

Local delivery of engineered exosomes promotes nerve regeneration post sacral nerve injury via macrophage polarization

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Abstract

Sacral nerve injury is a common form of peripheral nerve injury with limited treatment options. macrophage infiltration and activation play a vital role in nerve degeneration and regeneration preceded by, along with the release of inflammatory mediators. However, the effect of exosomes from induced neural stem cells (iNSCs) on sacral nerve injury has not yet been investigated. In this study, the authors induced neural stem cell (NSCs) from adipose derives stem cell (ADSCs) and further developed a novel nanotherapeutic system-3D-printed hydrogel encapsulating engineered exosomes (pGel@iNd^{NGF}-Exo) to manage sacral nerve injury. *In vivo* assessment demonstrated that pGel@iNd^{NGF}-Exo could apparently promote sacral nerve regeneration. Furthermore, *in vitro* and *in vivo* studies all demonstrated that iNd^{NGF}-Exo could improve the inflammatory activation by modulating macrophages polarization. This study indicated that this novel nanotherapeutic system presents a promising strategy for the effective management of sacral nerve injury.

Introduction

Sacral plexus injury is a serious peripheral nerve injury condition that can result from various diseases, including spinal bifida¹, tethered cord syndrome², anterior sacral meningocele³, sacral canal cyst and sacral fractures^{4,5}. 35% patients of sacral fracture alone has a probability of sacral nerve root fracture⁴, lumbosacral region tumors and iatrogenic factors (pelvic surgery) can all cause acute or chronic sacral nerve injury^{6,7}. In clinical situations, Patients with sacral nerve injury may experience neurogenic bladder and bladder dysfunction due to autonomic nerve injury within the sacral nerves^{3,6,8,9}. According to the published literatures, compared with other peripheral nerves, sacral nerves are less mentioned before. Previous studies almost focus on brachial, sciatic and optic nerves¹⁰⁻¹³. However, there are still many unsettled questions about the mechanism inside sacral nerve injury.

Owing to the potential of differentiation, multiple cytokines' secretion and less immune exclusion, the applications of stem cell in clinical trials are increasing¹⁴⁻¹⁷. For repair and regeneration of nerve, recent studies have reported encouraging evidence of a positive functional benefit after NSCs transplantation^{18,19}. NSCs can play a role of cell substitution and paracrine effects¹⁸. It is self-renewable and can generate neurons and glial cells during the development of the central nervous system. Furthermore, it can also secrete neurotrophic factors²⁰, which have been widely used to treat neurodegenerative diseases, nervous system tumors, nerve transplantation, nerve injury repair and other projects^{18,20,21}. Inconveniently, neural stem cells are rarely preserved and difficult to extract after adulthood, NSCs can be extracted from mouse embryos in experiments, which is time consuming with ethical problems. The above obstacles seriously slowed down the clinical application of NSCs. Consequently, it is worth taking efforts to explore new methods which resembles NSCs, such as gene reprogramming methods or cytokines activation.

ADSCs have the potential of neural differentiation and neuroendocrine ability, could be extracted from adipocytes¹¹. Previous researches have explored the therapeutic effect of ADSCs on nerve injury. ADSCs are applied it to nerve repair and many studies have achieved inspirational results²². Furthermore, previous studies have successfully induced NSCs and motor neuron from adipose-derived stem cell by culturing the ADSCs with specific neuronal medium¹¹. However, the ability of self-replication and immunomodulation has not been studied until now. In this study, the NSCs were induced from ADSCs and the characteristics of stemness, self-replication and immunomodulation were deeply studied. Stem cell-derived exosomes have emerged as a pivotal paracrine mediator of stem cell biological functions, serving as natural nanocarriers for the delivery of mRNAs, miRNAs, proteins and bioactive molecules to target cells, and exerting regulatory effects on tissue repair and immune microenvironment remodeling without the risks of cell transplantation such as tumorigenicity and immune rejection. ADSC-derived exosomes have been verified to possess pro-angiogenic, anti-inflammatory and tissue regenerative potentials, and can be engineered to load functional cargos (e.g., VEGF mRNA) for enhanced therapeutic efficacy in injury repair. Neural stem cell-derived exosomes also play a crucial role in blood-brain barrier repair, synaptogenesis and suppression of neurotoxic astrocyte activation by activating the Wnt/ β -catenin signaling pathway. Notably, exosomes can be stably combined with degradable hydrogels to achieve sustained local release, which effectively prolongs their biological activity at the injury site and avoids the rapid clearance of free exosomes in vivo, thus becoming an ideal delivery system for stem cell-based nerve injury repair strategies.

Neuroinflammation is critical to neuro restoration, curiously has opposite effects. Both M1/M2 polarization of macrophages affects this process²³. In the early stage, macrophages can phagocytize nerve residues and clear unrecoverable neurons, which is called the process of Wallerian's degeneration. We usually think that this is the role of M1 polarization, but excessive macrophage infiltration for a long time will affect the subsequent nerve repair^{23,24}. So, the appropriate modulation of inflammation would contribute to nerve repair. Some stem cells can inhibit the excessive inflammation^{25,26} after injury and shift the M1/M2 polarization²⁷. There is a complex interaction between the local inflammation response and the NSCs. According to previous studies, NSCs can modulate the local inflammation response during nerve injury repair by secreting some inflammatory factors²¹. Meanwhile, NSCs are also activated by such immune metabolites^{28,29}. But the therapeutic capability of exosomes from the induced NSCs from ADSCs in peripheral nerve injury is still unclear.

In this study, we utilized NSCs induced from ADSCs combined with degradable hydrogel as treatment measures in sacral nerve crush injury. The sacral nerve injury rat model was established. We test the immunomodulation effect of NSCs in vitro and in vivo, we performed a full assessment to evaluate the regeneration of the SNI model from functional. imaging, morphological and molecular level aspects (Fig. 1).

Preparation of Porous GelMA Hydrogel and Scanning Electron Microscopy (SEM) Characterization

Gelatin was dissolved in phosphate-buffered saline (PBS, pH 7.4) at 50°C under constant stirring until fully dissolved. Methacrylic anhydride was then slowly added dropwise under continuous stirring to synthesize gelatin methacryloyl (GelMA). The reaction was carried out under controlled temperature conditions and terminated by diluting the mixture with PBS (fivefold volume) to quench further methacrylation. The resulting solution was transferred into a dialysis membrane (molecular weight cutoff: 12–14 kDa) and dialyzed against deionized water for 5–7 days to remove unreacted reagents and low-molecular-weight byproducts. The deionized water was refreshed twice daily. The purified GelMA solution was subsequently lyophilized for long-term storage or stored at 4°C for immediate use. GelMA prepolymer solutions with final concentrations of 5% and 10% (w/v) were prepared. Porous GelMA (pGelMA) hydrogels were fabricated and subjected to microstructural characterization. For morphological analysis, hydrogels were freeze-dried, fractured, and sputter-coated prior to observation. The internal porous structure was examined using scanning electron microscopy (SEM, JEOL IT-100). Representative images were acquired, and pore size distribution was quantitatively analyzed using image analysis software from multiple randomly selected fields.

Rheological Analysis of GelMA Hydrogel

Rheological measurements were performed using a rotational rheometer (MCR302, Anton Paar, Austria). GelMA hydrogels with different concentrations (5%, 10%, and 15%, w/v) were analyzed to evaluate viscosity changes over time at a constant shear rate of 10 s^{-1} and 25°C. Measurements were recorded at 10 s intervals for 15 min. For GelMA-based composite hydrogels, dynamic mechanical properties were assessed under controlled strain and frequency conditions. The storage modulus (G') and loss modulus (G'') were recorded as functions of temperature and frequency. Temperature sweeps were conducted from 4°C to 40°C and then cooled back to 4°C, while frequency sweeps were performed over a range of 0–100 Hz.

Mechanical Testing

Hydrogel samples (diameter: 8 mm; height: 6 mm; $n = 3$) were subjected to compression testing using a mechanical testing system (Instron 5543A, USA) at a constant loading rate of 2 mm/min. The compressive modulus was determined from the slope of the linear region of the stress–strain curve.

Degradation Analysis

The biodegradation behavior of the hydrogels was evaluated using a gravimetric method. The initial dry mass of each hydrogel was recorded as W_0 . Samples were then immersed in 2 U/mL collagenase solution in PBS and incubated at 37°C. At predetermined time points, hydrogels were removed, gently

blotted, and weighed to obtain the remaining mass (W_1). The mass remaining ratio was calculated as follows: $\text{Mass remaining (\%)} = (W_1 / W_0) \times 100\%$

Isolation and Culture of Adipose Derived Stem Cells (ADSCs)

The ADSCs were isolated as follows. The subcutaneous adipose tissue was acquired from the bilateral inguinal region of Sprague Dawley rats of 3 days which were sacrificed and immersed in 75% ethanol for 15 min. Extracted adipose tissue were washed with PBS (Solarbio life science, Beijing, China) containing 10% penicillin-streptomycin (Gibco, 15140-122, USA) for 3 times. Then the tissue was transferred to a sterile vial with 7.5 ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco, 10567022, USA), 0.1% type-III collagenase (absin, abs47048000, China) and magnetic bead. The tissue was stirred for 30 min until digested into homogenate. The low-glucose DMEM containing 10% fetal bovine serum (FBS, Gibco, 10091148, USA) was then added to terminate the digestion. After the filtration, the filtrate was centrifuged at 1700 rpm for 5 min. Sediments containing ADSCs were re-suspended and transferred to a 75 cm² flask (Corning, 430641, USA). The cells were cultured at 37 °C with 5% CO₂ and refreshed with culture medium every other day.

NGF Plasmid Loading by CNP-Mediated Electroporation

Before neural induction, ADSCs were subjected to NGF plasmid loading using a cell membrane-engineered nanoporation/electroporation strategy according to the experimental design shown in the figure. Briefly, ADSCs were collected and mixed with a plasmid encoding nerve growth factor (NGF). Electrical stimulation was applied to facilitate intracellular delivery of NGF plasmids. The NGF plasmid-loaded ADSCs were subsequently transferred to fresh culture medium and used for neural induction.

The engineered exosomes generated from the induced cells were defined as iNSCs-derived engineered NGF-Exo and abbreviated as iNd^{NGF}-Exo in this study. This terminology was used consistently for subsequent exosome characterization, hydrogel loading, in vitro treatment, and in vivo administration.

Induction of the Neural Stem Cells (NSCs)

The medium used for the induction of NSCs consisted of Neurobasal medium (Gibco, 21103049, USA), 2% B27 supplement (Gibco, 17504044, USA), 20 ng/ml the epidermal growth factor (EGF, Gibco, PHG0314, USA) and 20 ng/ml the basic fibroblast growth factor (b-FGF, Gibco, PHG0264, USA). The ADSCs were cultured in treated Petri dishes (Corning, 430167, USA) with NM to allow the aggregation of cells and the formation of NSCs, followed by 4 days' culture in untreated ultra-low attachment Petri dishes (Corning, 3262, USA). The aggregation of ADSCs and the formation of neurospheres were observed by light field of inverted microscope (Olympus, IX53, Japan) everyday throughout the induction. ADSC-induced neural stem cell-like cells were referred to as induced neural stem cells (iNSCs) in this study.

Identification of the iNSCs

LIVE/DEAD Staining

The viability of cells was examined with LIVE/DEAD Fixable Dead Cell Stain Kits (ThermoFisher, L34957, USA) at the 7th day after the induction.

Cytological Immunofluorescent Staining

The proliferation of NSCs was tested by the immunofluorescent staining as the following procedures: The NSCs were plated on poly-L-Lysine (Sigma-Aldrich, Germany) coated cover slips inside a 12-well plate containing NM. The cells were fixed with 4% PFA for 30 min; washed with PBS solution for 3 times; blocked in 10% normal goat serum (Cayman Chemical, Ann Arbor, MI, USA) for 1 h; incubated with rabbit anti-Ki67 monoclonal antibody (Abcam, ab16667, 1:400) at 4 °C overnight, washed with PBS for 3 times, incubated with secondary antibody (Alexa Fluor 594 goat anti-rabbit, Abcam, Cambridge, UK) for 1h away from light, washed with PBS for 3 times, stained with DAPI (nuclear dye 4',6-diamidino-2'-phenylindole) for 15 min, washed with PBS for 3 times and cover with a cover glass at last.

Cytological Western Blot Assay

The NSCs harvested at 7th day after the induction were used for immunofluorescent staining (as described before) which verified the specificity of NSCs. The primary antibody applied included rabbit anti-Nestin polyclonal antibody (abcam, ab92391, 1:200), rabbit anti-SOX2 monoclonal antibody (Abcam, ab93689, 1:200) and rabbit anti-pax6 monoclonal antibody (Abcam, ab195045, 1:200) as NSC marker. Meanwhile, the protein of neurospheres were harvested with the RIPA Lysis Buffer (Beyotime, P0013C, China) and quantified with BCA Protein Assay kit (Beyotime, P0012S, China). The protein was separately by SDS-PAGE and transferred to a PVDF membrane, which was then blocked with 5% non-fat milk containing 0.05% Tween-20 for 1h. The membranes were incubated with primary antibodies at 4°C overnight: rabbit anti-Nestin polyclonal antibody, rabbit anti-SOX2 monoclonal antibody. Alexa Fluor 594 goat anti-rabbit (Abcam, Cambridge, UK) and Alexa Fluor 488 goat anti-rabbit (Abcam, Cambridge, UK) serve as the secondary antibodies. The protein bands were exposed to ECL hyperfilm and scanned with the densitometry (beyotime biotechnology, Shanghai).

Cytological Flow Cytometry

The NSCs were collected and resuspended for flow cytometry at the 3rd and 7th day after induction. The single cell suspension was permeabilized with Foxp3 staining Buffer Kit (eBioscience, 00-5523-00.USA) and incubated with mouse anti-SOX2 Alexa Fluor 647-conjugated antibody (R&D, IC2018-100UG) and mouse anti-Nestin PE-conjugated antibody (R&D, IC2736P) for 15min. The 50000 events for each samples were tested with Flow Cytometry (Beckman Coulter Life Sciences, DX FLEX, USA) and the data were analyzed using the software (Kaluza, German).

Isolation and Purification of iNSCs-Derived Engineered NGF-Exo

After neural induction, NGF plasmid-loaded iNSCs were cultured in exosome-depleted complete medium to minimize contamination from serum-derived exosomes. When neurospheres were stable and the cells were ready for collection, conditioned medium was collected for exosome isolation. Exosomes isolated from NGF-engineered iNSCs were termed iNSCs-derived engineered NGF-Exo (iNd^{NGF}-Exo). Exosomes isolated from non-engineered ADSCs were used as ADSC-Exo controls when required.

The collected supernatant was subjected to serial differential centrifugation to remove cells, debris, and large vesicles. Specifically, the medium was centrifuged at 300 × g for 10 min to remove intact cells, followed by 2000 × g for 10 min to eliminate cellular debris. The supernatant was filtered through a 0.22 µm sterile filter to further exclude large particles and apoptotic bodies.

Subsequently, the filtrate was centrifuged at 10,000 × g for 30 min at 4°C to remove larger vesicles. The resulting supernatant was ultracentrifuged at 100,000 × g for 70 min at 4°C to pellet exosomes. The exosome-containing pellet was resuspended in pre-cooled PBS and subjected to an additional ultracentrifugation step under the same conditions to improve purity. The final exosome pellet was resuspended in a minimal volume of PBS and stored at – 80°C until further use. All procedures were conducted at 4°C to preserve exosome integrity and biological activity.

Identification and Characterization of iNd^{NGF}-Exo

Morphological Characterization

The morphology of control exosomes and iNd^{NGF}-Exo was examined using transmission electron microscopy (TEM). Briefly, exosome suspensions were placed onto carbon-coated copper grids and negatively stained with phosphotungstic acid. After air-drying at room temperature, samples were observed under TEM to assess vesicle morphology and structural integrity.

Particle Size Distribution and Concentration Analysis

Nanoparticle tracking analysis (NTA) was performed to determine the size distribution and particle concentration of control exosomes and iNd^{NGF}-Exo. Each sample was measured in triplicate to ensure reproducibility, and the results were averaged for quantitative analysis.

Western Blot Analysis of Exosome Surface Markers

Exosome-associated protein markers were analyzed by Western blotting. Total protein from exosome samples and donor cells was extracted and separated via SDS-PAGE, followed by transfer onto PVDF membranes. Membranes were incubated with primary antibodies against canonical exosome markers, including CD9, CD63, TSG101, and HSP70, and the negative marker calnexin, followed by appropriate secondary antibodies. Protein bands were visualized using a chemiluminescence detection system, and band intensities were quantified using grayscale analysis.

Quantification of NGF mRNA in Exosomes

Total RNA was extracted from exosome samples using an appropriate RNA isolation method. The extracted RNA was reverse-transcribed into complementary DNA (cDNA), followed by quantitative real-time PCR (RT-qPCR) using NGF-specific primers. Relative NGF mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, with GAPDH serving as an internal reference for normalization. All reactions were performed in triplicate.

Zeta Potential and Protein Concentration Measurement

The surface charge properties of control exosomes and iNd^{NGF}-Exo were evaluated by measuring zeta potential using a nanoparticle zeta potential analyzer. Total protein concentration of exosome samples was determined using the bicinchoninic acid (BCA) assay. A standard protein calibration curve was generated for quantitative analysis, and measurements were performed according to the manufacturer's instructions.

Exosomes loading and sustained releasing test in vitro

To load exosomes, the exosome suspension was introduced into the hydrogel solution to achieve a final concentration of 10 mg/mL. Subsequently, a 3D-printed hydrogel patch was bio-fabricated using dynamic projection stereolithography, conforming to the shape of the sacral hiatus.

To establish the release profiles of primary and Exos loaded hydrogels, 100 μ L of exosome-loaded hydrogel (10 mg/mL, post UV exposure) was submerged in 500 μ L of PBS dissolution medium at 37°C. At defined time points, 500 μ L of the sample was collected, and an equal volume of pre-warmed PBS was added back. Exosome release was quantitatively assessed using the Micro BCA™ Protein Assay Kit (ThermoFisher, Rockford, USA) [12], and the release profile was constructed based on the obtained data.

Exosome Uptake Assay in Schwann Cells

Exosome uptake by Schwann cells was evaluated using fluorescent dye-labeled exosomes. Schwann cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO₂. Cells were seeded on coverslips and allowed to adhere before treatment.

ADSC-Exo and iNd^{NGF}-Exo were pre-labeled with Dil according to the manufacturer's instructions under light-protected conditions. PBS was processed in parallel as a negative control. The labeled preparations were washed with sterile PBS by ultracentrifugation to remove unbound dye and then resuspended in sterile PBS.

Schwann cells were divided into three groups: PBS, ADSC-Exo, and iNd^{NGF}-Exo. Labeled exosomes were added to the culture medium at the indicated concentration and co-incubated with Schwann cells for 6 h. Cells were then washed three times with PBS, fixed with 4% PFA for 30 min at room temperature, counterstained with a nuclear dye, mounted, and observed using confocal microscopy.

The Induction and Treatment of Rat Macrophages

Rat macrophages were acquired from bronchoalveolar lavage fluid and cultured in complete medium at 37°C with 5% CO₂. Macrophages were plated in six-well plates and randomized into the following groups: LPS/IFN group, LPS/IFN + ADSC-Exo group, and LPS/IFN + iNd^{NGF}-Exo group. For pathway inhibition experiments, an additional LPS/IFN + iNd^{NGF}-Exo + AS1517499 group was established.

Cells in the LPS/IFN group were treated with 300 ng/mL LPS and 100 ng/mL IFN-γ to induce an M1-like phenotype. Cells in the exosome-treated groups were additionally treated with ADSC-Exo or iNd^{NGF}-Exo, respectively. The induction lasted for 36 h, after which macrophages were harvested for Western blotting, qRT-PCR, immunofluorescence staining, and flow cytometry. AS1517499, a STAT6 phosphorylation inhibitor, was added to evaluate the role of STAT6 signaling in macrophage polarization.

The Examination of Polarization of rat Macrophages

Cytological Flow Cytometry

Surface antigenic expression of M1 and M2-polarized macrophages was analyzed by flow cytometry as described before. Macrophages were labeled antibodies for 15 min: cyanine5.5 anti-CD45 antibody (ELabscience, E-AB-F1227UJ-25UG) as leukocyte marker, FITC anti-CD86 antibody (BioLegend, 200305) as M1 macrophage marker, APC anti-CD163 antibody (Novus, NBP1-43341APC) as M2 macrophage marker. Followed by the rupture of cell membrane with Fcγ3 staining Buffer Kit, cells were stained with PE anti-CD68 antibody (Milenyi, 130-123-757) for 15 min as macrophage marker. The 50000 events for each samples were tested with Flow Cytometry(Beckman Coulter Life Sciences, DX FLEX, USA)and the data were analyzed using the software Kaluza (Kaluza, German).

Cytological Immunofluorescent Staining

The represented immunofluorescent staining included anti-CD68 polyclonal antibody (abcam, ab125212, 1:20000), rabbit anti-CD206 monoclonal antibody (CST, 24595, 1:1000), mouse anti-CD86 monoclonal antibody (Novus, BU63, 1:200). The expression of protein in macrophage was tested by Western Blot assay: rabbit anti-CD68 polyclonal antibody (Abcam, ab125212, 1:20000), rabbit anti-CD206 monoclonal antibody (CST, 24595, 1:1000), mouse anti-CD86 monoclonal antibody (Novus, BU63, 1:200), rabbit anti-VEGF polyclonal antibody (Abcam, ab46154, 1:1000), mouse anti-VEGFR2 monoclonal antibody (Abcam, ab9530, 1:500), rabbit anti-Ras polyclonal antibody (Abcam, ab52939, 1:10000), rabbit anti-ERK1/2 monoclonal antibody (Abcam, ab184699, 1:20000), rabbit anti-p-ERK1/2 monoclonal antibody (Abcam, ab201015, 1:1000), rabbit anti-STAT6 monoclonal antibody (Abcam, ab32520, 1:1000), rabbit anti-p-STAT6 monoclonal antibody (Abcam, ab188080, 1:1000).

Quantitative Real-Time Polymerase Chain Reaction (qRT PCR)

The macrophages were plated in a Petri dish at a density of 100000 cells/ml, cultured for 48 h and then added with 1 ml Trizol Reagent. The suspension was completely collected to a 1.5 ml EP tube with 300 μl

Tris-saturated phenol to extracted the total RNA. Centrifugated at 12600 rpm for 15 min, the upper aqueous phase was transferred and fully blended into isometric pre-cooled isopropanol, which was centrifugated again at 12000 rpm for 10 min afterwards. Sediments alone was dissolved in 1ml 75% ethanol to get sequential centrifugation: 7900 rpm for 4 min, 12000 rpm for 1 min. 20 ~ 55 µl diethyl pyrocarbonate-treated water (DEPC-water) was used to dissolve the dried RNA particles. Reverse transcription was made using ReverTra Ace® qPCR RT Kit (FSQ-201, TOYOBO, Japan) at 37 °C for 15 min and at 85 °C for 5 s. The qPCR was performed using StepOne™ Real-Time PCR System (Applied Biosystems) :95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C, 15 s at 65 °C. The sequences of primers for TNF-α and IL-4 were listed in Table 1. The relative expressions were determined by the normalizing expression of each Ct value to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Ct value.

Table 1
Primers sequences for quantitative RT-PCR

Gene		Primer Sequence (5' to 3')	Temperature (°C)
TNF-α	Forward:	CCAGGTTCTCTTCAAGGGACAA	56
	Reverse:	GGTATGAAATGGCAAATCGGCT	
IL-4	Forward:	ACCTTGCTGTACCCTGTTCT	58
	Reverse:	AGCTCGTTCTCCGTGGTG	

Surgical procedure and treatment

All animal experiments were conducted under the U.S. National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. The study was approved by the Administration Committee of Experimental Animals, Chinese PLA General Hospital, China. Animals were provided by the Experimental Animal Center of Chinese PLA General Hospital.

Female Sprague–Dawley rats weighing 220–225 g were anesthetized by intraperitoneal injection of sodium pentobarbital before surgery. At the predetermined experimental endpoint, the rats received sodium pentobarbital (50 mg/kg, intraperitoneally). After deep anesthesia was confirmed by the absence of a pedal withdrawal reflex, cervical dislocation was performed by trained personnel. Death was confirmed by the cessation of respiration and heartbeat before tissue collection.

After posterior incision, the bilateral second sacral vertebrae were exposed and carefully removed to reveal the sacral nerve root. Crush injuries of the bilateral sacral nerves were produced twice using maximal clamping with a microneedle holder for 10 s each time, with a 10 s interval; the pressure was maintained at approximately 10 N.

Rats were randomized into four groups: Sham (n = 6), Crush (n = 6), ADSC-Exo (n = 6), and iNd^{NGF}-Exo (n = 6), sample size was decided for statistical analysis. In the Sham group, the sacral nerve roots were

exposed without crush injury. In the Crush group, rats received an equivalent volume of PBS or blank pGel at the injury site. In the ADSC-Exo group, ADSC-Exo incorporated in pGel was applied locally to the crushed nerve. In the iNd^{NGF}-Exo group, pGel@iNd^{NGF}-Exo was applied locally to the crushed nerve immediately after injury. Group names in figures were abbreviated as ADSC-Exo and iNd^{NGF}-Exo. All animals were included at the baseline. No experimental units were excluded. Since no a priori removal criteria were established, animals that experienced major unforeseen accidents or severe illness were excluded post-hoc from the data analysis.

All rats were housed with free access to water and food. Bladders were emptied by the Crede procedure once daily for 4 weeks until euthanasia. To minimising potential confounders, animal cages were randomly placed on the racks, and the order of treatments and measurements was randomized. All experimenter conducting was blinded to group allocation.

Urodynamic Test

The retrograde urodynamic test was carried out at the end of the observation period as previously described with some modifications. In brief, the rats were anesthetized by urethane (10%, 1 g/kg), the dose of which was adjusted according to the toe pinching reflex. The sterile PE-50 catheter was retro-inserted into the bladders through the urethras. The bladder was then perfused with normal saline 0.9% by an infusion pump (Harvard Apparatus, Cambridge, MA, USA) at a rate of 5 ml/h. An electrophysiology recorder (Millar, Texas, USA) with a pressure transducer was connected to the PE-50 for cystometrogram with the initial bladder pressure rectified to zero. Urodynamic parameters for each rat (leakage point pressure, maximum bladder volume) were collected directly from the respective urination cycles.

And the bladder compliance was calculated with the bellowed formula:

$$\text{bladder compliance} = \frac{\text{maximum bladder volume}}{\text{leakage point pressure}}$$

High-frequency microultrasoundgraphy

The measurement of postvoid residual (PVR) were performed as an evaluation of urination function by a portable digital ultrasound imaging system (Lanmage, Shenzhen, China). At the 2 weeks and 4 weeks points after the surgical procedures, the rats of four groups were anesthetized with inhalant isoflurane (RWD Life Science Co., Shenzhen, China) delivered by a gas anesthesia machine (Medical Supplies & Services Int. LTD, UK). The lower abdomen was unhaired with depilatory paste and covered with coupling agent (Jinya Co, Tianjin, China). The lower abdomen was scanned with a high frequency linear probe (65 channels, 10 MHz, 38 mm). The axial and sagittal sectional images with the largest area were captured. Firstly, the probe was in axial position to capture the oval-shaped bladder and measure the width (D1) and depth (D3) simultaneously. Then, with probe rotated 90 degrees, a wedgy-shaped bladder in sagittal position was captured and the length (D3) was measured. The bladder volume could be estimated with the formula:

$$Volume = \frac{D1 \times D2 \times D3}{2}$$

Voiding Pattern Analysis

Voiding pattern analysis serve as a noninvasive evaluation of urination function. All rats of four groups were placed solitarily in cages (27 cm * 17 cm) with iron gauze at the bottom. The adaptive period last for 6 h and then a filter paper was fixed onto the gauge. After 4 h, the filter paper was collected and area of urine vestige were measured under the ultraviolet light (UV). The area of voiding was analyzed with ImageJ 1.52a.

Histopathological Evaluation

The rats of all groups were humanely euthanized at the end of the study subsequently, the injury sites of sacral nerves and bladders were harvested. The images of gross appearance of bladders were captured. The nerves and bladders were fixed in 4% PFA and dehydrated with 30% sucrose solution. After that, all tissues were embedded in OCT Tissue Tek (Sakura, USA) and soon afterwards, sectioned at 10µm thickness. All slide of the bladder were stained with hematoxylin&eosin (H&E, Solarbio life sciences, Beijing, China) and Masson's trichrome staining (Solarbio life sciences, Beijing, China). The thickness of the bladder wall was measured and the ratio of the muscle and collagen were calculated, Slides of sacral nerve were stained with H&E. All images were analyzed with ImageJ 1.52a.

Tissue Immunofluorescent Staining

The slide of bladder and sacral nerve were obtained in accordance with the method in "Histopathological evaluation". Slides with tissues were processed as follows: fixed in 4% PFA for 30 min; washed with PBS solution (Solarbio life sciences, Beijing, China)for 3 times; blocked in 10% normal goat serum (Cayman Chemical, Ann Arbor, MI, USA)for 1 h; incubated with a primary antibody at 4 °C overnight; washed with PBS for 3 times ; incubated with a secondary antibody at RT away from light for 2 h; washed with PBS for 3 times ; stained with DAPI(nuclear dye 4',6-diamidino-2'-phenylindole) for 15 min and cover with a cover glass at last. The primary antibodies included rabbit anti-S100 polyclonal antibody (ab11428, 1:200; Abcam, Cambridge, UK), mouse anti-neurofilament antibody (ab215903, 1:200; Abcam, Cambridge, UK), rabbit anti-collagen Ⅰ monoclonal antibody (ab270993, 1:100; Abcam, Cambridge, UK), mouse anti-collagen Ⅰ monoclonal antibody (ab6310, 1:100; Abcam, Cambridge, UK). The secondary antibodies used were Alexa Fluor 594 goat anti-rabbit (Abcam, Cambridge, UK), Alexa Fluor 594 goat anti-mouse (Abcam, Cambridge, UK), Alexa Fluor 488 goat anti-rabbit (Abcam, Cambridge, UK) and Alexa Fluor 488 goat anti-mouse (Abcam, Cambridge, UK). All slides were examined and imaged using a fluorescence microscope (Leica, Wetzlar, Hessen, Germany). The average intensity of immunofluorescence was calculated by an image analysis system (ImageJ 1.52a).

Tissue Western Blot Analysis

Sacral nerves were harvested and homogenized by Tissue Lyser. With Cell Lysis Buffer Kit. Afterwards, the proteins were quantified with BCA kit and electrophoresed with SDS-PAGE. The separate proteins were transferred to a PVDF membrane, which was then blocked with 5% non-fat milk containing 0.05% Tween-20 for 1h. The membranes were incubated with primary antibodies at 4°C overnight: rabbit anti-CD68 polyclonal antibody (Abcam, ab125212, 1:20000), rabbit anti-CD206 monoclonal antibody (CST, 24595, 1:1000), mouse anti-CD86 monoclonal antibody (Novus, BU63, 1:200), rabbit anti-VEGF polyclonal antibody (Abcam, ab46154, 1:1000), mouse anti-VEGFR2 monoclonal antibody (Abcam, ab9530, 1:500), rabbit anti-Ras polyclonal antibody (Abcam, ab52939, 1:10000), rabbit anti-ERK1/2 monoclonal antibody (Abcam, ab184699, 1:20000), rabbit anti-p-ERK1/2 monoclonal antibody (Abcam, ab201015, 1:1000), rabbit anti-STAT6 monoclonal antibody (Abcam, ab32520, 1:1000), rabbit anti-p-STAT6 monoclonal antibody (Abcam, ab188080, 1:1000), anti- β -actin monoclonal antibody (Immunoway, YM3028, 1:5000). The protein bands were exposed to ECL hyperfilm and scanned with the densitometry.

RNA sequence and quantification of cytokine concentration

Sacral nerve tissue was sequenced on an Illumina HiSeq™ 2500 desktop sequence. RNA sequencing libraries were prepared using the TruSeq Small RNA Sample Prep Kits (Illumina, USA). Differential expression analysis and target gene enrichment analysis were conducted using ACGT101-miR (v4.2). Venn diagram and volcano diagram were used to demonstrate the differentially expressed genes. Functional enrichment analyses were conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database to identify pathways related to nerve regeneration and inflammation. Gene Ontology (GO) enrichment analyses were used according to p-value < 0.05 of exclusion criteria. The BioPlex rat cytokine assay was adopted for quantifying of cytokine concentrations at acute phase. Sacral nerves were harvested at 3rd day after injury. Tissues were firstly washed by PBS and dissociated by RIPA and Tissue Grinder. The lysis was separated by centrifugation and stored at the degree of -80°C before analysis. Luminex liquid suspension chip detection was implemented by Wayen Biotechnologies (Shanghai, China). The BioPlex protein array (Bio-Rad, Hercules, CA, USA) and Luminex 100 (Mirai Bio, Alameda, CA, USA) were adopted according to the manufacturer's instruction. A cytokine panel of rat (Bio-Plex Pro Rat 23-plex Cytokine Grp Panel, #12005641) was used to measure 23 cytokines, including IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, IL-18, G-CSF, GM-CSF, GRO/KO, INF, MCP-1, MIP-1 α , MIP-3 α , M-CSF, RANTES, TNF- α and VEGF. All lysates were detected in triplicate. The lower limits of cytokine detection were different for each experiment and cytokine. The detection ranges from 0.01 to 1.0 pg/ml. Protein-protein interaction (PPI) networks were constructed for the differentially expressed cytokines to identify potential interactions among the detected proteins. All procedures were performed according to the manufacturers' protocols and standard bioinformatics pipelines.

Tissue Flow Cytometry

Surface antigenic expression of M1 and M2-polarized macrophages was analyzed by flow cytometry. The sacral nerves were cut into pieces and enzymolyzed to single cell suspension. Cells were then

incubated with fluorescence labeled antibodies for 15 min: cyanine5.5 anti-CD45 antibody (ELabscience, E-AB-F1227UJ-25UG) as leukocyte marker, FITC anti-CD86 antibody (BioLegend, 200305) as M1 macrophage marker, Allophycocyanin anti-CD163 antibody (Novus, NBP1-43341APC) as M2 macrophage marker. Followed by the rupture of cell membrane with Fcγ3 staining Buffer Kit, cells were stained with PE anti-CD68 antibody (Miltenyi, 130-123-757) for 15 min as macrophage marker. Then cells were washed and suspended in PBS. The 100000 events for each samples were tested with Flow Cytometry(Beckman Coulter Life Sciences, DX FLEX, USA)

Statistics

Statistical analyses were conducted using GraphPad Prism 11.0 (GraphPad Software, San Diego, CA, USA). Comparisons between two groups were performed using Student's t-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Data are presented as mean ± standard error of the mean (SEM). P values < 0.05 were considered statistically significant.

Results

ADSCs were derived into iNSCs via inducible factors and neurobasal medium.

ADSCs (P3) were harvested and re-suspended in NSCs-induce medium (Fig. 1A), cells were started to aggregate after 3 days and the neurosphere was formed at 7th day (Fig. 1C). The results of western blot assay showed that the expression of nestin and SOX2 were significantly up-regulated after induction (Fig. 1B). The immunofluorescence staining showed the similar results that the density of neurosphere surface markers including nestin, SOX2 and PAX6 were higher in induced neurosphere (Fig. 1E), however the expression of these markers was much lower in ADSCs (Fig. S1). Live/Dead staining showed that over 80% percentage cells were alive in sphere (Fig. 1D), which mean the good vigour during induction. Maintaining the reproducibility during induction was also important for neurosphere, the Ki-67 staining showed that the neurosphere possessed good reproducibility during induction (Fig. 1F). Flow cytometry was also performed for identifying the induced-NSCs at 3rd and 7th day, the results showed that the nestin+SOX2 + cells were increased during the induction process (Fig. 1G and H). The nestin positive cells and SOX2 positive cells were also increased separately during induction process (Fig. 1I). Neural-associated protein expression was negative in ADSCs (Fig. S1)

Preparation and characterizatiion of iNSCs-derived engineered NGF-Exo

iNSCs-derived engineered NGF-Exo (called iNd^{NGF}-Exo in the following article) were initially collected (Fig. 2A). To prepare iNSCs-derived Exo loaded with NGF mRNA, the Cell membrane-engineered nanoparticles (CNP) technique was employed, which involved transfecting NGF plasmids into ADSCs.

The morphology of ADSC-Exo, ADSC^{NGF}-Exo and iNd^{NGF}-Exo secreted post-CNP was examined by TEM. Both types exhibited similar sizes and the typical cup-shaped structures with smooth bilayer membranes (Fig. 2B). NTA revealed that the size distributions of iNd^{NGF}-Exo were ranging from ≈ 50 –250 nm (Fig. 2E). The result of zeta potential and OD value determined by BCA after quantification further proves that there is no difference in potential between the two (Fig. S2). Western blotting analysis showed significantly higher expression levels of CD9, HSP70, and TSG101 in the iNd^{NGF}-Exo group. To investigate whether iNd^{NGF}-Exo could be effectively internalized by Schwann cells, PKH26-labeled exosomes were co-cultured with Schwann cells for 48 h and 72 h, respectively. Confocal laser scanning microscopy (CLSM) analysis revealed minimal red fluorescence in the PBS control group, indicating negligible nonspecific background signal (Fig. 2C and S3). In contrast, robust PKH26 fluorescence (red) was clearly observed in the cytoplasm of Schwann cells treated with both ADSC-Exo and iNd^{NGF}-Exo at both 48 h and 72 h time points, demonstrating efficient cellular uptake of exosomes. Notably, the fluorescence intensity and intracellular distribution patterns were comparable between the ADSC-Exo and iNd^{NGF}-Exo groups at each corresponding time point, with no statistically significant difference detected (Fig. 2F). To evaluate the effects of iNSC-derived exosomes on Schwann cell viability and proliferation, CCK-8 assays were performed. At the initial time point (0 h), no significant difference in optical density (OD) values was observed among the control, ADSC-Exo, and iNd-NGF-Exo groups, confirming comparable baseline cell seeding densities across all experimental conditions. By 72 h, the iNd^{NGF}-Exo group exhibited a significantly elevated OD value compared to the ADSC-Exo and control groups (** $p < 0.01$), indicating robust enhancement of Schwann cell proliferation. (Fig. S4)

Preparation and characterization of pGel @ iNd^{NGF}-Exo

To find the appropriate for iNd^{NGF}-Exo envelopment for nerve regeneration, we configured two different concentrations (5% and 10% (w:v)) of pGel, then Exo was then mixed into pGel. After photo-crosslinked and lyophilization, the structure were observed by SEM (Fig. 2G and 2J). The morphology of pGel scaffold exhibits a continuous and interconnected porous three-dimensional network structure (Fig. 2H and K). The degradation rate of the hydrogels was measured every 5 days during immersion in the culture medium (Fig. 2I). The relationship between compressive stress and strain was revealed in Fig. 2L. Rheology is the science of material deformation and flow (continuous deformation). The profile of viscosity versus time under constant shear rate 10/s was illustrated. The temperature-dependent modulus (G' and G'') change and frequency-dependent modulus (G' and G'') change was inspected (Fig. 2M, H and O). To assess the release kinetics of iNd^{NGF}-Exo from the delivery system, the cumulative release percentage was monitored over a 9-day period. As shown in Fig. S5, the release curve exhibiting a sustained and progressive upward trend over the subsequent time points and achieved nearly complete release (~ 70 –75%) by day 9. Such a sustained release characteristic is favorable for maintaining therapeutic concentrations of NGF-loaded exosomes at the target site and may contribute to the prolonged biological effects observed in Schwann cell proliferation assays.

iNd^{NGF}-Exo suppressed M1 inflammatory cytokine production and promoted M2 phenotype switch

The combination of Interferon (IFN- γ , 100 ng/ml) and Lipopolysaccharide (LPS, 300 ng/ml) induction cause an M1 macrophage phenotype, cells was induced with ADSC-exo and iNd^{NGF}-Exo respectively. The observation period lasted 36 hours, Cells were harvested 36h after LPS/IFN induction for WB, qPCR and flow cytometry. iNd^{NGF}-Exo and ADSC-exo could, to a certain extent, reverse this M1 polarization and cause a switch toward M2 phenotype (Fig. 3A, B and C), and the qPCR results demonstrated that LPS/IFN could promote the increase of the M1 prototypical cytokines compared with + ADSC-exo group and + iNd^{NGF}-Exo group including TNF- α (Fig. 3D, $P < 0.001$, $P < 0.0001$). However, the +ADSC-exo/+ iNd^{NGF}-Exo could partly suppress this process and promote the expression of M2-related cytokine: IL-4 (Fig. 3E, $P < 0.05$, $P < 0.001$). The different expression of CD86 and CD206 was also confirmed by Western blot assay and flow cytometry. The results of WB showed that the expression of CD86 was relatively higher in LPS/IFN induction and the expression of CD206 was upregulated in ADSC-exo and iNd^{NGF}-Exo group (Fig. 3H). Flow cytometry demonstrated the similar results that the percentage of CD68⁺CD86⁺CD206⁻ cells was higher and CD68⁺CD86⁻CD206⁺ cells was lower in LPS/IFN group compared with ADSC-exo, iNd^{NGF}-Exo group (Fig. 3F and G). The results above demonstrated that the iNd^{NGF}-Exo could reverse the M1 polarization by LPS/IFN and promote the M2 polarization.

3D printed hydrogel@iNd^{NGF}-Exo implantation enhanced the functional recovery and radiological evaluation after sacral nerve injury

Urodynamic tests of the lower urinary tract are regarded as the gold standard for functional evaluation (Fig. 4A). After 4 weeks, the urodynamic measurements showed that the leakage point pressure (LPP) was apparently higher in crush group compared to other groups (Fig. 4B, $P < 0.001$). However, the administration of iNd^{NGF}-Exo promote the function recovery of bladder (Fig. 4B, $P < 0.05$). Meanwhile, the maximum bladder volume (MBV) and the compliance of bladder were also significantly increased in crush group compared to other groups (Fig. 4C and D, $P < 0.0001$, $P < 0.001$). The results above demonstrated that the administration of iNd^{NGF}-Exo at the local site of injury could promote the sacral nerve regeneration and further improve the bladder function. Ultrasonography showed that the volume of residual urine was apparently much higher in crush group compared to other groups at 2 weeks (Fig. 4F, $P < 0.05$) and 4 weeks (Fig. 4F, $P < 0.01$, $P < 0.001$). The postvoid residual was a vital parameter for evaluating bladder function, results above demonstrated that PVR was improved after the administration of iNd^{NGF}-Exo. The voiding pattern analysis revealed an increased area on the filter paper after 2 weeks and 4 weeks in the crush group compared to other groups (Fig. 4H and I). And NSC/ADSC could partially reverse this change.

3D printed hydrogel@iNd^{NGF}-Exo implantation promote sacral nerve regeneration by upregulating the M2/M1 cell ratio.

To obtain deeper insight into the positive effect of iNd^{NGF}-Exo on sacral nerve regeneration, we quantified the number of axons and schwann cell by using antibodies NF200 and S100, we also quantified the number of macrophages in nerves by using antibodies CD68, CD86 and CD206. Immunofluorescence

images showed that the number of SCs and axons was significantly reduced in Crush group compared with Sham group (Fig. 5A, $P < 0.0001$) and iNd^{NGF}-Exo caused a significantly upregulation of SCs and axon density (Fig. 5B and C, $P < 0.001$, $P < 0.05$). In addition, we found that the mean density of CD68 + CD86+ cells was higher in Crush group compared to nerves in ADSC-Exo and iNd^{NGF}-Exo group (Fig. 6A, C and D, $P < 0.0001$, $P < 0.01$), however, the number of CD68 + CD206+ cells was higher in ADSC-Exo and iNd^{NGF}-Exo group (Fig. 6B, C and E, $P < 0.05$, $P < 0.01$). The macrophage plays an important in the nerve regeneration at the site of injury. CD68⁺CD206⁺ macrophages could build an anti-inflammation environment which is essential for axon spreading and remyelination, and CD68⁺CD86⁺ macrophages would cause pro-inflammation which would in turn suppress the nerve regeneration after injury. So, we found that iNd^{NGF}-Exo could promote the sacral nerve regeneration after injury by increasing the M2/M1 cell ratio.

(A) The immunofluorescence image (NF200, S100) of the sacral nerves among the four groups. (B) The mean density of NF200 (corrected to DAPI) among four groups (Sham, Crush, ADSC-Exo and iNd^{NGF}-Exo). (C) The mean density of S100 (corrected to DAPI) among four groups (Sham, Crush, ADSC-Exo and iNd^{NGF}-Exo). The data were presented as means $\pm$ SEM of three separate experiments ($n = 3$ per group). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. $P > 0.05$.

3D printed hydrogel@iNd^{NGF}-Exo implantation promote the sacral nerve regeneration and further suppress fibrosis and collagen remodeling of bladder

H&E staining method was used to evaluate the bladder wall thickness. In the crush group, bladder wall thickness was increased apparently compared to sham, ADSC-Exo and iNd^{NGF}-Exo group (Fig. 7A and B, $P < 0.05$, $P < 0.05$, $P < 0.01$). Masson's staining was used to distinguish the smooth muscle and fibrosis (collagen). According to Masson's staining, the ratio of muscle to collagen in crush group was much lower compared to sham group (Fig. 7C and D, $P < 0.0001$). iNd^{NGF}-Exo administration could reverse this pathological process, the ratio of muscle to collagen was lower higher than crush group (Fig. 7C and D, $P < 0.05$, $P < 0.01$). These results mean that the administration of iNd^{NGF}-Exo could promote the regeneration of sacral nerve and further suppress the fibrosis and collagen remodeling of bladder. Immunofluorescence staining of Col-1 and Col-3 of bladder also demonstrated the similar results, urine retention and muscle denervation always cause bladder fibrosis and collagen remodeling, so, the ratio of Col-1 to Col-3 could represent the degree of fibrosis of bladder. In the crush group, the expression of Col-1 was higher than other three groups (Fig. 7E and F, $P < 0.01$, $P < 0.05$ and $P < 0.01$), and the expression of Col-3 was lower by contrast (Fig. 7E and G, $P < 0.01$, $P > 0.05$ and $P < 0.05$). However, in the iNd^{NGF}-Exo group, the expression of Col-1 was lower and Col-3 was higher compared to crush group, which mean that the administration of NSC and ADSC could suppress the collagen remodeling process of bladder after sacral nerve injury.

3D printed hydrogel@iNd^{NGF}-Exo implantation modulated the microenvironment immune-response and M1/M2 phenotype of sacral nerve.

Flow cytometry of sacral nerve was used to analyse the macrophage phenotype. As shown in Fig. 8A, B and C, the CD45⁺CD68⁺CD86⁺CD163⁻ cells (M1) and the CD45⁺CD68⁺CD86⁻CD163⁺ cells (M2) were gated step by step. The ratio of M1/M2 was statistically lower in iNd^{NGF}-Exo group compared to Crush group (Fig. 8D, $P < 0.05$). Luminex analysis showed the acute cytokines reaction at the injury site of sacral nerves (Fig. 9).

(A) The Venn diagram demonstrated the common differential genes of Crush vs ADSC-Exo and Crush vs iNd^{NGF}-Exo group. (B) PCA demonstrated the (C) The volcano diagram showed the differential expressed genes between ADSC-Exo and iNd^{NGF}-Exo group. (D and E) KEGG showed the enrichment pathway of nerve regeneration and inflammatory. (F) Selected GO biological processes were significantly overrepresented ranked by enrichment foldchange between crush and iNd^{NGF}-Exo group. (G and H) Differential expression of cytokines of sacral nerves in three groups: Crush, ADSC-Exo and iNd^{NGF}-Exo group. Luminex liquid suspension chip analysis of sacral nerves in Crush, ADSC-Exo and iNd^{NGF}-Exo group. Schematic representation of full-length and truncated domains of cytokines detected in this study. PPI showed the interaction between these cytokines.

pGel@iNd^{NGF}-Exo suppressed M1 polarization and upregulated M2 polarization via RAS-ERK and STAT6 signaling pathway

Above results have demonstrated the immunomodulation effect of iNd^{NGF}-Exo and its conditioned medium in vivo and in vitro, however, the molecular mechanism inside the immunomodulation was not clear. To further define the signaling pathway inside the M1/M2 switching, we did Western blot assay and we found that the RAS-ERK signaling pathway was activated in LPS/IFN induction in vitro and Crush in vivo (Fig. 10A and C). the expression of RAS (Fig. 10B, $P < 0.05$, $P < 0.01$), p-ERK/ERK (Fig. 10B, $P < 0.05$) was suppressed and p-STAT6/STAT6 (Fig. 10B, $P < 0.05$, $P < 0.01$) was upregulated in iNd^{NGF}-Exo group in vitro. Meanwhile, the similar results were confirmed in studies in vivo. The expression of Ras (Fig. 10D, $P > 0.05$, $P < 0.01$), CD86 (Fig. 10D, $P < 0.05$, $P < 0.01$) and the ratio of p-ERK/ERK (Fig. 10D, $P < 0.01$, $P < 0.001$) was increased in the injury site of sacral nerve in Crush group, these proteins were suppressed by iNd^{NGF}-Exo in vivo and the protein of CD206 (Fig. 10D, $P < 0.05$, $P < 0.001$), p-STAT6/STAT6 (Fig. 10D, $P < 0.05$, $P > 0.01$) were upregulated after the iNSC/ADSCs treatment in the injury site. What's more, in order to further confirm the role of p-STAT6/STAT6 in the process of iNSC/ADSCs on M2 polarization, we use the special p-STAT6 inhibitor (AS1517499, MedChemExpress), AS1517499 was added into the culture of iNd^{NGF}-Exo group, and the WB results showed that the expression p-STAT6 was suppressed after administration of AS1517499 and the expression of CD86/CD206 was also reversed after AS1517499 (Fig. 10E and F, $P < 0.05$, $P < 0.01$, $P < 0.001$), which mean that iNd^{NGF}-Exo promote the switching of M2/M1 via p-STAT6/STAT6 signaling pathway.

iNd^{NGF}-Exo promote the VEGF/VEGFR2 expression in vivo and in vitro

Furthermore, we tested the expression of VEGF in vitro and VEGF/VEGFR2 in vivo by WB. The WB results showed that the expression of VEGF was increased by adding iNd^{NGF}-Exo, as we know, that the M2 macrophage could secret VEGF, so, the VEGF expression was increased in iNd^{NGF}-Exo administrating group (Fig. 10A, and B). The similar results were found in vivo, at the injury site of sacral nerve, the VEGF and VEGFR2 were upregulated after iNd^{NGF}-Exo local injection (Fig. 10C and D). These results demonstrated that iNd^{NGF}-Exo (conditioned medium) could promote the local up-regulation of pro-angiography protein and have positive effect on nerve regeneration.

iNd^{NGF}-Exo cause M1/M2 switching and promote nerve regeneration via RAS-ERK and STAT6 signaling pathway. (A) Western blot assay of molecular signaling of RAS-ERK, (p-)STAT6 and VEGF in vitro. (B) The expression of p-ERK/ERK, p-STAT6/STAT6, Ras and VEGF in cells harvested from culture of LPS/IFN, +ADSC-Exo and + iNd^{NGF}-Exo group. (C) Western blot assay of molecular signaling of RAS-ERK, (p-)STAT6, CD86/CD206 and VEGF/VEGFR2 in vivo. (D) The expression of CD86, CD206, Ras, p-ERK/ERK, p-STAT6/STAT6 and VEGF/VEGFR2 of Crush, ADSC-Exo and iNd^{NGF}-Exo group in vivo. (E) Western blot assay of p-STAT6 and CD86/CD206 after AS1517499 administration. (F) The expression of p-STAT6, CD86 and CD206 of cells harvested from culture of LPS/IFN, + iNd^{NGF}-Exo and AS1517499 group. The data were presented as means $\pm$ SEM of three separate experiments (n = 3 per group). ***P < 0.001, **P < 0.01, *P < 0.05, n.s. P > 0.05.

Collectively, our data clearly demonstrated that iNd^{NGF}-Exo could promote the switching of macrophage into M2 phenotype via Ras-ERK and STAT6 signaling pathway and iNd^{NGF}-Exo may act as an immunoregulator to modulate microenvironment inflammatory reaction in future.

Discussion

Peripheral nerve injury is a serious disease that often cause paralysis and organ denervation. For many years, researches have explored the treatment for peripheral nerve injury such as sciatic nerve and facial nerve. However, few studies elaborated the severity of sacral nerve injury. The reasons for sacral nerve injury are complex, which could happen because of fracture, tethered syndrome, tumor and so on. In our previous studies, we have established the sacral nerve injury rat model with neurogenic bladder which mimics the clinical conditions. Before us, some studies have established the neurogenic bladder rat model by pudendal nerve injury or spinal nerve injury. However, no studies have systematically elaborated the sacral nerve injury combined with neurogenic bladder rat model. In this study, we used this model to evaluate the therapeutic effect of hydrogel-loaded ADSC-Exo and iNSCs-derived engineered NGF-Exo (iNd^{NGF}-Exo) on sacral nerve regeneration.

In previous studies, many researchers have investigated the therapeutic effect of stem cells for spinal cord injury and peripheral nerve injury. Many different kinds of stem cells such as ADSCs, NSCs or iPSCs could promote nerve regeneration when engrafted into the injury site of nerve. However, although many

studies have investigated nerve regeneration, few have focused on exosome-based therapy for sacral nerve regeneration.

In this study, we investigated the therapeutic effect of ADSC-Exo and iNd^{NGF}-Exo delivered by biodegradable hydrogel in a rat model of sacral nerve injury. The hydrogel may help retain exosomes at the injury site and prolong their local bioactivity. The results indicate that exosome-loaded hydrogel accelerated the recovery of sacral nerve injury and improved bladder function. Furthermore, we explored the underlying mechanism and found that ADSC-Exo and iNd^{NGF}-Exo may promote nerve regeneration by modulating macrophage polarization.

ADSCs can be obtained from adipose tissue and passaged in vitro, the resources of adipose-derived stem cells are abundant and the obtainment process is safe. ADSC was regarded as potential cures for some diseases and has been investigated on nerve injury, skin wound³⁰, tumor³¹ and so on. With the progress of research, as for the treatment of nerve injury relative diseases, NSC performs better therapeutic effect compared to ADSC. However, there are still limitations associated with the direct use of primary NSCs, including limited cell sources and ethical concerns, which restrict their clinical translation. In this study, we induced the ADSCs into iNSCs by using some inducible factors and neurobasal medium. The induced iNSCs stably expressed neural markers including Nestin, SOX2 and PAX6 and showed self-renewal capacity, which supported their use as donor cells for engineered exosome production. The induced iNSCs stably expressed neural markers including Nestin, SOX2 and PAX6 and showed self-renewal capacity, which supported their use as donor cells for engineered exosome production^{32,33}. In the present study, NGF plasmid loading was further introduced before neural induction, and the resulting iNSCs-derived engineered NGF-exosomes were used as the main therapeutic component. Some studies have demonstrated methods for inducing NSC-like cells from ADSCs; however, few studies have evaluated iNSCs-derived engineered exosomes for sacral nerve regeneration. In this study, ADSC-induced iNSCs were used to generate engineered NGF-exosomes, and pGel@iNd^{NGF}-Exo promoted sacral nerve regeneration and further improved bladder function. NF200 and S100 was the specific marker for the axons and schwann cells respectively, which can reflect the level of repairment of peripheral nerves. In the Crush group, the density of NF200 and S100 was apparently lower than that in the exosome-treated groups. These results suggested the positive effect of exosome-based treatment on sacral nerve regeneration. Meanwhile, when bladder was denervated, urine retention would happen and the bladder would experience muscle atrophy, collagen remodeling and fibrosis. In this model, pathological processes above were observed by histological evaluation too. And the administration of ADSC-Exo or iNd^{NGF}-Exo could reverse these pathological changes and improve bladder function. Urodynamic test and postvoid residual are also two important functional detection methods for evaluating the bladder function. Previous studies have demonstrated the utility value of these methods³⁴⁻³⁶, in this study, we found that bladder function was improved after administration of exosome-loaded hydrogel.

Regeneration of axons is highly influenced by the recruited macrophages, many previous studies have demonstrated the role that macrophages play during the nerve regeneration^{37–40}. When peripheral nerve is injured, macrophages recruited and polarized into two different subtypes: M1, which is considered pro-inflammation, would accelerate the Wallerian degeneration and M2, which is regarded as anti-inflammation, could promote the subsequent regeneration^{41,42}. However, too often, recruited macrophages are excessively polarized into M1 phenotype, which means the extreme inflammation reaction at local site. And excessive and long-lasting inflammation reaction would inhibit the regeneration process and finally cause poor functional recovery⁴³. So, we consider, if the macrophages could be induced into M2 phenotype at early stage after injury, it could have better functional recovery. Previous studies have demonstrated that the IL-10 deficiency could result in both structural and functional deficits compared to WT mice⁴⁴. Ran Li et al. reported that the bone marrow mesenchymal stem cell could treat spinal injury via promoting M2 macrophage polarization⁴⁵ Pao-Jen Kuo et al. reported that exosomes from ADSCs could treat spinal injury via modulating the polarization of macrophages⁴⁶. However, few studies have elaborated the role of macrophage polarization in sacral nerve regeneration based on exosome-mediated therapy. In this study, we demonstrated the local macrophage recruitment in sacral nerve after crush injury by using immunofluorescence, WB and tissue flowcytometry. Our results demonstrated that ADSC-Exo and iNd^{NGF}-Exo could apparently modulate macrophage polarization at the injury site. Further, we demonstrated by western blotting that ADSC-Exo and iNd^{NGF}-Exo promoted M2 polarization through ERK and STAT6 signaling pathways both in vitro and in vivo. In LPS/IFN and crush group, the phosphorylation state of ERK was upregulated. Conversely, the phosphorylation of STAT6 was downregulated. However, in the ADSC-Exo and iNd^{NGF}-Exo groups, the phosphorylation pattern was reversed, indicating that exosome-based treatment could modulate macrophage polarization through ERK and STAT6 signaling pathways. STAT6 is a subtype of STAT family proteins and its phosphorylation state is known to promote macrophage M2 polarization⁴⁷. Meanwhile, the activated ERK was recognized to promote M1 polarization. Previous studies have demonstrated the role of ERK and STAT6 signaling pathway of macrophages polarization in the tissue regeneration, however, none studies have demonstrated the macrophages modulation of iNSC/ADSCs in sacral nerves. In this study, we showed that ADSC-Exo and iNd^{NGF}-Exo could suppress M1 polarization and promote M2 polarization via ERK and STAT6 signaling pathways. It is widely accepted that intercellular communication is critical for the regenerative effects of stem cell-derived therapies. However, the role of ADSC-Exo and iNSCs-derived engineered NGF-Exo in sacral nerve regeneration through modulation of macrophage polarization has not been fully clarified. Here, we provided evidence that ADSC-Exo and iNd^{NGF}-Exo modulate macrophage polarization in response to sacral nerve injury. Furthermore, ERK and STAT6 are confirmed as the critical regulatory factors in the process of macrophages polarization and the molecular mechanisms are clearly studied in this investigation. Compared with direct stem cell transplantation, exosome-based acellular therapy may reduce concerns related to immune rejection, uncontrolled differentiation and tumorigenicity.^{19,48}. In the present study, the incorporation of engineered exosomes into GelMA hydrogel further enabled local delivery at the injured sacral nerve site. Nevertheless, the specific exosomal cargos responsible for macrophage modulation and nerve

regeneration, as well as the release kinetics of exosomes from GelMA hydrogel⁴⁹, require further investigation.

Conclusion

In summary, we demonstrate insights into the immune-modulation effect and mechanism inside of novel nanotherapeutic system during sacral nerve regeneration. The results suggest that 3D printed hydrogel encapsulated iNd^{NGF}-Exo could modulate the macrophage polarization and promote nerve regeneration post sacral nerve injury. These findings provide a novel insight in the understanding of macrophages related to nerve regeneration. Our work provided a novel therapeutic strategies based on pGel@ iNd^{NGF}-Exo to improve nerve regeneration.

Abbreviations

ADSC

adipose derived stem cell

APC

allophycocyanin

b-FGF

basic fibroblast growth factor

Col

collagen

DAPI

nuclear dye 4',6-diamidino-2'-phenylindole

DEPC

diethyl pyrocarbonate

DMEM

Dulbecco's Modified Eagle Medium

EGF

Epidermal Growth Factor

EP

eppendorf

FBS

Fetal Bovine Serum

FITC

Fluorescein

GAPDH

glyceraldehyde-3-phosphate dehydrogenase

G-CSF

Granulocyte-Colony Stimulating Factor

GM-CSF
Granulocyte/macrophage-Colony Stimulating Factor
GRO
growth-related oncogene/
H&E
hematoxylin-eosin
IFN
Interferon
IL
interleukin
LPP
leakage point pressure
LPS
Lipopolysaccharide
MBV
maximum bladder volume
MCP
Membrane cofactor protein
M-CSF
Macrophage-Colony Stimulating Factor
NF200
neurofilament 200
iNSC
induced neural stem cell
PBS
phosphate buffer saline
PE
P-phycoerythrin
PE-50
Polyethylene-50
PFA
Paraformaldehyde
PVR
postvoid residual
qRT PCR
Quantitative Real-Time Polymerase Chain Reaction
RNA
Ribonucleic Acid
SCs
schwann cells

SD

Sprague Dawley

TNF- α

tumor necrosis factor- α

UV

ultraviolet light

VEGF

vascular endothelial growth factor

VEGFR2

vascular endothelial growth factor receptor 2

WB

Western Blot

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. These rats were obtained from and maintained by the Animal Laboratory Center of the Fifth Medical Center, Chinese PLA General Hospital, under license number SCXK (Military) 2007-004. All experimental procedures were conducted with the approval of the Ethics Committee of the Animal Facility of the Chinese PLA General Hospital and adhered to the established guidelines for the care of laboratory animals. The study was reported in accordance with the ARRIVE 2.0 guidelines. Consent to participate was not applicable because no human participants were involved. The authors declare that they have not use AI-generated work in this manuscript"

Ethic approval and consent to participate: These rats were obtained from and maintained by the Animal Laboratory Center of the Fifth Medical Center, Chinese PLA General Hospital, under license number SCXK (Military) 2007-004. All experimental procedures were conducted with the approval of the Ethics Committee of the Animal Facility of the Chinese PLA General Hospital and adhered to the established guidelines for the care of laboratory animals.

Consent for publication: All authors agree to the publication of this article.

Availability of data and material: All data supporting the findings of this study are available within the article and its Supplementary Materials. Additional information related to the study is available from the corresponding author upon reasonable request.

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Figures

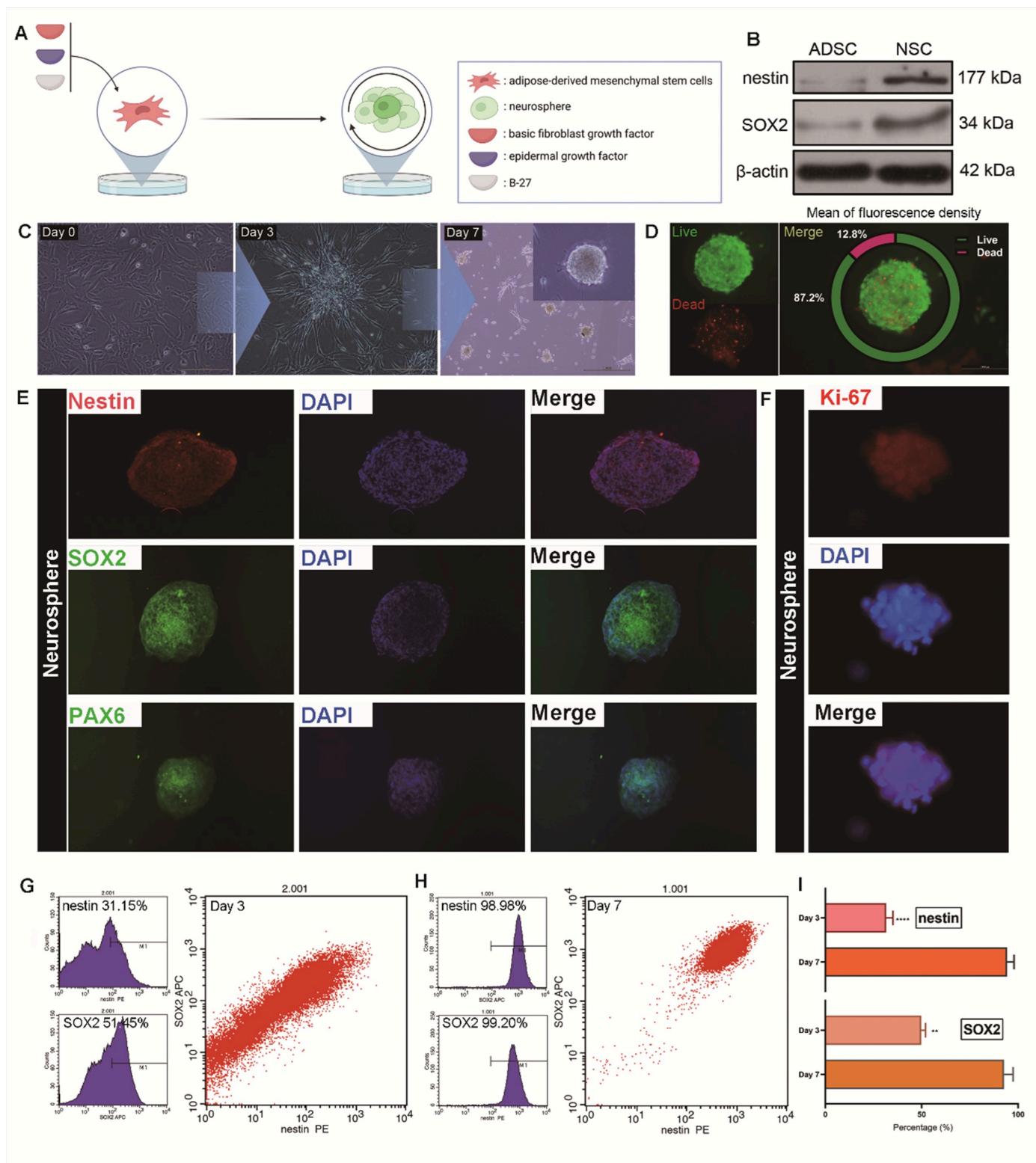


Figure 1

Identification of neural stem cells (NSCs) derived from Adipose-derived Mesenchymal Stem Cells (ADSCs). (A) Illustration of induction procedure of NSCs from ADSCs. (B) Western blot assay of the ADSCs induced neurosphere at 3rd day. Nestin and SOX2 were detected. (C) Photomicrograph showing the morphological change of ADSCs after 3 days and 7 days in NSC-induce culture medium. The cells started to gather after 3 days, and the neurospheres were formed with further culture at 7th day. (D)

Live/Dead staining of Neurosphere at 7th day. Green fluorescent represents the live cells, red fluorescent represents the dead cells. (E) Immunofluorescent staining of Nestin, SOX2 and PAX6 of the neurosphere at 7th day, ADSCs were stained with same antibody as control. (F) Immunofluorescent staining of Ki-67 of the neurosphere at 7th day. (G-I) The neural surface markers Nestin and SOX2 of neural stem cells were analyzed using flow cytometry at 3rd day (G) and 7th day (H), the nestin positive cells and SOX2 positive cells at 3rd day and 7th day were showed as column chart (I). The data were presented as means $\pm$ SEM of three separate experiments (n = 3 per group). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, n.s. P > 0.05.

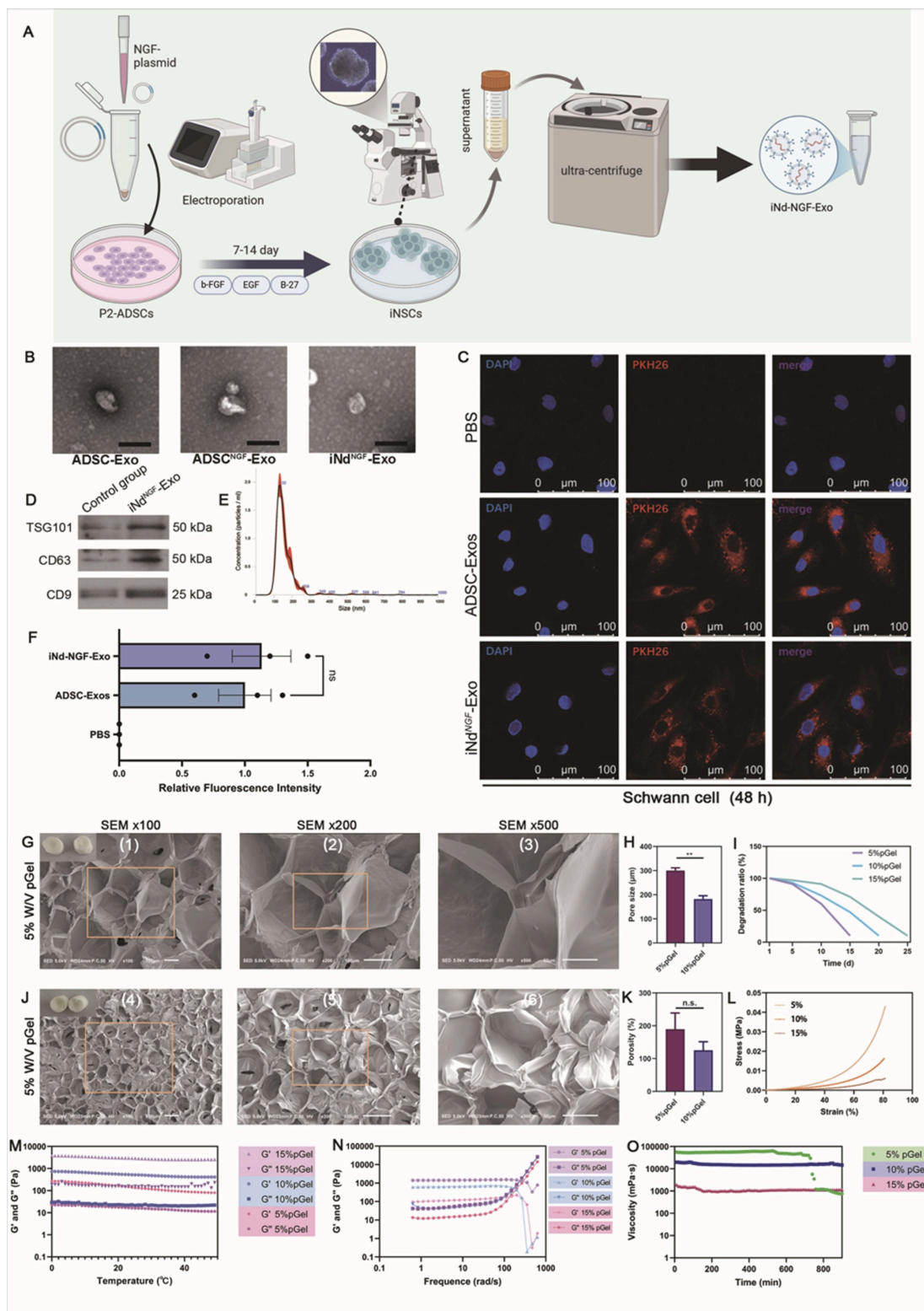


Figure 2

Preparation and characterization of iNd^{NGF}-Exo and pGel@iNd^{NGF}-Exo. Schematic illustration of the preparation of iNd^{NGF}-Exo. (A) Illustration of exosome extraction. (B) TEM images of ADSC-Exo, ADSC^{NGF}-Exo and iNd^{NGF}-Exo showing typical cup-shaped structures. Scale bar = 200 nm (C) Schwann cells co-culture with PKH26-labeled exosomes for 48 h. DAPI (blue), PKH26 (red), and merged images

are shown. Scale bar = 100 μm . (D) Western blot assay of iNd^{NGF}-Exo. (E) NTA revealed that the size distributions of iNd^{NGF}-Exo. (F) Fluorescence intensity and intracellular distribution patterns. (G and J) SEM images of pGel scaffolds at 5% and 10% (w:v) concentrations showing interconnected porous structures, scale bar = 100 μm in (1,2,4,5), scale bar = 50 μm in (3,6). (I) Degradation rate of pGel hydrogels measured every 5 days in culture medium. (L) Compressive stress-strain curves of pGel scaffolds. (M) Temperature-dependent modulus (G' and G'') changes. (N) Frequency-dependent modulus (G' and G'') changes. (O) Rheological analysis showing viscosity versus time under constant shear rate (10/s).

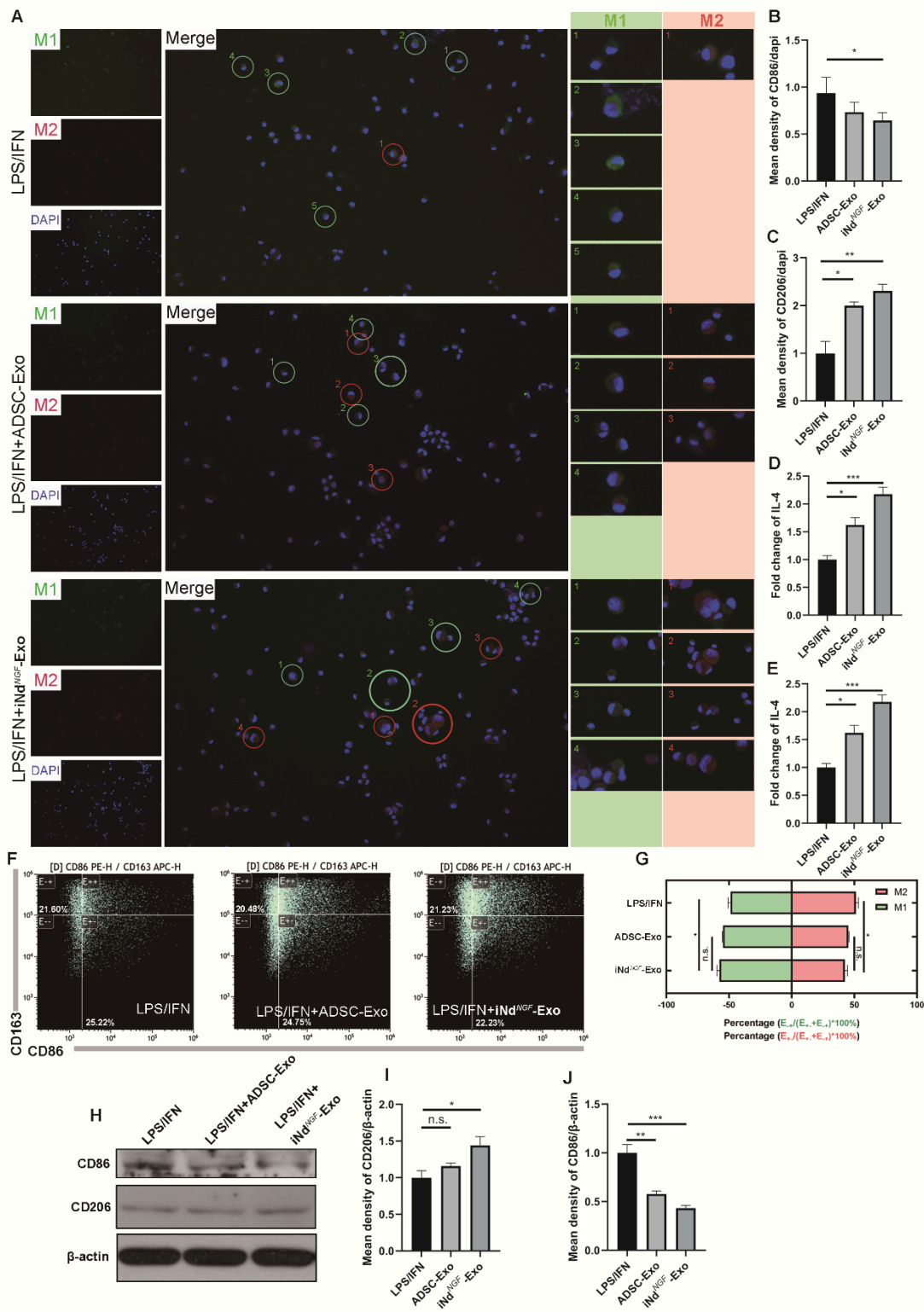


Figure 3

iNdn^{NGF}-Exo promote M2/M1 switching. (A) The immunofluorescence staining of CD86 (M1) and CD206 (M2) after induction. (B and C) The mean density of CD86 and CD206 after induction. (D and E) The expression of TNF- α and IL-4 of macrophages after induction. (F) The flow cytometry of CD86/CD163 of macrophage after induction. (G) The panel of percentage of CD86+CD163- cells and percentage of CD163+CD86- cells after induction. (H) The WB bands of CD86 and CD206 of macrophage after

induction and the mean density of CD206 (I), CD86 (J) after induction. The data were presented as means $\pm$ SEM of three separate experiments (n = 3 per group). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, n.s. P > 0.05.

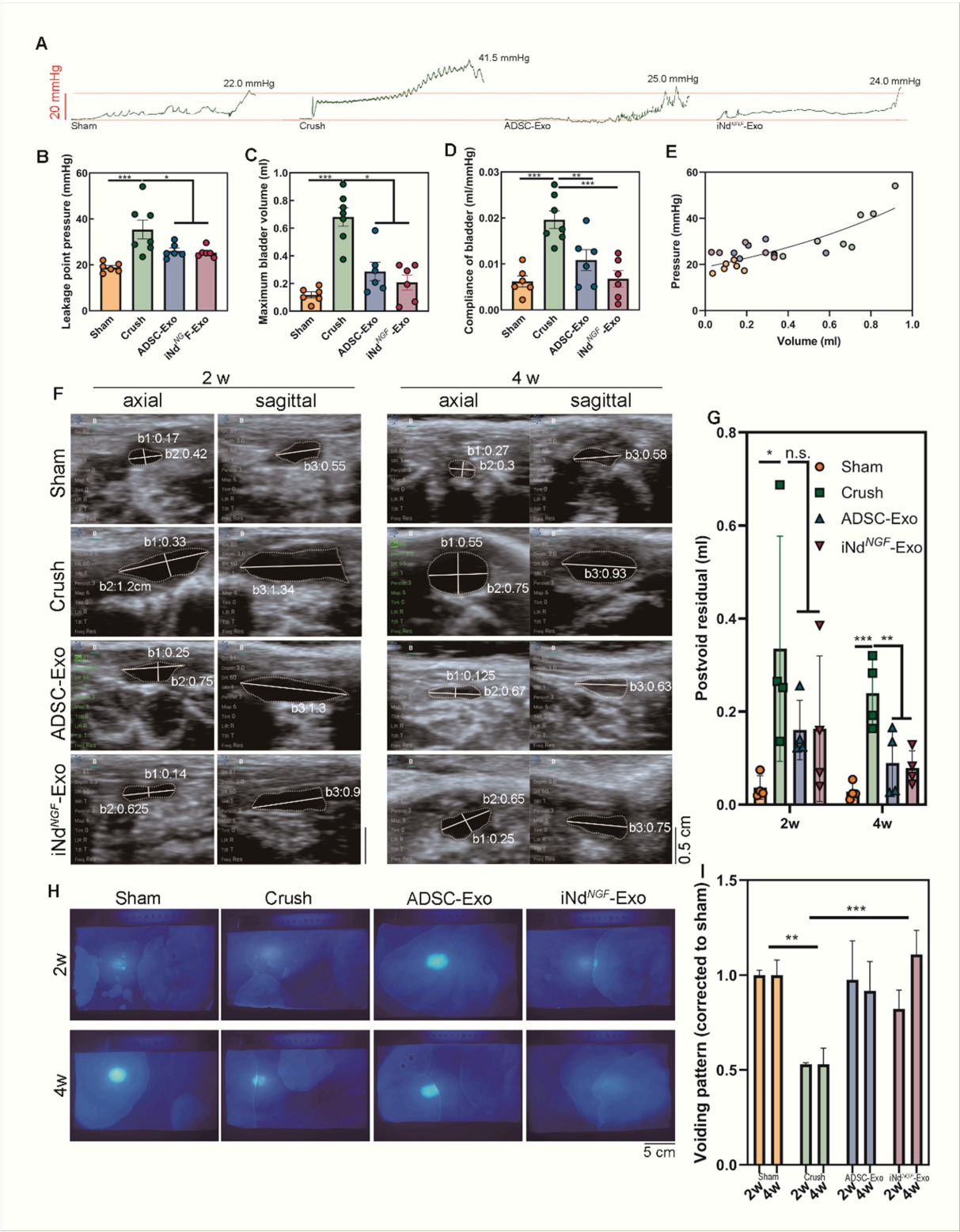


Figure 4

Urodynamic test, ultrasonography and voiding pattern analysis results. (A) The urodynamic test curve of four groups. (B) Leakage point pressure of four groups at 4-week. N = 6 in sham, NSC and ADSC group; N = 7 in crush group. (C) Maximum bladder volume of the four groups at 4 weeks. N = 6 in sham, iNd^{NGF}-Exo and ADSC-Exo group; N = 7 in crush group. (D) Compliance of the bladder in the four groups at 4 weeks. N = 6 in sham, iNd^{NGF}-Exo and ADSC-Exo group; N = 7 in crush group. (E) the pressure – volume curve. (F) The ultrasonography images of bladder at 2 w and 4 w. (G) The calculated volume of postvoid volume according to ultrasonography at 2 w and 4 w. (H) The images of voiding pattern analysis among four groups. (I) The area of voiding among four groups at 2 w and 4 w. The data were presented as means ± SEM. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, n.s. P > 0.05.

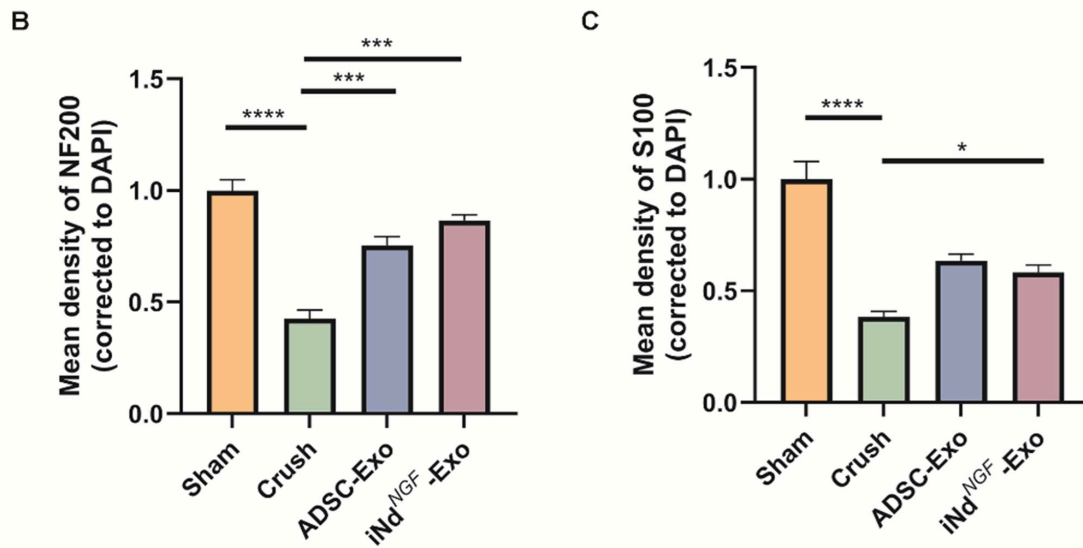
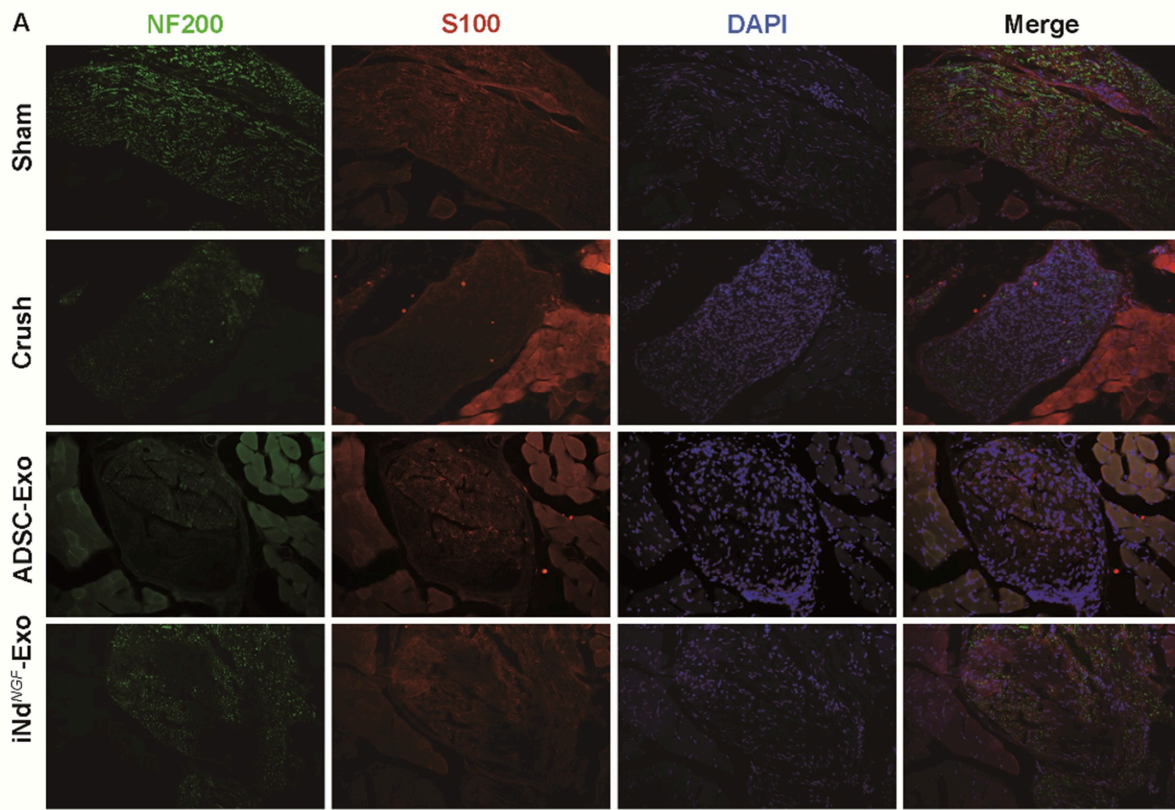


Figure 5

iNd^{NGF}-Exo promotes sacral nerve regeneration in vivo.

(A) The immunofluorescence image (NF200, S100) of the sacral nerves among the four groups. (B) The mean density of NF200 (corrected to DAPI) among four groups (Sham, Crush, ADSC-Exo and iNd^{NGF}-Exo). (C) The mean density of S100 (corrected to DAPI) among four groups (Sham, Crush, ADSC-Exo and iNd^{NGF}-Exo).

iNd^{NGF}-Exo). The data were presented as means ± SEM of three separate experiments (n = 3 per group). ***P < 0.001, **P < 0.01, *P < 0.05, n.s. P > 0.05.

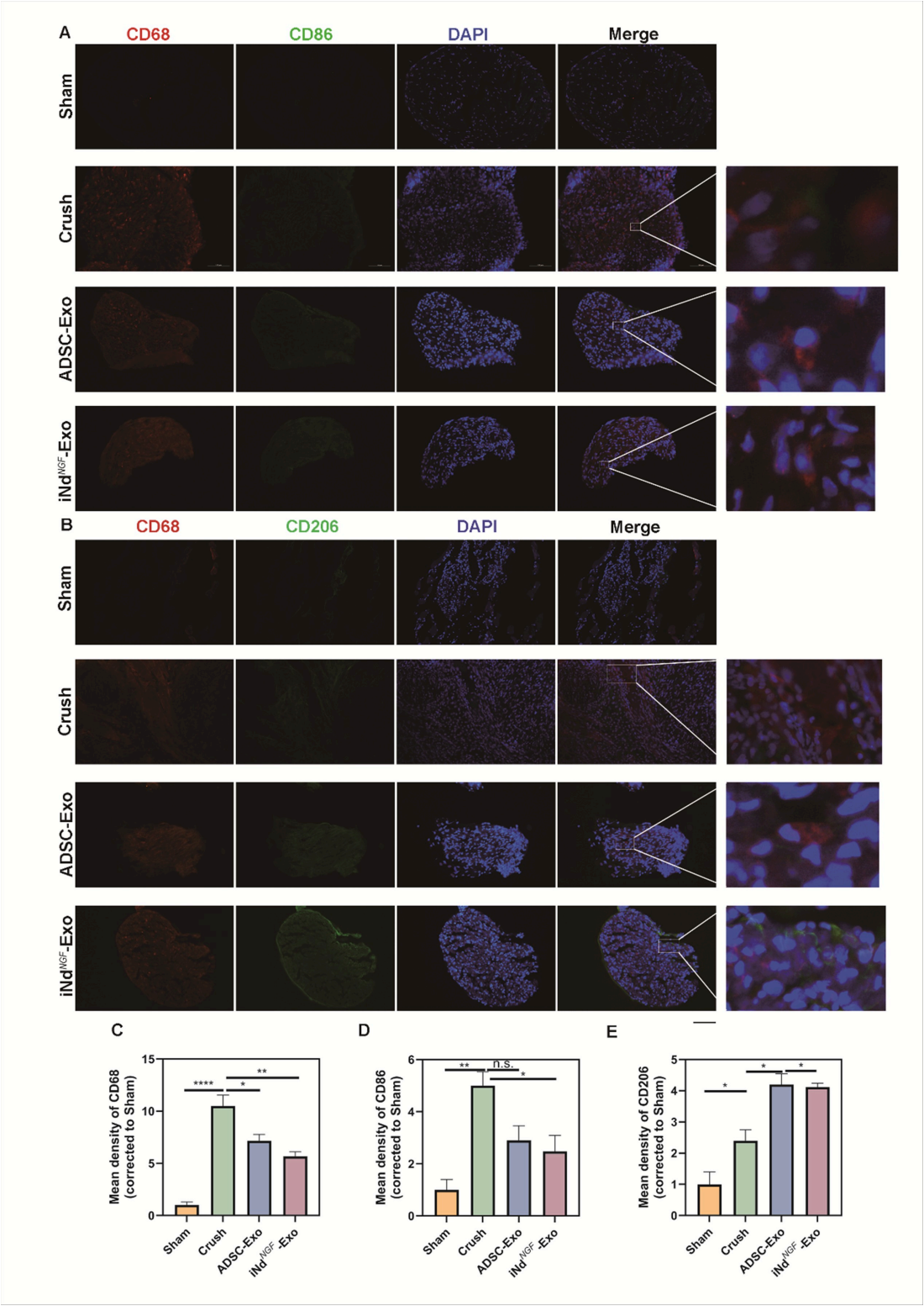


Figure 6

iNd^{NGF}-Exo promote sacral nerve regeneration by upregulating the M2/M1 cell ratio. (A) The immunofluorescence image (CD68, CD86) of the sacral nerves among the four groups. (B) The

immunofluorescence image (CD68, CD206) of the sacral nerves among the four groups. (C) The mean density of CD68 (corrected to Sham) among four groups. (D) The mean density of CD86 (corrected to Sham) among four groups. (E) The mean density of CD206 (corrected to Sham) among four groups. The data were presented as means $\pm$ SEM of three separate experiments (n = 3 per group). ***P < 0.001, **P < 0.01, *P < 0.05, n.s. P > 0.05.

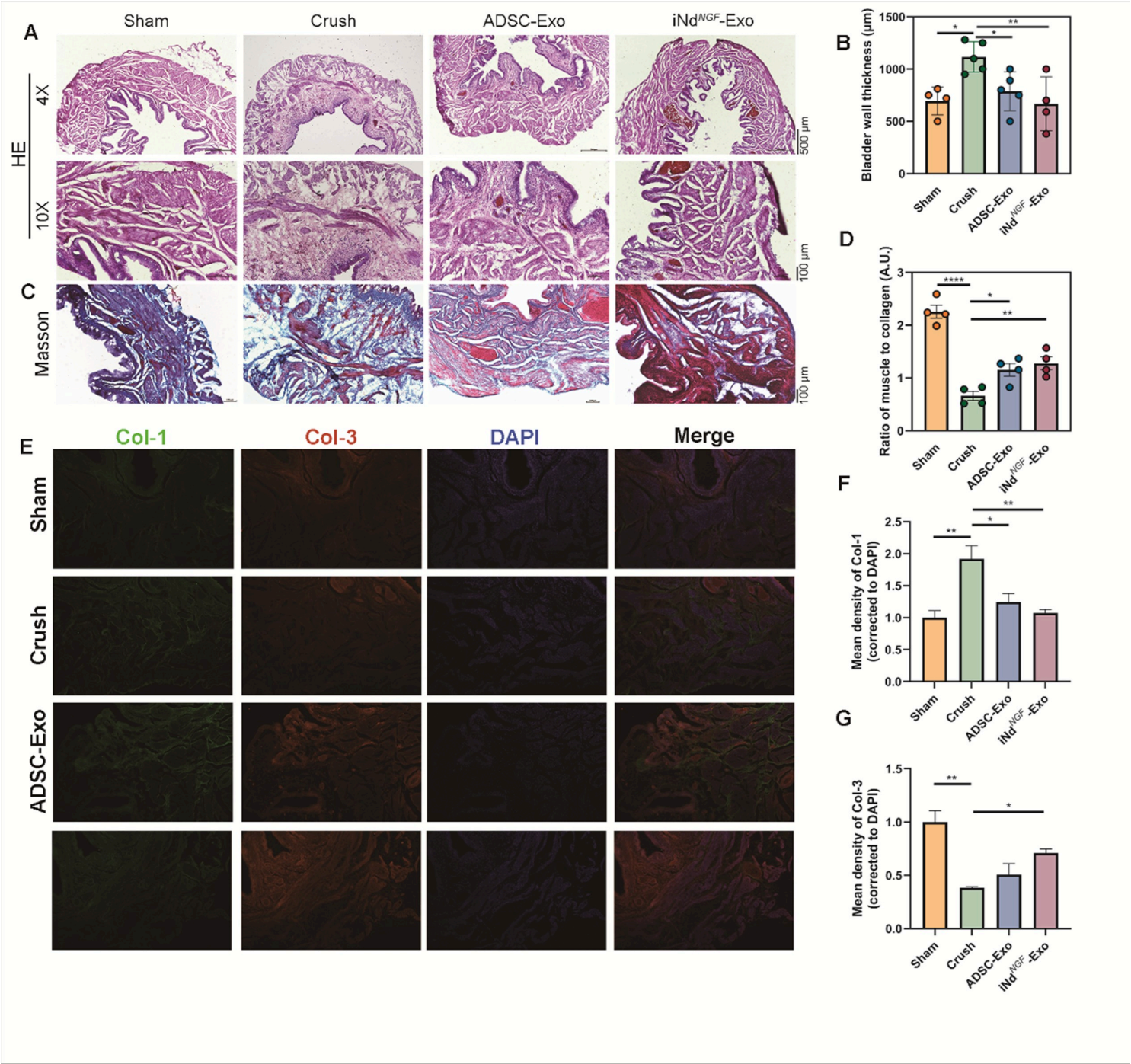


Figure 7

iNdnGF-Exo promote the sacral nerve regeneration and further suppress fibrosis and collagen remodeling of bladder. (A) H&E staining of bladder among four groups. (B) The panel of the wall thickness of bladder

among four groups. (C) Masson's staining of bladder among four groups. (D) The panel of the ratio of muscle to collagen among four groups. (E) The immunofluorescence staining of Col-1/Col-3 of bladder among four groups. (F and G) The panel of the mean density of Col-1 and Col-3 of bladder among four groups. The data were presented as means $\pm$ SEM of three separate experiments ($n = 3$ per group). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. $P > 0.05$.

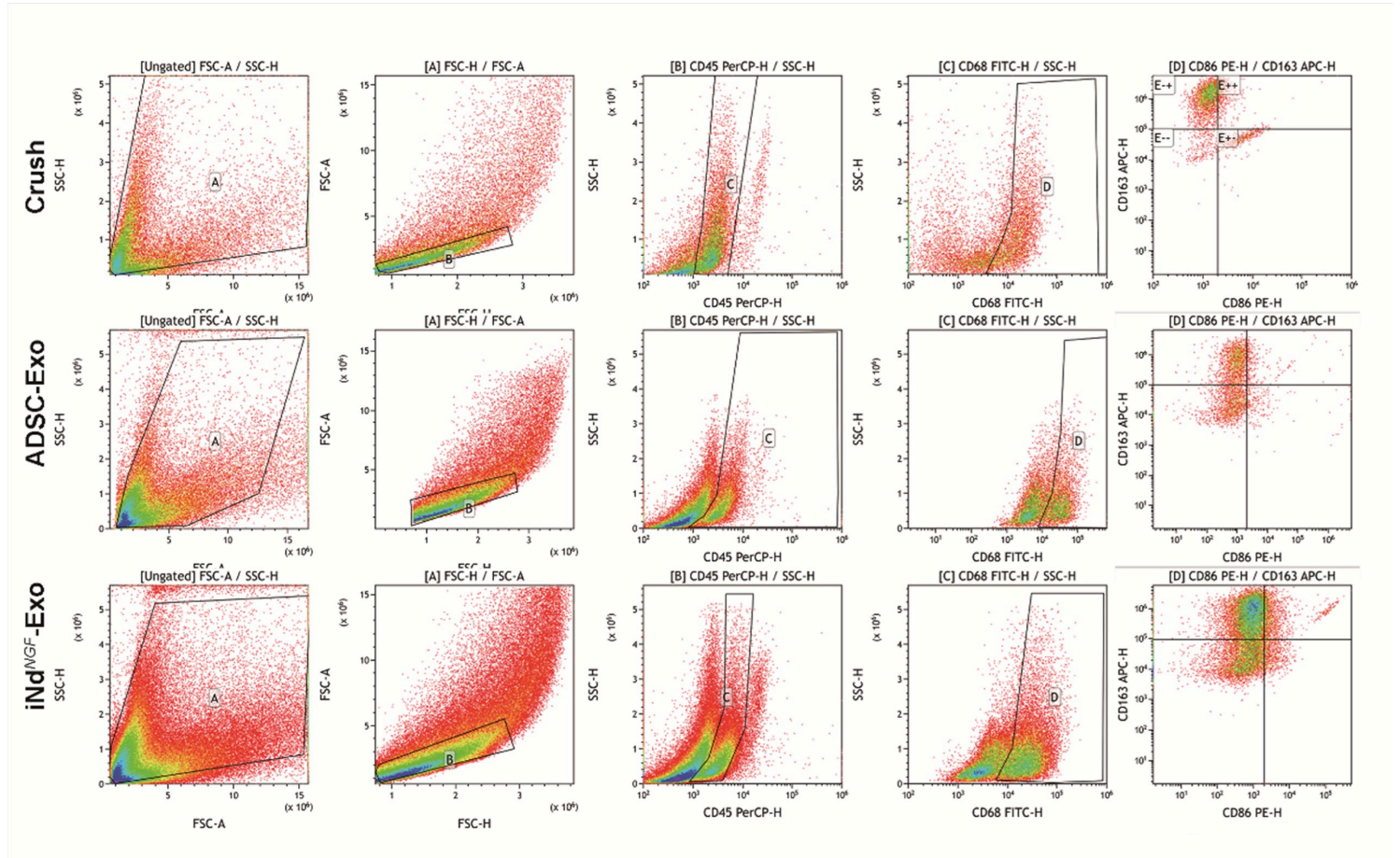


Figure 8

iNd^{NGF}-Exo modulated the M1/M2 phenotype of sacral nerve. (A-C) The flow cytometry chart of M1 and M2 cells in sacral nerves. (D) The column diagram of the ratio of M1 (CD86+CD163-) and M2 (CD86-CD163+) in sacral nerve. The data were presented as means $\pm$ SEM of three separate experiments ($n = 3$ per group). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. $P > 0.05$.

nerve regeneration and inflammatory. (F) Selected GO biological processes were significantly overrepresented ranked by enrichment foldchange between cursh and iNd^{NGF}-Exo group. (G and H) Differential expression of cytokines of sacral nerves in three groups: Crush, ADSC-Exo and iNd^{NGF}-Exo group. Luminex liquid suspension chip analysis of sacral nerves in Crush, ADSC-Exo and iNd^{NGF}-Exo group. Schematic representation of full-length and truncated domains of cytokines detected in this study. PPI showed the interaction between these cytokines.

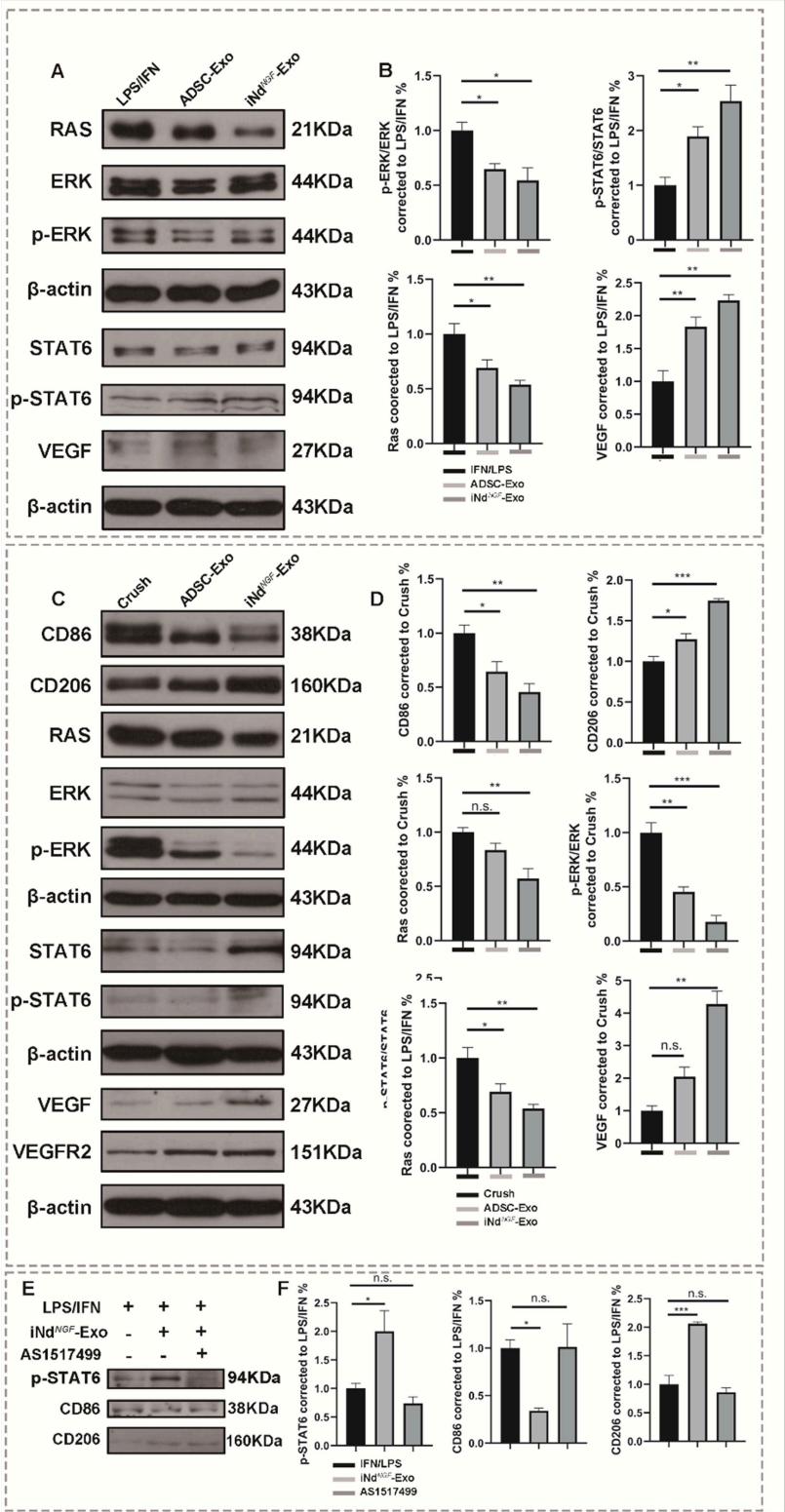


Figure 10

iNd^{NGF}-Exo cause M1/M2 switching and promote nerve regeneration via RAS-ERK and STAT6 signaling pathway. (A) Western blot assay of molecular signaling of RAS-ERK, (p-)STAT6 and VEGF in vitro. (B) The expression of p-ERK/ERK, p-STAT6/STAT6, Ras and VEGF in cells harvested from culture of LPS/IFN, +ADSC-Exo and + iNd^{NGF}-Exo group. (C) Western blot assay of molecular signaling of RAS-ERK, (p-)STAT6, CD86/CD206 and VEGF/VEGFR2 in vivo. (D) The expression of CD86, CD206, Ras, p-ERK/ERK, p-STAT6/STAT6 and VEGF/VEGFR2 of Crush, ADSC-Exo and iNd^{NGF}-Exo group in vivo. (E) Western blot assay of p-STAT6 and CD86/CD206 after AS1517499 administration. (F) The expression of p-STAT6, CD86 and CD206 of cells harvested from culture of LPS/IFN, + iNd^{NGF}-Exo and AS1517499 group. The data were presented as means $\pm$ SEM of three separate experiments (n = 3 per group). ***P < 0.001, **P < 0.01, *P < 0.05, n.s. P > 0.05.

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