sup> showed that ablation of YAP/TAZ alters the expression of transcription regulators Cc2d1b and Pur $\beta$  known to regulate SCs myelination. Other researchers also have illustrated the relationship of YAP/TAZ with additional proteins in SCs such as G $\alpha$ s protein,<sup>[17]</sup> Pmp22,<sup>[38]</sup> and laminin receptors<sup>[34]</sup> which are key regulators for normal SCs function. In the present study, we do find upregulation of TAZ and YAP after LiESWT administration in SCs. More TAZ and YAP proteins are believed to translocate into the nucleus and act as one integrated transcriptional complex for related gene expression modification. The phosphorylation status of TAZ and YAP (p-TAZ and p-YAP) which are their inactive forms are not affected by LiESWT administration. With TAZ knockdown in the *in vitro* studies, increased p-YAP/YAP ratio after LiESWT might indicate the increased degradation process for relative surplus YAP protein, because no adequate TAZ protein could bind with YAP to perform its function.

SCs have surprising plasticity. It has been proven that SCs possess the ability to dedifferentiate from myelinated SCs to become repair SCs or Bungner cells with c-Jun-dependent myelin protein loss and p75 protein upregulation after axon injury.<sup>[3,19]</sup> The p75 protein is a kind of neurotrophin receptor located in the cell membrane which





**Figure 5:** A hypothetical scheme of the Hippo signaling pathway affected by LiESWT. BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell-derived neurotrophic factor; LiESWT: Low-intensity extracorporeal shock wave therapy; NGF: Nerve growth factor; p: Phosphorylation; PNFs: Perineurial fibroblasts; SCs: Schwann cells; TAZ: Transcriptional co-activator with a PDZ-binding domain; YAP: Yes-associated protein.

could bind to various neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor, and glial cell-derived neurotrophic factor to induce SCs fate shift.<sup>[39]</sup> The dedifferentiated SCs then undergo redifferentiation, proliferation, and maturation. Myelination of growing axons by SCs is a complicated process with a strict polarity that has not been fully understood.<sup>[3]</sup> An apicobasal polarity is initially established by the axon and ECM and with the growth of axon, a longitudinal polarity is established marked as nodes of Ranvier, compact myelin, and uncompact myelin (Schmidt-Lanterman incisures).<sup>[3]</sup> In the present study, we find upregulated SCs dedifferentiate marker p75 protein and cell proliferation marker Ki67 after LiESWT treatment indicating promoted SCs activation process *in vitro*. This might explain the improved myelination (marked as MBP expression) and axon regeneration (marked as NF expression) process in SNI rats after 3 weeks' LiESWT treatment which finally lead to better performance in sensory-motor coordination test and tactile sensitivities test *in vivo*. The SCs activation process, however, is significantly suppressed in the circumstances of TAZ knockdown *in vitro* indicating that intact Hippo signaling pathway might be the “bridge” between LiESWT

administration and SCs activation [Figure 5]. The SCs activation process is also not active if it is not needed *in vivo*. This could explain why the upregulation of TAZ and YAP in the Sham + LiESWT group (indicating mechano-transduction does happen) was not associated with increased p75 expression (indicating no SCs activation process followed).

Last but not least, we should be aware that tissue function arises from the coordinated behavior of cells in both time and space.<sup>[29]</sup> A cell's response to external stimuli is largely dictated by its interactions with the ECM, neighboring cells, and soluble factors from the microenvironment *in vivo*. The study of molecules and signaling out of context *in vitro* is therefore only partially reflect the true basic structure-function relationships of molecular interactions *in vivo*.<sup>[40]</sup> SCs activation process led by the non-invasive mechanical stimulus of a certain magnitude of LiESWT involves the orchestration of mechanosensors and mechano-transduction that might be different to some extent for *in vivo* and *in vitro* situations.<sup>[17,41]</sup> Based on our present results, SCs also responded more dramatically to LiESWT with more obvious changes in the expression of YAP/TAZ

protein than PNFs. We believe that the LiESWT application would be beneficial for SCs activation and axon regeneration in clinical scenarios involving PNI. The activity of PNFs is another vital factor involved in nerve regeneration as excessive fibrous scar caused by over-activation of PNFs could hinder axonal passages which is a common reason for failure functional recovery in the clinical field. We might explore more on the biology of PNFs influenced by LiESWT as well as other mechanosensitive signaling pathways that might also be activated in SCs in the future.

In conclusion, LiESWT promotes histological and functional recovery of SNI in a rat model. This process is associated with the upregulation of the YAP/TAZ signaling pathway and the activation process of SCs with increased expression of p75 and Ki67.

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### Conflicts of interest

None.

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