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TGF-β1/P-Snail1-NGF signaling axis mediates the repair of peripheral nerve injury in rats

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Abstract

In the repair process of peripheral nerve injury, Schwann cells rapidly migrate to the injury site, where they form a neural sheath and secrete nerve growth factor (NGF) to facilitate nerve regeneration. Enhancing Schwann cell migration and boosting NGF secretion from Schwann cell are thus critical for effective nerve repair. Our study demonstrates that in the presence of transforming growth factor-beta 1 (TGF-β1), NGF secretion by Schwann cells is significantly upregulated in a concentration- and time-dependent manner. Importantly, we reveal that TGF-β1 through the TGF-β1/Smad3 signaling pathway promotes the phosphorylation of Snail1 as transcription factor. Snail1 phosphorylation has been shown to enhance its binding affinity to the NGF promoter, thereby directly upregulating NGF transcription. This mechanism is critical for promoting peripheral nerve regeneration and repair processes in response to injury.The use of Snail1 phosphorylation inhibitors, such as LiCl, has demonstrated a significant reduction in NGF expression, highlighting the necessity of Snail1 phosphorylation for the upregulation of NGF transcription.Moreover,the results also show that TGF-β1 promotes the expression of NGF and contributes to the repair of sciatic nerve damage and functional recovery in vivo.Collectively, our findings elucidate the TGF-β1/Smad3-p-Snail1-NGF signaling axis as a key regulator of peripheral nerve injury repair.

Introduction

Nerve injury repair is a complex, multi-phased biological process that involves intricate interactions among various cellular components and molecular mechanisms. This process is critical for restoring sensory and motor functions in affected individuals. Depending on the location of injury, nerve repair is classified into peripheral nerve injury (PNI) repair and central nervous system (CNS) injury repair. PNI occurs within the peripheral nervous system (PNS) and is triggered by trauma, compression, metabolic disorders, infections, intoxication, and autoimmune reactions. Mechanistically, PNI often results in axonal transection, initiating Wallerian degeneration at the distal end of neurons and retrograde degeneration at the proximal end. This cascade of damage underlies sensory and motor dysfunction, autonomic neuropathies, and neuropathic pain[1]. peripheral nerve injury (PNI) exhibits a partial capacity for self-repair, although the axonal regeneration rate is relatively slow, averaging 1–3 mm per day. A notable gap exists in clinically effective interventions to accelerate this process[2]. While PNI treatment often results in slow and incomplete functional recovery, exploring innovative technologies for PNI repair and elucidating their mechanisms could help overcome these clinical challenges.

Following PNI, nerve growth factor (NGF) emerges as a crucial mediator in nerve repair[3]. After axonal transection, reactive Schwann cells surrounding the axons undergo dedifferentiation and markedly upregulate NGF expression. Enhancing NGF expression capabilities facilitate axonal regeneration and myelination primarily through two receptors: tropomyosin receptor kinase A (TrkA) and p75 neurotrophin receptor (p75NTR). Within the PNI repair framework, NGF activates axonal growth via the MAPK-Ras-Erk pathway or through phospholipase Cγ1, PI3K, and SNT pathways mediated by neuronal TrkA receptors. Additionally, NGF enhances Schwann cell autophagy through the p75NTR/AMPK/mTOR signaling

pathway, expediting myelin debris clearance and promoting neural regeneration[4,5]. However, clinical intravenous injection of NGF can induce adverse side effects, including systemic neuropathic pain, cutaneous hyperalgesia, weight loss, and immune-related complications[6–9]. Thus, exploring the upregulation of endogenous NGF expression as a means to support PNI repair presents a promising and clinically relevant alternative. Future research should focus on developing targeted delivery systems for NGF to enhance its therapeutic efficacy while minimizing side effects.Following peripheral nerve injury (PNI), NGF is significantly upregulated in neurons, and TGF-β1 levels are also elevated within the injured neural microenvironment[10,11]. Emerging evidence indicates a regulatory interaction between TGF-β1 and NGF. Specifically, TGF-β1 signaling has been shown to mediate a substantial increase in NGF secretion in pulmonary artery cells under conditions of oxidative stress and inflammation. Similarly, TGF-β1 enhances NGF expression and secretion in human dental pulp cells[12,13]. In intervertebral disc injury models, TGF-β1 has been demonstrated to induce NGF expression, although the precise mechanisms underlying this effect remain to be fully elucidated[14]. In Schwann cells, the impact of TGF-β1 signaling on NGF mRNA levels remains a topic of ongoing debate[15,16].Clinical research has demonstrated that activation of the TGF-β1 pathway in macrophages can alleviate neuropathic pain. Furthermore, animal studies have revealed that TGF-β1 treatment enhances Schwann cell-mediated axonal regeneration in chronic nerve injury models[17,18]. These findings collectively position TGF-β1 as a promising therapeutic agent for PNI, suggesting that modulating NGF expression via TGF-β1 signaling may offer novel insights into PNI repair strategies.

In the context of PNI repair, the regulation of endogenous NGF secretion is of paramount importance. NGF is primarily expressed by neuroglial cells, including Schwann cells, astrocytes, and oligodendrocytes, and exerts its effects on neuronal cells through paracrine mechanisms. Among these cells, Schwann cells play a crucial role in PNI repair by transitioning into diverse reparative phenotypes that support axonal and myelin regeneration, clear myelin debris, form nerve bridges, and engage in immune responses[19]. However, the pro-regenerative properties of Schwann cells appear to be time-limited; in chronic PNI, a decline in Schwann cell reparative phenotypes and reduced neurotrophic factor expression may contribute to unsuccessful nerve regeneration[20,21]. Recent studies indicate that TGF-β1 significantly influences Schwann cell function. For instance, tumor-produced TGF-β1 mediates prostaglandin E secretion by Schwann cells, thereby curbing tumor progression via immune pathways. Similarly, another research reported that TGF-β1 enhances Schwann cell migration and invasion through matrix metalloproteinase MMP-2 and MMP-9 pathways[22,23]. Additionally, cAMP activators have been shown to induce NGF expression, and morphine can increase NGF mRNA levels in rat hippocampi, although the precise mechanisms regulating NGF expression are not fully defined[22,24].

The regulation of Schwann cell-derived NGF via transcription factors has not been extensively reported[25,26]. The classical TGF-β1/Smad3 pathway impacts transcription factors like Snail1, β-catenin, and KLF6, yet similar regulatory effects in Schwann cells remain unverified[27–29]. NGF secretion is modulated bymultiple molecular mechanisms, particularly those involving cAMP, phospholipase C (PLC),

phospholipase A2 (PLA2), and Ca^{2+} mobilization[30]. The maturation and proNGF/NGF secretion ratios are influenced by cell type and the physiological context[31,32]. In vivo studies show that NGF at 20 ng/ml promotes Schwann cell-mediated nerve regeneration and myelin debris clearance[4]. Drugs like rosiglitazone and dexamethasone significantly inhibit NGF expression in white adipocytes[33]. This study revealed the mechanism by which TGF-β1 regulates NGF expression and secretion in rat Schwann cells, thereby demonstrating the existence of a TGF-β1/P-Snail1-NGF signaling axis. Moreover, our findings uncovered that the phosphorylation of Snail1 plays a critical role in modulating NGF expression in these cells. These insights not only deepen our understanding of Schwann cell biology in peripheral nerve repair but also provide a valuable foundation for the development of novel therapeutic strategies.

Materials and methods

Animal Experiment

The rat strain used was Sprague-Dawley (SD) male. The rats were purchased from the Animal Experiment Center of Wenzhou Medical University, with a cleanliness level of SPF. All surgical operations and animal care procedures were carried out in accordance with appropriate guidelines and ethical standards. The experimental operations and animal feeding processes were approved by the Animal Ethics Committee of the university. All surgical procedures were performed under aseptic conditions.

A surgical latex glove was cut into 6 mm × 6 mm and 6 mm × 8 mm pieces. These pieces were immersed in 75% ethanol in a sterilized Petri dish for 30 minutes and then washed three times in sterile water for 5 minutes each to minimize inflammation. Rats were anesthetized via intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg). The piriformis muscle was exposed through blunt dissection of the muscularis. The inferior edge of the piriformis muscle was separated, and the sciatic nerve was exposed and gently isolated. A piece of latex glove was placed under the nerve to ensure it was neither stretched nor twisted. Before applying **NBCA**, the nerve and latex were soaked in fetal bovine serum (Gibco, New York, NY, USA). A volume of 5 μL of **NBCA** glue was carefully applied to the sciatic nerve and rubber sheet. After the NBCA had coagulated, another piece of surgical glove latex was placed on top of the target area and allowed to adhere to the first piece, forming a sandwich-like complex.

Cell Model

The DH5α strain was removed from the − 80°C refrigerator. A 1.5 mL aliquot of bacterial solution in the logarithmic growth phase was taken and suspended in pre-cooled 0.1 mol/L $CaCl_2$ solution to obtain fresh competent cells. One microliter of plasmid was added to 80 μL of competent cells, followed by the addition of 1 mL of LB liquid medium without antibiotics. The mixture was cultured for 120 minutes and then plated on LB solid medium containing ampicillin for overnight incubation. Plasmids were extracted according to the instructions of the Endotoxin-Free Plasmid Mini Extraction Kit (Tiangen Biotech, Beijing, China).

When the HEK293T cell density reached 70–80%, the target plasmid, PSPAX, and PMD2G were mixed in a ratio of 4:3:1 (V:V:V). Specifically, 8.0 μg of the target plasmid, 6.0 μg of PSPAX, 2.0 μg of PMD2G, and 24 μg of PEI were combined in 200 μL of DMEM and added to a Petri dish. After 16–18 hours, the original culture medium was discarded and replaced with fresh complete medium. Supernatants containing viral particles were collected at 48 and 72 hours, filtered through a 0.45 μm filter membrane, and used to infect RSC96 cells. The virus solution was prepared at a ratio of virus liquid:fresh complete medium:polybrene (V:V:V) = 1000:1000:2. After mixing, the solution was added to RSC96 cell cultures. After 16 hours, the mixture was discarded, and 2 mL of fresh complete medium was added. After 72 hours, puromycin solution was added to select the stable cell line.

Bioinformatic analyses

A database(https://www.ncbi.nlm.nih.gov/, http://genome.ucsc.edu/index.html) is used to search for sequences that may contain target gene promoters, and then another database(https://www.fruitfly.org/seq_tools/promoter.html) is used to predict this sequence to obtain short sequences of suspected promoters. Then, by using a database

(http://bioinfo.life.hust.edu.cn/AnimalTFDB/#!/) query to obtain the binding sites of transcription factors, the short sequences of suspected promoters are compared with the binding sites of transcription factors to confirm whether they overlap.

Western Blot

Western Blot
RSC96 cells were lysed using RIPA buffer supplemented with protease inhibitor and phosphatase inhibitor. A total of 5 μg of protein was separated on native 12% Bis-Tris Protein Gels and transferred using standard procedures (100 V, 1 h). Nitrocellulose membranes (BioRad) were blocked in TBST (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Tween 20) containing 5% non-fat dry milk. The membranes were then incubated overnight at 4°C with specific primary antibodies NGF Antibody(Bioways, CY5411), Phospho-SNAI1 (Bioways, Ser246) Antibody（Bioways, CY6488）, SNAI1 Antibody（Bioways, CY3066）, Phospho-Smad3 (S423 + S425) Antibody（Bioways, CY5140）, Smad3 Antibody（Bioways, CY5013）, GAPDH Antibody（Bioways, AB200）, β-Actin Mouse Monoclonal Antibody（Bioways, AY0573）, then in TBST supplemented with 5% non-fat dry milk, followed by washing with TBS. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h. After washing with TBST, the membranes were developed using either the Millipore Classico or Kindle Biosciences ECL kit for chemiluminescence detection and imaged using a Bio-rad ChemiDoc XRS (1708265).

RT-PCR

The RNeasy kit (Qiagen, Hilden, Germany) was utilized to isolate total RNA. For cDNA synthesis, the SuperScript™ III First-Strand Synthesis SuperMix kit (Invitrogen) was employed for reverse transcription. RT-PCR was conducted in a 96-well thermal cycler (Thermo Scientific) using DreamTaq Green PCR Master Mix (Thermo Scientific) and an optimized program as per the manufacturer's instructions. The resulting RT-PCR products were analyzed on a 1% agarose gel and visualized using MIDORI Green staining (Nippon Genetics, Düren, Germany). The primers utilized in this study can be found in Supplementary Table 2.

Total RNA isolation and real-time quantitative RT-PCR

The TRIzol Reagent (15596-026, Invitrogen) was used to isolate total RNA, and 1 μg of the total RNA was used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (QIAGEN, 205311). qRT-PCR was conducted using SYBR Green mix (Thermo Fisher Scientific, A25743) and analyzed on the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Each biological sample was subjected to at least three replicates, and the expression values of each replicate were normalized against β-actin cDNA using the $2^{-\Delta\Delta CT}$ method. The primers employed in this study can be found in Supplementary Table 2.

Chromatin immunoprecipitation (ChIP)

ChIP assays were conducted using BeyoChIP™ ChIP Assay Kit(BeyoChIP).Lowing the manufacturer's instructions. To initiate cross-linking of proteins to DNA, 1% formaldehyde was directly added to RSC96 cells. The cell pellet was then lysed and sonicated. After centrifugation, the resulting supernatant was incubated overnight at 4°C with antibodies against Snail1 and normal rabbit IgG. Following immunoprecipitation using protein A and G beads, the antibody/protein/DNA complex was subjected to a 4-hour incubation at 65°C to reverse the protein/DNA cross-links. The DNA was purified using the PCR Purification kit (Qiagen) and utilized as a template for PCR amplification. Different pairs of NGF promoter primers (Supplementary Table 2) were employed for amplification. The PCR products were separated on 1% agarose gels and visualized after staining with ethidium bromide.

Woudhealing

The RSC96 cell lines were cultured until a monolayer forms. Artificial wounds were created on the cell monolayer, then capture the images of wound healing at 0, 72 h, and compare the images to quantify the cell migration rate.

Transwell

A cell suspension with a concentration of 5×10^4 cells/ mL was seeded into the upper chamber of the Transwell inserts (6.5mm diameter inserts, 8.0μm pore size) using serum-free media. The lower wells were filled with 10% FBS. After 24 hours of migration at 37°C, the cells on the upper surface of the

membrane filter were carefully removed. The migrated cells that passed through the inserts were then fixed with 4% PFA and stained with crystal violet before being counted.

Elisa

The measurement of NGF levels in culture supernatants was conducted using the Rat Nerve factor ELISA Kit according to the manufacturer's instructions. In brief, samples were added to the plate, followed by the addition of detection antibodies, and incubated for 1 hour at 37°C. Between each step, the plate was carefully washed with a wash solution containing 0.05% Tween 20 in PBS. Subsequently, the plate was treated with TMB (BD Bioscience) to develop the color, and the reaction was stopped using 0.01 N H_2SO_4. Finally, the Optical Density at 450 nm was measured using a microtiter plate reader within 15 minutes.

Immunocytochemistry

Cells were first cultured on a coverslip, followed by a 20-minute wash with 4% Paraformaldehyde in PBS. After that, three wash steps with PBS were performed, and blocking with 5% BSA in 0.4% PBST (PBS-Triton) at 37°C for 1 hour was carried out. Subsequently, the cells were treated with 0.5% Triton X-100 for 10 minutes. Following this, the cells were incubated overnight at 4°C with a primary antibody diluted in 0.4% PBS-T. The next day, three wash steps with 0.4% PBST were conducted, followed by a 1-hour incubation at 37°C with a secondary antibody. After three additional washes with 0.4% PBST, a nuclear marker, 0.5–10 μg/ml DAPI, was added and incubated for 20 minutes. The cells were then washed three times with PBS. Finally, the coverslips were mounted on microscope slides using fluorescence mounting medium, and confocal images were acquired using the Leica SP8 DMI8 confocal microscope. Image processing was performed using ImageJ.

Catwalk

Catwalk were performed as an automatic system for gait detection and analysis[33]. The rats with nerve injury were equally divided into three groups and injected with DMEM,TGF-β1and Liposome TGF-β1 respectively. Through the footprint information left by the slender channel, the walking cycle, step width, footprint area and speed of rat footprints were automatically identified and analyzed. Each rat was tested at least three times at a time, and each time they must walk continuously through the set length range of the plate.

Electrophysiology method

As in the gait experiment group mentioned above, after weighing, the rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium solution 40 mg/kg then fixed in prone position to expose bilateral sciatic nerves. The electrode was used to stimulate the distal 2 cm of the injured sciatic nerve, and the changes of electrical signals were recorded in the proximal 2 cm. Every data point in amplitude and interval was the mean of 3–4 cycles.

H&E Staining

Skeleton muscles tissues from different experimental groups were fixed with 4% buffered formalin (Fisher), paraffin embedded, and sectioned at 5 μm before staining. The prepared sections were stained with Hematoxylin&Eosin (H&E) for us to observe the shape of muscle under light microscope.

Transmission electron microscopy (TEM)

TEM was performed to observe ultrastructure. 1mm^3 sciatic nerve tissue specimens were fixed with 3% glutaraldehyde at 4°C for 2 hours, then washed with PBS for 3 times, fixed in 1% osmium tetroxide at room temperature for 2 hours. The fixed sample was embedded with 30min in acetone and EPON812 resin (1:1) through gradient ethanol. After infiltrating into the resin for an extra 2 hours, the ultrathin sections were cut into ultra-thin sections and placed on a 200-mesh copper net. It was stained with uranium acetate and lead nitrate for 25 min. Finally, the sections were observed by TEM.The sections were observed with a transmission electron microscope and were measured the thickness of myelin sheath in electron microscope photos.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software v9.5, San Diego, US). Normally distributed data were analyzed using one-way ANOVA for multiple comparisons or unpaired t tests for comparisons of two groups, unless indicated otherwise. Significance level was defined at 0.05. Significance levels are indicated in the figures as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

Results

The expression of NGF in Schwann cells is correlated with the concentration and stimulation time of exogenous TGF-β1

Previous studies have shown that adding TGF-β1 to the repair of peripheral nerve injury improves axon regeneration[34], TGF-β1 significantly increases the migration and invasion of Schwann cells[35]. Therefore, we explored the effects of TGF-β1 on the expression levels of NGF and P-Smad3 by dose-response experiment, and treated RSC96 cells with TGF-β1 at a working concentration of 10 ng/mL for 48 h to explore the effects of TGF-β1 on the expression levels of NGF protein and mRNA. In dose-response experiments, Western Blot demonstrated that TGF-β1 treatment increased the expression levels of NGF and P-Smad3 in a concentration - and time-dependent manner (Fig. 1a,b,c and d). Then, RSC96 cells were treated with TGF-β1 at a working concentration of 10 ng/mL for 48 h, and immunofluorescence staining showed that the expression level of NGF protein was significantly increased (Fig. 1f and g). Similarly, qPCR experiment proved that the expression level of NGF mRNA increased significantly (Fig. 1e). Transwell and Wound Healing experiments showed increased cell mobility in the treatment group compared to the control group (Fig. S1a,b). These results indicated that TGF-β1 promoted RSC96 cell migration and increased NGF protein expression.

Blocking TGF-β1/Smad3 signaling pathway can downregulate the expression of NGF in Schwann cells

Our evidence suggests that the expression of NGF is concentration- and time-dependent in response to TGF-β1. However, it remains unclear whether the TGF-β1 signal regulates NGF expression through the classical TGF-β1/Smad3 signaling pathway or through a paracrine pathway[12]. Therefore,we generated RSC96 cell lines overexpressing Smad7 and primary RSC96 cells treated with the Smad3 phosphorylation inhibitor SIS3[36]. The data shows that expression of NGF was reduced by 40% in both OE Smad7 and SIS3, compared with RSC96 (Fig. 2a, b, c, and d). The ratio of P-Smad3/Smad3 in the sarkosyl-soluble (SS) lysates was significantly decreased in OE Smad7; Similarly, the NGF mRNA was reduced by 40% in both OE Smad7 and SIS3, compared with RSC96 (Fig. 2e). Furthermore, the secretion of NGF was measured using the Rat Nerve factor ELISA kit. The results demonstrated a significant reduction in the levels of NGF in the culture medium of SIS3-treated RSC96 cells (Fig. 2f). At the cellular level, immunofluorescence results confirm the effective blockade of TGF-β1-induced upregulation of NGF expression by overexpressed Smad7 (Fig. 2g). Subsequently, transwell assays and wound healing assays (Fig. S2a, b) were performed, demonstrating that the dephosphorylation of Smad3 caused by Smad7 can effectively inhibit both individual migration and population migration induced by TGF-β1. The aforementioned vitro experiments provided initial evidence supporting the role of the classical TGF-β1/Smad3 signaling pathway in promoting RSC96 cell migration and stimulating NGF expression.

Snail1 mediates the regulation of NGF by the TGF-β1/Smad3 signaling pathway in Schwann cells

Previous studies have shown that TGF-β1 promotes the expression of Snail1 in kidney and lung cancer tissues[37,38] while Blaney Davidson EN et al found that TGF-β1 promotes the expression of NGF in chondrocytes[39]. We also observed a similar phenomenon in previous experiments. So, is it possible that TGF- β1 affects the secretion of NGF by regulating Snail1 protein? First of all, using Western Blot and qPCR (Fig. 3a,b,c), we determined that the best treatment conditions for the increase of Snail1 protein and nucleic acid expression induced by TGF-β1 were 48 h and 10 ng/mL. In order to explore the existence of TGF-β1/Snail1/NGF signal axis, we blocked Snail1 expression by establishing cell models of knocking down Snail1 at sites # 1 and # 2, respectively. Through Western Blot and qPCR experiments, we found that # 1 knockdown model had better effect (Fig. S3c,d), and used as a follow-up experiment, named sh Snail1. The analysis of knock-down cell model showed that after the down-regulation of Snail1 expression, the expression level of NGF also decreased, suggesting that Snail1 is indeed the upstream protein (Fig. S3c,d) of NGF.Combined with our existing experimental data, we can confirm that there are TGF-β1/ Smad3/NGF signal axis (Fig. 1,2), TGF- β1/Snail1 signal axis and Snail1 is the upstream protein of NGF, so is there a TGF- β1/Smad3/Snail1/NGF signal axis? Knockdown of Snail1 did not affect the phosphorylation (Fig. 3d) of Smad3 induced by TGF-β1, suggesting that Smad3 is the upstream protein of Snail1. Knocking down Snail1 could counteract the synchronous increase of Snail1,P-Snail1 protein

level induced by TGF-β1, and the expression of NGF protein decreased with the trend of Snail1,P-Snail1 change (Fig. 3d), suggesting that there is still a positive linear relationship between NGF and Snail1, P-Snail1 under the action of TGF- β1, and Snail1 is the upstream protein of NGF. The results of qPCR and immunofluorescence experiments also support this conclusion (Fig. 3e,g). We can conclude that there is a TGF-β1/Smad3/Snail1/NGF signal axis, but whether dephosphorylated Snail1 or phosphorylated Snail1 regulates NGF remains to be studied. TGF-β1 can not only promote the expression of NGF in RSC96, but also promote the secretion of NGF out of cells. However, knocking down Snail1 can effectively inhibit the positive effect of TGF-β1 (Fig. 3f). Transwell and Woundhealing experiments show that TGF-β1 regulates population migration and individual migration of RSC96 cells through Snail1. After Snail1 knockdown, the migration of RSC96 cells induced by TGF-β1 was inhibited (Fig. S3e,f). In summary, TGF-β1 regulates NGF expression and secretion through TGF-β1/Smad3/Snail1/NGF signal axis, and affects RSC96 cell migration through Snail1, but the effect of Snail1 modification on the above mechanisms and functions remains tos be explored.

TGF-β1 promotes the phosphorylation of Snail1 to upregulate the transcription of NGF in vitro in Schwann cells .

Our evidence suggests that in the effect of TGF-β1 on nerve injury repair, Snail1 plays an important role in mediating the TGF-β1/Smad3/NGF signaling axis. However, whether TGF-β1 is affected by the increase of expression of Snail1 or the increase of phosphorylation Snail1 level to inflence the expression of the downstream NGF protein levels remains unclear. Our experimental results indicate that there is a correlation between TGF-β1 and the phosphorylation level of Snail1, that is, the level of phosphorylated Snail1 can increase approximately 50% when 10 ng/mL TGF-β1 treats RSC96 cells. We speculate that Snail1 regulates NGF through phosphorylation, so we hope to do further research on this speculative result. Lithium chloride is a white crystal, easily soluble in water. Indeed,In a research of GSK3β-dependent Snail phosphorylation, JongInYook et al found that the phosphorylation of Snail could be blocked by LiCl (20 mM)[40]. Our research also shows that in Western Blot experiments, as the working concentration of LiCl increases, the phosphorylation level of Snail1 decreases significantly, and 40 mM LiCl aqueous solution has obtained good results. At the same time, contrary to the phosphorylation level of Snail, the dephosphorylated Snail1 increased with the increase of the working concentration of LiCl, excluding the effect of the decrease of the total amount of Snail1 caused by LiCl (Fig. 4a, b). Next, we investigated in that Snail1 mediates the TGF-β1/Smad3/NGF signaling axis through phosphorylation, we treated RSC96 cells with LiCl and TGF-β1. The results of Western Blot and qPCR showed that under the condition of Snail1 phosphorylation was inhibited, TGF-β1 could not promote the high expression of NGF protein levels and mRNA levels (Fig. 4c,d,e), while ELISA results indicated that TGF-β1 could not promote the secretion of NGF under the condition that Snail1 phosphorylation was inhibited (Fig. 4f). So far, we have demonstrated that TGF-β1 promotes RSC96 cell migration and promotes NGF expression by promoting Snail1 phosphorylation rather than upregulating Snail1 expression (Fig. S4b,c). More significantly,direct or indirect regulatory effect of phosphorylated Snail1 on NGF deserves to be further explored. Yan Liu et al found that Snail1 binds to the promoter of fatty acid synthase and recruits

HDAC1/2 to induce the deacetylation of H3K9 and H3K27, thereby inhibiting the activity of the promoter of fatty acid synthase[41].

In another research, Mana Taki et al found that Snail which most likely through direct binding to the promoters upregulates CXCR ligands through NF-kB pathway[42]. By using the http://genome.ucsc.edu/index.html database and the https://www.ncbi.nlm.nihgov/ database to obtain sequence that may contain the NGF gene promoter of rat respectively. By using BLAST, sequences were compared with each other and compared with the rat NGF gene library. Amazingly,the homology reaches 99%. In addition, https://www.fruitfly.org/seq_tools/promoter.html is an unparrelleled website to predict the promoters sequence, and gain promoter sequences successfully and respectively. Then used the http://bioinfo.life.hust.edu.cn/AnimalTFDB/#!/ database to query the three binding sites of the rat Snail1 transcription factor, compared the binding sites with the promoter sequence. The transcription factor binding site coincides with one of the promoter sequences support our prediction that Snail1 can regulate the expression of NGF. Meanwhile, the results of agarose gel electrophoresis and Western blot of the Ch-IP experiment proved that P-Snail1 plays a direct role with the promoter of NGF (Fig. 4g,h). In order to explore whether P-Snail1 enters the nucleus in the form of P-Smad3/P-Snail1 complex or a single protein to regulate the promoter, we conducted a CO-IP experiment after cytoplasm-nuclear separation and found that the P-Smad3/P-Snail1 complex exists in both the cytoplasm and the nucleus, suggesting that P-Snail1 is more likely to enter the nucleus in the form of a complex to regulate NGF gene transcription (Fig. 4i). In summary, TGF-β1 promoted the migration of RSC96 cells by promoting the phosphorylation of Snail1 rather than up-regulating the expression of Snail1 and directly binding to the promoter sequence of NGF in the nucleus to promote NGF expression.

In vivo TGF-β1 promotes the expression of NGF and contributes to the repair of neural damage.

We have demonstrated the effect of TGF-β1 on the repair of nerve injury and the existence of Snail1-mediated TGF-β1/Smad3/NGF signal axis at the cellular level. However, in order to make TGF-β1 more promising, we need to carry out vivo experiments, and through the detection of morphology and function to get more reliable conclusions.Hematoxylin and eosin (HE) staining was used to detect the morphometry clearer of skeletal muscles which were dominated by related nerves. Nerves nourish the muscles at their disposal, so when the nerve is damaged, the muscle density decreases. On the contrary, when the nerve injury is repaired, the muscle density increases. When TGF-β1 was injected into the injured sciatic nerve compression site, the muscle density increased with no significant difference, but the TGF-β1 entrapped by liposome could achieve ideal effect, which may be related to the sustained release effect of liposome[43] (Fig. 5a,b). Next, an electrode for real-time electrophysiology method was fixed to muscle to record sciatic nerve activity after a stimulus every 2 seconds. The conclusion is obvious that both TGF- β1 and TGF-β1 entrapped by liposome could extremely promote the repairment of nerve injury (Fig. 5c,d). We next analyzed the myelin cross section by transmission electronic microscopy (TEM). Axonal membrane are distinctly visible under TEM as concentric circle. Adding TGF-β1 and TGF-β1 entrapped by liposome to the injured nerve fibers can significantly thicken the axon membrane, that is, the nerve injury can be repaired to a certain extent (Fig. 5e,f). Immunofluorescence

experiment can well prove the expression of NGF in the cytoplasm, which is similar to the results of cell experiment. Injection of TGF-β1 can increase the expression of NGF in rat glial cells, and explain the recovery of nerve in the aspects of structure and function (Fig. 5g,h). To further evaluate the recovery of motor function, on the 30th day after compression damage surgery, 3 groups of rats‘ gait changes were detected by automated gait analysis system Catwalk XT. Gait obstacle were characterized by decline in temporal asymmetry, running speed,especially footprint areas. In order to quantify the footprint data,we examined the maxiumum print areas. When the nerve injury was repaired, which means the pain was reduced, the force of the injured side was greater and the contact area with the measured surface was smaller. The results suggest that both TGF-β1 and TGF-β1 entrapped by liposome could increase the footprint area and repair nerve injury, and the effect of the latter was better (Fig. 5i,j).

Discussion

This study has thoroughly investigated the mechanisms of TGF-β1 in the repair of peripheral nerve injury (PNI) through both in vitro and in vivo experiments. Previous studies have demonstrated that TGF-β1 significantly promotes axonal regeneration and enhances the migration and invasion capabilities of Schwann cells[17,22,44–46]. Additionally, nerve growth factor (NGF) plays a crucial role in nerve injury repair. Our study further elucidates the correlation between TGF-β1 and NGF secretion. Through dose-response and time-course experimental designs, we found that TGF-β1 significantly upregulates NGF protein and mRNA expression levels and enhances the migratory capacity of RSC96 cells (Figs. 1 and S1). These results indicate that TGF-β1 exerts important regulatory functions in PNI repair, likely through the promotion of NGF expression and secretion.Although previous research has indicated that TGF-β1 regulates NGF expression, the specific mechanisms remain unclear[47]. In this study, we constructed RSC96 cell lines overexpressing Smad7 and utilized the Smad3 phosphorylation inhibitor SIS3 to further explore the regulatory role of the TGF-β1/Smad3 signaling pathway in NGF expression. Experimental results showed that both Smad7 overexpression and SIS3 treatment significantly reduced NGF protein and mRNA expression levels (Figs. 2a-e) and inhibited TGF-β1-induced NGF secretion (Fig. 2f). These findings suggest that TGF-β1 regulates NGF expression through the canonical TGF-β1/Smad3 signaling pathway, with Smad3 phosphorylation being a key step in this process.

This study further explored whether TGF-β1 influences NGF secretion by regulating Snail1 expression. Previous studies have shown that TGF-β1 promotes Snail1 expression in various tissues[48,49]. Our experimental results demonstrated that TGF-β1 significantly upregulates Snail1 protein and mRNA expression levels (Figs. 3a-c). Additionally, Snail1 knockdown significantly inhibited NGF expression (Figures S3c-d) and TGF-β1-induced RSC96 cell migration (Figures S3e-f). These results indicate that Snail1 plays a crucial regulatory role in the TGF-β1/Smad3/NGF signaling axis, and TGF-β1 likely affects NGF secretion through the regulation of Snail1 expression.To further explore the regulatory mechanisms of Snail1 in NGF expression, we employed LiCl, a GSK3β-dependent Snail1 phosphorylation inhibitor[50,51]. The results indicated that LiCl significantly attenuated the phosphorylation levels of Snail1 (Figs. 4a-b) and blocked the upregulation of NGF protein and mRNA induced by TGF-β1 (Figs. 4c-e). Moreover, ELISA

assays revealed that LiCl treatment inhibited TGF-β1-induced NGF secretion (Fig. 4f). These findings suggest that Snail1 phosphorylation plays a pivotal role in the TGF-β1/Smad3/NGF signaling axis, and TGF-β1 likely regulates NGF expression by promoting Snail1 phosphorylation rather than upregulating its expression.Through bioinformatics analysis and Ch-IP experiments, we identified that the binding sites of Snail1 highly matched the NGF promoter sequence (Figs. 4g-h), and P-Snail1 could directly bind to the NGF promoter. Additionally, CO-IP experiments demonstrated the presence of P-Smad3/P-Snail1 complexes in both the cytoplasm and nucleus, suggesting that P-Snail1 likely enters the nucleus as a complex to directly regulate NGF gene transcription (Fig. 4i). These results further substantiate the existence of the TGF-β1/Smad3/Snail1/NGF signaling axis and reveal the direct regulatory role of Snail1 in NGF expression.

To further validate the role of TGF-β1 in nerve injury repair, we conducted in vivo experiments. HE staining results showed that both TGF-β1 and liposome-encapsulated TGF-β1 significantly increased muscle density (Figs. 5a-b), indicating a certain degree of nerve injury repair. Real-time electrophysiological experiments revealed that both TGF-β1 and liposome-encapsulated TGF-β1 significantly promoted nerve injury repair (Figs. 5c-d). Furthermore, TEM analysis showed that TGF-β1 treatment significantly thickened the axonal membrane (Figs. 5e-f), further confirming the repair effect on nerve injury. Immunofluorescence experiments indicated that TGF-β1 significantly increased NGF expression in rat glial cells (Figs. 5g-h), consistent with the in vitro results. Additionally, automated gait analysis using the Catwalk XT system showed that both TGF-β1 and liposome-encapsulated TGF-β1 significantly increased footprint area and improved motor function (Figs. 5i-j). These results demonstrate that TGF-β1 has significant therapeutic effects on in vivo nerve injury repair, with liposome-encapsulated TGF-β1 showing better repair effects due to its sustained-release action. Snail1 phosphorylation plays a significant role in the regulation of nerve growth factor (NGF) expression, particularly in the context of nerve injury repair and cellular signaling pathways. Recent studies have highlighted the importance of Snail1 phosphorylation in modulating NGF expression through the TGF-β1/Smad3 signaling axis. Specifically, the phosphorylation of Snail1 is crucial for its transcriptional activity and stability, which in turn affects the expression levels of NGF.Snail1 phosphorylation has been shown to enhance its binding affinity to the NGF promoter, thereby directly upregulating NGF expression. This mechanism is critical for promoting nerve regeneration and repair processes in response to injury.The use of GSK3β-dependent Snail1 phosphorylation inhibitors, such as LiCl, has demonstrated a significant reduction in NGF expression, highlighting the necessity of Snail1 phosphorylation for NGF upregulation.The TGF-β1/Smad3 pathway is a key regulator of Snail1 phosphorylation. TGF-β1-induced Smad3 phosphorylation promotes the formation of P-Smad3/P-Snail1 complexes, which can translocate to the nucleus and directly bind to the NGF promoter. This interaction enhances NGF transcription and secretion, facilitating nerve repair and regeneration. In vivo experiments have shown that TGF-β1, particularly when delivered via liposome-encapsulated formulations, significantly promotes nerve repair by enhancing NGF expression through Snail1 phosphorylation. This approach has demonstrated therapeutic potential in improving nerve function and structure following injury. The regulation of Snail1 phosphorylation and its downstream effects on NGF expression offers a promising therapeutic target for

nerve injury repair. By modulating Snail1 phosphorylation, it is possible to enhance NGF production and promote nerve regeneration, potentially improving outcomes in patients with peripheral nerve injuries. In summary, Snail1 phosphorylation is a critical regulatory mechanism for NGF expression, mediated through the TGF-β1/Smad3 signaling pathway. This process is essential for nerve repair and regeneration, with potential applications in clinical settings for enhancing nerve injury recovery.

Despite the insights provided by this study into the molecular mechanisms by which TGF-β1 promotes RSC96 cell migration and NGF expression through the TGF-β1/Smad3/Snail1/NGF signaling axis, several limitations remain. First, the precise mechanisms by which Snail1 phosphorylation regulates NGF expression require further investigation. Second, the role of TGF-β1 in nerve injury repair in vivo and its potential for clinical application need to be validated through additional experimental and clinical studies. Future research directions may include: (1) elucidating the direct regulatory mechanisms of Snail1 phosphorylation on NGF expression; (2) exploring the crosstalk between TGF-β1 and other signaling pathways (such as PI3K/Akt and MAPK) and their synergistic effects in nerve injury repair[52,53]; and (3) developing novel neurorepair drugs and therapeutic strategies based on TGF-β1 and Snail1, with validation in preclinical models.This study, through both in vitro and in vivo experiments, has elucidated the significant role and underlying molecular mechanisms of TGF-β1 in the repair of peripheral nerve injury. TGF-β1 promotes the migration of RSC96 cells and the expression of NGF via the TGF-β1/Smad3/Snail1/NGF signaling axis, thereby accelerating nerve repair. These findings not only provide novel insights into the role of TGF-β1 in nerve injury repair but also offer a theoretical foundation for the development of neurorepair strategies based on TGF-β1. Future research will further explore the specific mechanisms of Snail1 phosphorylation and validate the clinical potential of TGF-β1.

Declarations

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References

1. Hussain, G., et al.: Current status of therapeutic approaches against peripheral nerve injuries: a detailed story from injury to recovery. Int. J. Biol. Sci. **16**, 116–134 (2020)
2. Wei, C., et al.: Advances of schwann cells in peripheral nerve regeneration: from mechanism to cell therapy. Biomed. Pharmacotherapy = Biomedecine Pharmacotherapie. **175**, 116645 (2024)
3. Madduri, S., Gander, B.: Schwann cell delivery of neurotrophic factors for peripheral nerve regeneration. J. Peripheral Nerv. System: JPNS. **15**, 93–103 (2010)
4. Li, R., et al.: Nerve growth factor activates autophagy in schwann cells to enhance myelin debris clearance and to expedite nerve regeneration. Theranostics. **10**, 1649–1677 (2020)
5. Sofroniew, M.V., Howe, C.L., Mobley, W.C.: Nerve growth factor signaling, neuroprotection, and neural repair. Annu. Rev. Neurosci. **24**, 1217–1281 (2001)
6. Denk, F., Bennett, D.L., McMahon, S.B.: Nerve growth factor and pain mechanisms. Annu. Rev. Neurosci. **40**, 307–325 (2017)
7. Petty, B.G., et al.: The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. Ann. Neurol. **36**, 244–246 (1994)
8. Price, R.D., Milne, S.A., Sharkey, J., Matsuoka, N.: Advances in small molecules promoting neurotrophic function. Pharmacol. Ther. **115**, 292–306 (2007)
9. Thoenen, H., Sendtner, M.: Neurotrophins: from enthusiastic expectations through sobering experiences to rational therapeutic approaches. Nat. Neurosci. 5 Suppl., 1046–1050 (2002)
10. Chen, Z.L., Yu, W.M., Strickland, S.: Peripheral regeneration. Annu. Rev. Neurosci. **30**, 209–233 (2007)
11. Clements, M.P., et al.: The wound microenvironment reprograms schwann cells to invasive mesenchymal-like cells to drive peripheral nerve regeneration. Neuron. **96**, 98–114e7 (2017)
12. Bouchet, C., et al.: Inflammation and oxidative stress induce NGF secretion by pulmonary arterial cells through a TGF-β1-dependent mechanism. Cells. **11**, 2795 (2022)
13. da Rosa, W.L., O, Piva, E., da Silva, A.F.: Disclosing the physiology of pulp tissue for vital pulp therapy. Int. Endod. J. **51**, 829–846 (2018)
14. Yokozeki, Y., et al.: TGF-β regulates nerve growth factor expression in a mouse intervertebral disc injury model. BMC Musculoskelet. Disord. **22**, 634–644 (2021)
15. Matsuoka, I., Meyer, M., Thoenen, H.: Cell-type-specific regulation of nerve growth factor (NGF) synthesis in non-neuronal cells: comparison of schwann cells with other cell types. J. Neuroscience: Official J. Soc. Neurosci. **11**, 3165–3177 (1991)
16. Rogister, B., et al.: Transforming growth factor beta as a neuronoglial signal during peripheral nervous system response to injury. J. Neurosci. Res. **34**, 32–43 (1993)

17. Sulaiman, W., Nguyen, D.H.: Transforming growth factor beta 1, a cytokine with regenerative functions. Neural Regeneration Res. **11**, 1549–1552 (2016)
18. Zeboudj, L., et al.: Silencing miR-21-5p in sensory neurons reverses neuropathic allodynia via activation of TGF-β-related pathway in macrophages. J. Clin. Invest. **133**, e164472 (2023)
19. Bosch-Queralt, M., Fledrich, R., Stassart, R.M.: Schwann cell functions in peripheral nerve development and repair. Neurobiol. Dis. **176**, 105952 (2023)
20. Höke, A., Gordon, T., Zochodne, D.W., Sulaiman, O.A.R.: A decline in glial cell-line-derived neurotrophic factor expression is associated with impaired regeneration after long-term schwann cell denervation. Exp. Neurol. **173**, 77–85 (2002)
21. Jessen, K.R., Mirsky, R.: The success and failure of the schwann cell response to nerve injury. Front. Cell. Neurosci. **13**, 33 (2019)
22. Muscella, A., Vetrugno, C., Cossa, L.G., Marsigliante, S.: TGF-β1 activates RSC96 schwann cells migration and invasion through MMP-2 and MMP-9 activities. J. Neurochem. **153**, 525–538 (2020)
23. Shurin, G.V., et al.: Tumor-induced T cell polarization by schwann cells. Cells. **11**, 3541 (2022)
24. Rouhani, F., Khodarahmi, P., Naseh, V.: NGF, BDNF and arc mRNA expression in the hippocampus of rats after administration of morphine. Neurochem. Res. **44**, 2139–2146 (2019)
25. Ertosun, M.G., Hapil, F.Z., Osman Nidai, O.: E2F1 transcription factor and its impact on growth factor and cytokine signaling. Cytokine Growth Factor Rev. **31**, 17–25 (2016)
26. Riccio, A., Pierchala, B.A., Ciarallo, C.L., Ginty, D.D.: An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. Science. **277**, 1097–1100 (1997)
27. Chen, W., et al.: Blocking interleukin-6 trans-signaling protects against renal fibrosis by suppressing STAT3 activation. Theranostics. **9**, 3980–3991 (2019)
28. Fan, M., et al.: Lactate promotes endothelial-to-mesenchymal transition via Snail1 lactylation after myocardial infarction. Sci. Adv. **9**, eadc9465 (2023)
29. Hu, Y., et al.: Tartrate-resistant acid phosphatase 5 promotes pulmonary fibrosis by modulating β-catenin signaling. Nat. Commun. **13**, 114 (2022)
30. Lipnik-Stangelj, M.: Multiple role of histamine H1-receptor-PKC-MAPK signalling pathway in histamine-stimulated nerve growth factor synthesis and secretion. Biochem. Pharmacol. **72**, 1375–1381 (2006)
31. Al-Shawi, R., et al.: Neurotoxic and neurotrophic roles of proNGF and the receptor sortilin in the adult and ageing nervous system. Eur. J. Neurosci. **27**, 2103–2114 (2008)
32. Mysona, B.A., et al.: Imbalance of the nerve growth factor and its precursor as a potential biomarker for diabetic retinopathy. Biomed Research International. 571456(2015). (2015)
33. Peeraully, M.R., Jenkins, J.R., Trayhurn, P.: NGF gene expression and secretion in white adipose tissue: regulation in 3T3-L1 adipocytes by hormones and inflammatory cytokines. Am. J. Physiol. Endocrinol. Metab. **287**, E331–339 (2004)

34. Zahednezhad, F., et al.: Liposome and immune system interplay: challenges and potentials. J. Controlled Release: Official J. Controlled Release Soc. **305**, 194–209 (2019)
35. Sulaiman, W., Dreesen, T.D.: Effect of local application of transforming growth factor-β at the nerve repair site following chronic axotomy and denervation on the expression of regeneration-associated genes. Laboratory investigation. J. Neurosurg. **121**, 859–874 (2014)
36. Muñoz, M.D., de la Fuente, N., Sánchez-Capelo, A.: TGF-β/Smad3 signalling modulates GABA neurotransmission: implications in parkinson's disease. Int. J. Mol. Sci. **21**, 590 (2020)
37. Li, H., et al.: Specific inhibitor of Smad3 (SIS3) alleviated submandibular gland fibrosis and dysfunction after dominant duct ligation in mice. J. Dent. Sci. **18**, 865–871 (2023)
38. Li, S., Yu, L., He, A., Liu, A.: Klotho inhibits unilateral ureteral obstruction-induced endothelial-to-mesenchymal transition via TGF-β1/Smad2/Snail1 signaling in mice. Front. Pharmacol. **10**, 348 (2019)
39. Liu, J., et al.: Suppression of SCARA5 by Snail1 is essential for EMT-associated cell migration of A549 cells. Oncogenesis. **2**, e73 (2013)
40. Blaney Davidson, E.N., et al.: TGF-β is a potent inducer of nerve growth factor in articular cartilage via the ALK5-Smad2/3 pathway. Potential role in OA related pain? Osteoarthr. Cartil. **23**, 478–486 (2015)
41. Yook, J.I., et al.: Wnt-dependent regulation of the E-cadherin repressor snail. J. Biol. Chem. **280**, 11740–11748 (2005)
42. Liu, Y., et al.: Insulin/Snail1 axis ameliorates fatty liver disease by epigenetically suppressing lipogenesis. Nat. Commun. **9**, 2751 (2018)
43. Taki, M., et al.: Snail promotes ovarian cancer progression by recruiting myeloid-derived suppressor cells via CXCR2 ligand upregulation. Nat. Commun. **9**, 1685 (2018)
44. Luo, S.X., et al.: TGF-β signaling in dopaminergic neurons regulates dendritic growth, excitatory-inhibitory synaptic balance, and reversal learning. Cell. Rep. **17**, 3233–3245 (2016)
45. McNamara, N.B., et al.: Microglia regulate central nervous system myelin growth and integrity. Nature. **613**, 120–129 (2023)
46. Rosner, B.I., Hang, T., Tranquillo, R.T.: Schwann cell behavior in three-dimensional collagen gels: evidence for differential mechano-transduction and the influence of TGF-beta 1 in morphological polarization and differentiation. Exp. Neurol. **195**, 81–91 (2005)
47. Yongchaitrakul, T., Pavasant, P.: Transforming growth factor-beta1 up-regulates the expression of nerve growth factor through mitogen-activated protein kinase signaling pathways in dental pulp cells. Eur. J. Oral. Sci. **115**, 57–63 (2007)
48. Batlle, R., et al.: Snail1 controls TGF-β responsiveness and differentiation of mesenchymal stem cells. Oncogene. **32**, 3381–3389 (2013)
49. Peinado, H., Quintanilla, M., Cano, A.: Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. J. Biol. Chem. **278**,

21113–21123 (2003)

50. Tanthaisong, P., et al.: Enhanced chondrogenic differentiation of human umbilical cord wharton's jelly derived mesenchymal stem cells by GSK-3 inhibitors. PLOS One. **12**, e0168059 (2017)
51. Makoukji, J., et al.: Lithium enhances remyelination of peripheral nerves. Proc. Natl. Acad. Sci. U.S.A. **109**, 3973–3978 (2012)
52. El Baassiri, G.: Nerve growth factor and burn wound healing: update of molecular interactions with skin cells. Burns: J. Int. Soc. Burn Injuries. **49**, 989–1002 (2023)
53. Zhuo, Y., et al.: TGF-β1 mediates hypoxia-preconditioned olfactory mucosa mesenchymal stem cells improved neural functional recovery in parkinson's disease models and patients. Military Med. Res. **11**, 48 (2024)

Figures

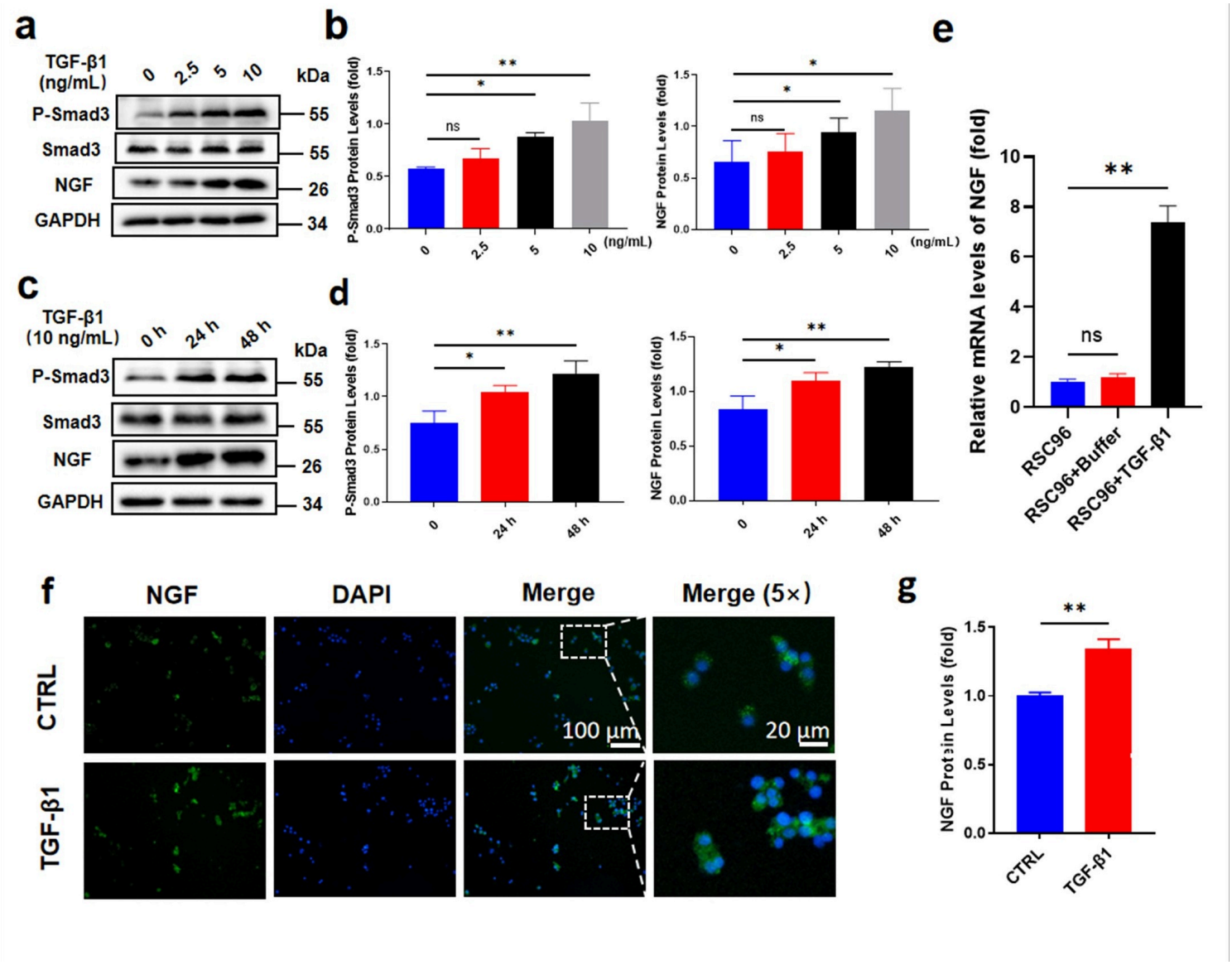


Figure 1

The expression of NGF in Schwann cells is correlated with the concentration and stimulation time of exogenous TGF-β1. a Western blot analysis of the corresponding proteins expressed in RSC96 using antibodies against P-Smad3, Smad3, and NGF after treatment with TGF-β1 for 48 h at concentrations of 0 ng/mL, 2.5 ng/mL, 5 ng/mL, and 10 ng/mL. **b** Densitometric measurement of three biological replicates of Western blots using antibodies against P-Smad3 and NGF in a. **c** Western blot analysis of the corresponding proteins expressed in RSC96 using antibodies against P-Smad3, Smad3, and NGF after treatment with TGF-β1 for 0 h, 24 h, and 48 h at a concentration of 10 ng/mL. **d** Densitometric measurement of three biological replicates of Western blots using antibodies against P-Smad3 and NGF in c. **e** qPCR was used to test the expression intensity of NGF mRNA in RSC96 cells with no treatment, only cytokine solvent buffer control group, and 10 ng/mL optimal TGF-β1 concentration treatment group for 48 h. **f**Immunostaining of Ctrl and TGF-β1 10 ng/mL experimental groups after 48 h of treatment using antibodies against NGF, and DAPI nuclear staining. **g** Analysis of the fluorescence density of anti-NGF antibody protein in f. Data represent mean±SEM, n=3 (three biologic replicates), One-way ANOVA test, *p<0.05; **p<0.01; ***p<0.005; ns, not significant. a.u. arbitrary unit.

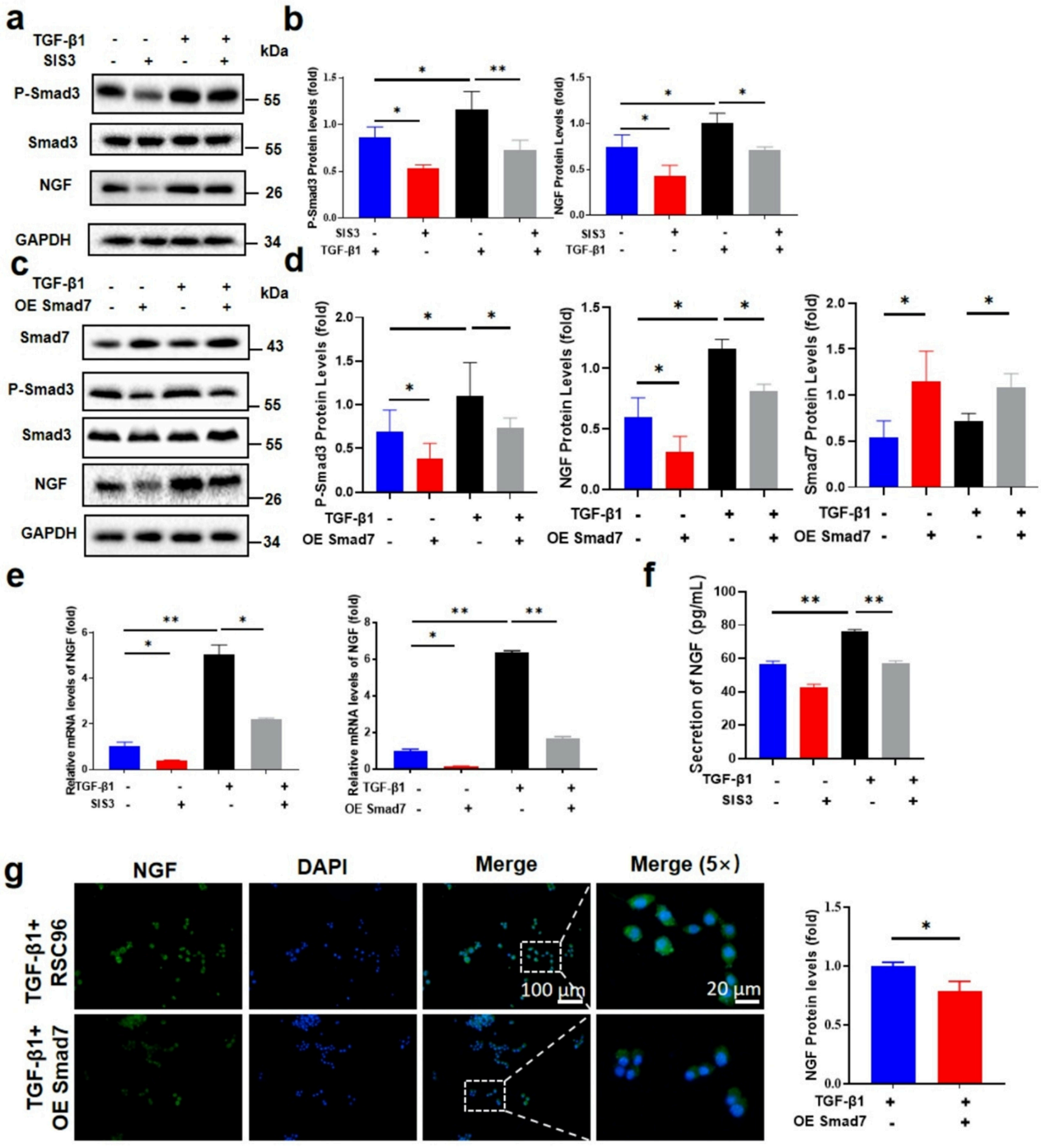


Figure 2

Blocking TGF-β1/Smad3 signaling pathway can downregulate the expression of NGF in Schwann cells. a RSC96 cell line was treated with TGF-β1 (10 ng/mL, 48 h) or SIS3 (3 μM, 1 h) or a combination of drugs, and the corresponding proteins expressed in RSC96 were analyzed by Western blot using antibodies against P-Smad3, Smad3, and NGF. **b** The density of three biological replicates of Western blot was measured using antibodies against P-Smad3 and NGF in a. **c** Control TGF-β1 (10 ng/mL, 48 h) treatment,

and the corresponding proteins expressed in Ctrl and OE Smad7 cell lines were analyzed by Western blot using antibodies against P-Smad3, Smad3, and NGF. **d**The density of three biological replicates of Western blot was measured using antibodies against P-Smad3, NGF, and Smad7 in c. **e** The same treatment method as a and c, using qPCR, to test the nucleic acid expression intensity of NGF mRNA in cells under each treatment condition. **f** The same treatment method as c, using ELISA to measure the NGF content in the culture medium of RSC96 cells under different culture conditions.**g** Ctrl and OE Smad7 cell lines were immunostained with antibodies against NGF after treatment with TGF-β1 (10 ng/mL, 48 h), and nuclear staining with DAPI was performed. Data represent mean±SEM, n=3 (three biologic replicates), One-way ANOVA test, *p<0.05; **p<0.01; ***p<0.005; ns, not significant. a.u. arbitrary unit.

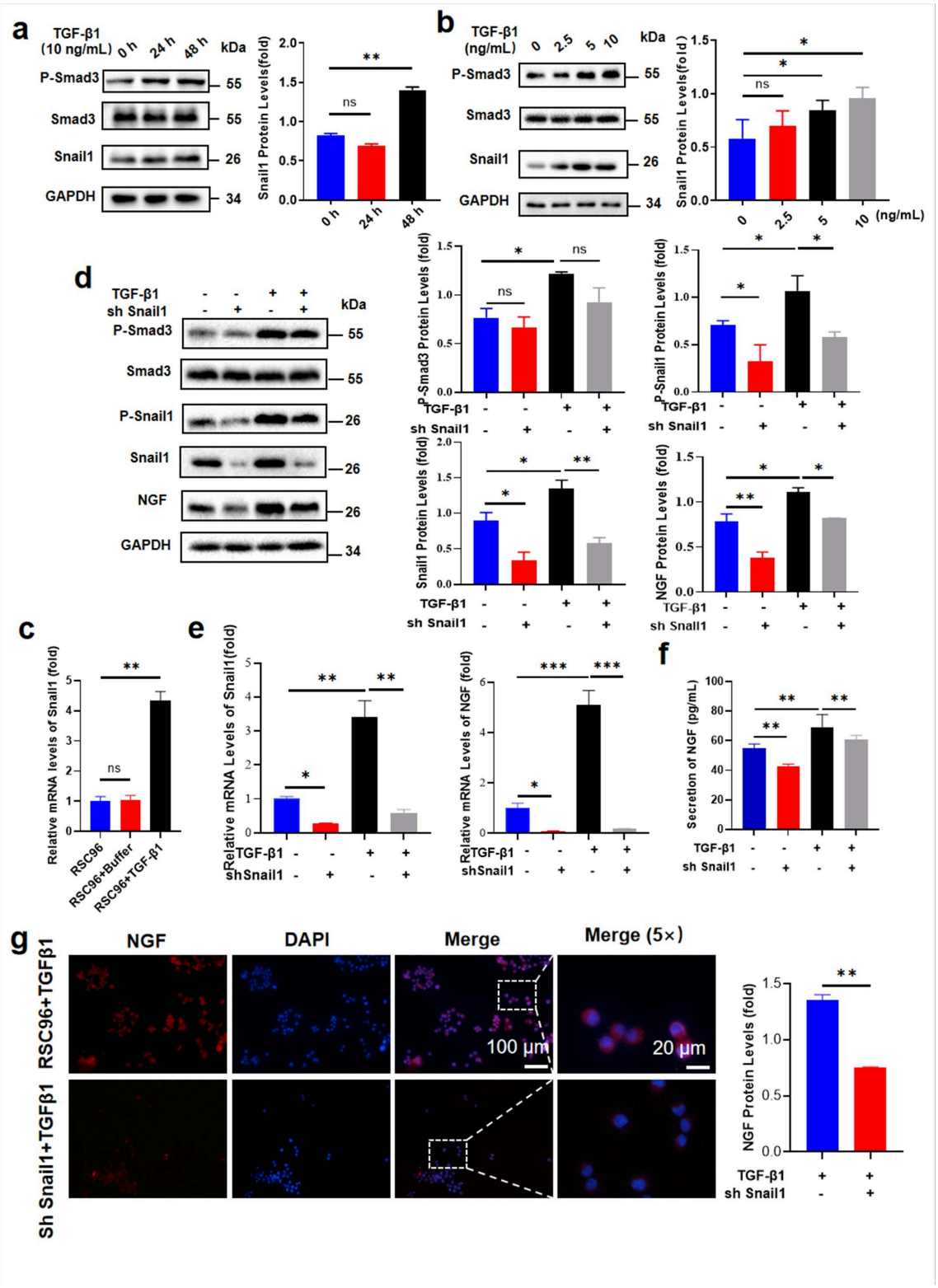


Figure 3

Snail1 mediates the regulation of NGF by the TGF-β1/Smad3 signaling pathway in Schwann cells. **a**Western blot analysis of the corresponding proteins expressed in RSC96 was performed using antibodies against P-Smad3, Smad3, and Snail1 after TGF-β1 treatment for 0 h, 24 h, and 48 h at a concentration of 10 ng/mL. The density of the protein blot of Snail1 antibody was measured in three biological replicates. **b** Western blot analysis of the corresponding proteins expressed in RSC96 was

performed using antibodies against P-Smad3, Smad3, and Snail1 after TGF-β1 treatment for 48 h at concentrations of 0 ng/mL, 2.5 ng/mL, 5 ng/mL, and 10 ng/mL. The density of the protein blot of Snail1 antibody was measured in three biological replicates. **c** qPCR was used to test the expression intensity of Snail1 mRNA in RSC96 cells with no treatment, only cytokine solvent buffer control group, and 10.0 ng/mL optimal TGF-β1 concentration treatment group for 48 h. **d** Control TGF-β1 (10 ng/mL, 48 h) treatment, the corresponding proteins expressed in Ctrl and shSnail1 cell lines were analyzed by western blot using antibodies against P-Smad3, Smad3, Snail1, P-Snail1, and NGF. The density of three biological replicates of the protein blots of P-Smad3, Snail1, P-Snail1, and NGF antibodies was measured. **e**The same treatment method as d, using qPCR, to test the nucleic acid expression intensity of Snail1 mRNA in cells under each treatment condition. **f** The same treatment method as d, using ELISA to measure the NGF content in the culture medium of RSC96 cells under different culture conditions. **g**After TGF-β1 (10 ng/mL, 48 h) treatment, the Ctrl and shSnail1 cell lines were immunostained using antibodies against NGF, and DAPI nuclear staining was performed. Data represent mean±SEM, n=3 (three biologic replicates), One-way ANOVA test, *p<0.05; **p<0.01; ***p<0.005; ns, not significant. a.u. arbitrary unit.

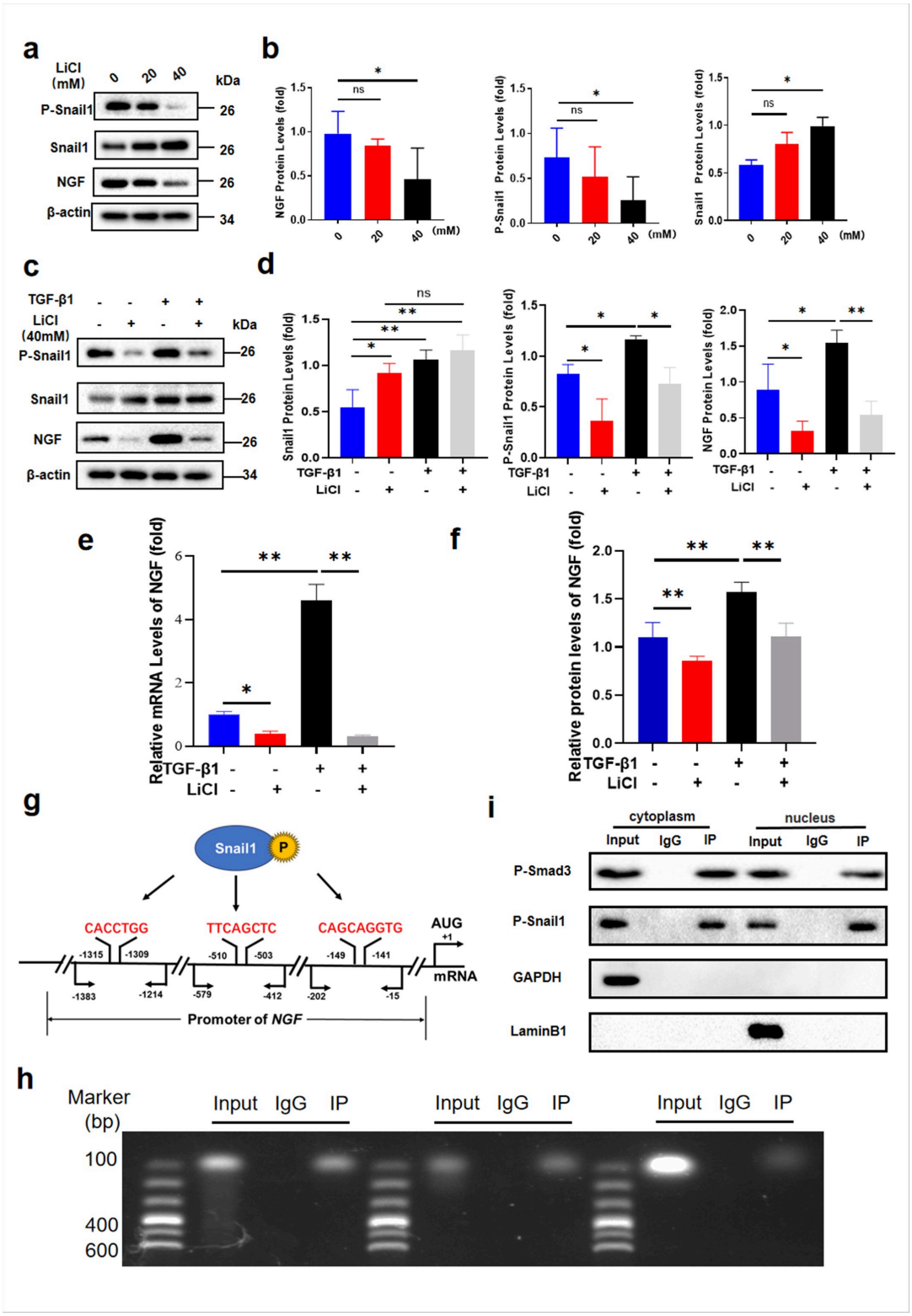


Figure 4

TGF-β1 promotes the phosphorylation of Snail1 to upregulate the transcription of NGF in vitro in Schwann cells. a RSC96 cells were treated with TGF-β1 at a concentration of 10 ng/mL for 0h, 24 h, and 48 h, respectively, followed by western blot analysis using antibodies specific for P-Snail1, Snail1, and NGF to assess their expression levels. **b** Density measurements of the western blots were conducted with triplicate biological repeats to ensure reproducibility. **c** RSC96 cells were treated with either TGF-β1

(10 ng/mL) for 48 hours, LiCl (40 mM), or a combination of both, and western blot analysis was performed to evaluate the expression of P-Snail1, Snail1, and NGF under these conditions. **d** Density measurements repeated three times for biological reproducibility.**e** qPCR was employed to quantify the expression intensity of NGF mRNA within the cells under each treatment condition. **f** ELISA method was used to measure the NGF content in the culture media of RSC96 cells to assess secretion levels. **g** Potential promoter sequence binding sites were identified using bioinformatics database. **h**These sequences were further validated using Ch-IP assays, with the resulting Ch-IP samples analyzed by western blot and agarose gel electrophoresis to confirm the binding of specific proteins to the predicted binding sites. **i** After RSC96 cytoplasm and nuclei were separated, CO-IP experiments were performed with antibodies against P-Snail1 and P-Smad3. Data represent mean±SEM, n=3 (three biologic replicates), One-way ANOVA test, *p<0.05; **p<0.01; ***p<0.005; ns, not significant. a.u. arbitrary unit.

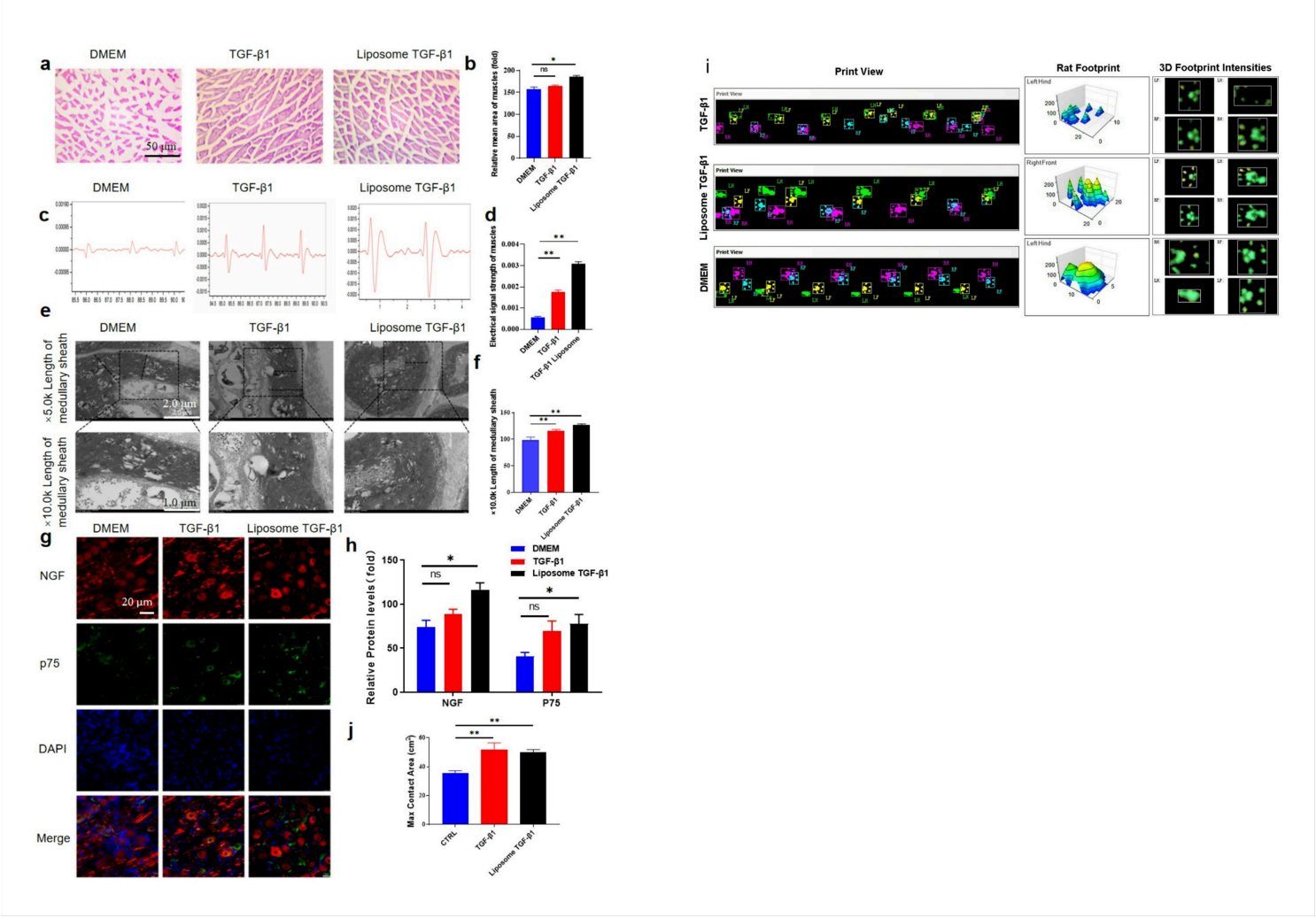


Figure 5

In vivo TGF-β1 promotes the expression of NGF and contributes to the repair of sciatic nerve damage. a H&E staining of thigh muscles dominated bysciatic nerve 30 days after surgery. H&E staining of DMEM group under the light microscope, showing that the number of fibroblasts was extremely small, while theTGF-β1 group and Liposome TGF-β1 group had larger number of fibroblasts. **b** The area covered by muscle cells in the field of view was observed under a 40 × microscope and counted. **c** The damage

surgery is the same as **a**, and the potential changes at the distal end of the muscle are measured using an electrophysiological device. **d** The difference between the peak and valley of the potential changes is statistically analyzed. **e-f** The myelin sheath of the nerve is observed by using 5 k and 10 k electron microscopes, and its thickness is measured using software called image j. **g** Use antibodies against NGF for immunostaining of cells and DAPI nuclear staining h Quantification of relative NGF, p75 protein levels. **i** On the 30th day after surgery, the CatWalk XT system recorded the real - time walking footprints of each group of experimental animals (left). It also presented the representative footprints of rats (right) and detected the 3D signal intensity of these footprints (middle). **j** Quantification of Print area (cm^2), Max Contact Area in each group (**p < 0.01).

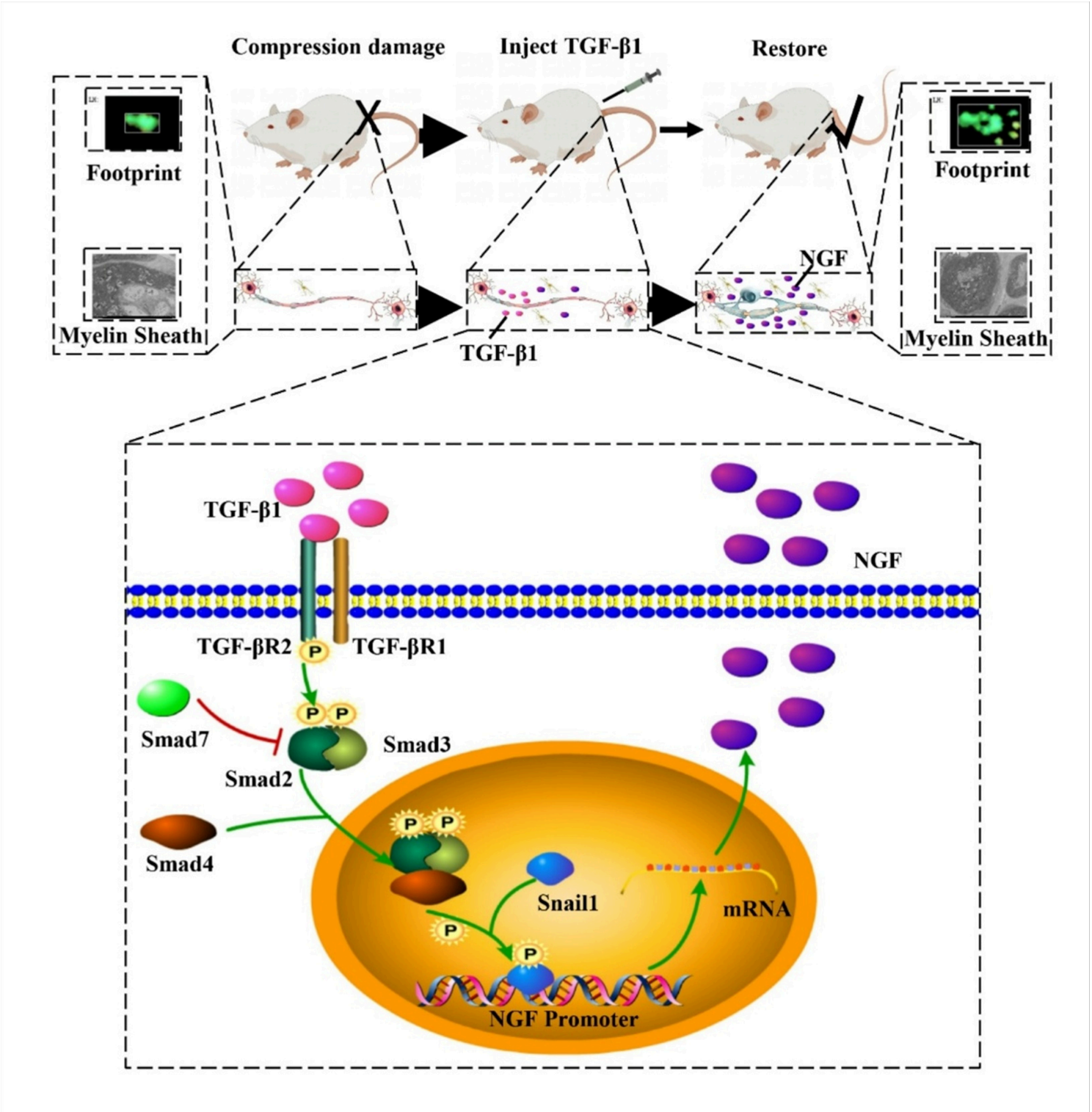


Figure 6

Work model of our mechanical study of the expression of NGF in Schwann cells is correlated with TGF-β1. In the rat experiment, the dorsal root nerve was damaged by compression injury. When TGF-β1 was injected, compared with the control group, the experimental group achieved better repair of the dorsal root nerve in terms of morphology and function. To elucidate the reasons for this phenomenon, we conducted cellular and molecular level signaling pathway studies and found that exogenous TGF-β1 can induce phosphorylation of Smad3 in cells by binding to membrane receptors. Smad complexes can

regulate the transcription of NGF in DNA fragments by phosphorylating the transcription factor Snail1. Specifically, phosphorylated Snail1 can bind to the promoter sequence of DNA to trigger transcription. When NGF is expressed and secreted more, the repair of the dorsal root nerve occurs.

Supplementary Files

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