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Isolation, culture, and characterization of primary endothelial cells and pericytes from mouse sciatic nerve

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ABSTRACT

Background: The recovery of injured peripheral nerves relies on angiogenesis, where newly formed blood vessels act as pathways guiding Schwann cells across the wound to support axon regeneration. While some research has examined this process, the specific mechanisms of angiogenesis in peripheral nerve healing remain unclear. *In vitro* models are vital tools to investigate these mechanisms; however, no current *in vitro* culture methods exist for isolating vascular cells, such as endothelial cells (ECs) and pericytes, specifically from sciatic nerves.

New method: We developed a straightforward and reliable technique for isolating ECs and pericytes from injured sciatic nerves, optimized for use in *in vitro* studies. Cell types were characterized using specific markers and phenotypic assessments, with flow cytometry confirming cell identity and determining cell purity.

Results: Our method successfully isolated high-purity ECs and pericytes from injured sciatic nerves. Immunofluorescence analysis showed that primary cultured ECs exhibited strong positive staining for CD31, while pericytes stained strongly for NG2 and PDGFR β . Flow cytometric analysis confirmed that ECs achieved a purity of 90.22%, and pericytes reached a purity of 92.01%. Both cell types were capable of forming organized capillary-like structures, and in co-culture systems, pericytes effectively wrapped around ECs.

Comparison with existing methods: Current isolation methods for ECs and pericytes from sciatic nerves are limited. Although techniques exist for isolating these cells from other tissues, they often rely on enzymatic digestion, which can damage cell surface proteins and reduce cell viability. Our method allows for the efficient isolation of intact ECs and pericytes from sciatic nerve tissue without such drawbacks, providing a robust platform for *in vitro* studies.

Conclusions: This newly developed method offers an effective approach to isolate ECs and pericytes from the sciatic nerve, contributing a valuable tool for investigating the function and pathology of angiogenesis in the context of sciatic nerve injury recovery.

1. Introduction

Peripheral nerve injury is a significant health concern that frequently results in profound and persistent functional and physiological impairments (Dong et al., 2019; Lopes et al., 2022). The etiology of peripheral neuropathy includes trauma, infection, metabolic dysregulation, genetic predispositions, and toxic exposures (Tusnim et al., 2024). Although the

peripheral nervous system retains limited self-repair capacity, this alone is often insufficient for complete recovery, necessitating the use of therapeutic adjuvants to facilitate nerve regeneration (Ma et al., 2014; Wang et al., 2020). Current treatment modalities—including surgical interventions, electrical stimulation, pharmacotherapy, and physical therapy—have demonstrated some efficacy in promoting nerve regeneration. However, as reported by Kalli et al., successful outcomes are

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achieved in only half of the cases following secondary nerve suturing and nerve grafting (Kallio and Vastamaki, 1993; Vastamaki et al., 1993). The structural complexity of peripheral nerves, which require long-distance regeneration and coordinated actions across multiple cell types, presents further challenges. Neurons, neuronal axons, Schwann cells, inflammatory cells, and vascular cells each play critical roles in this process, but their specific mechanisms of action in nerve repair are still not fully understood and remain under active investigation (Bhandari, 2019; Cattin et al., 2015). Consequently, in-depth *in vivo* and *in vitro* models are essential to elucidate these mechanisms and advance therapeutic strategies.

The sciatic nerve, the largest peripheral nerve in the human body, composed of both motor and sensory fibers, has become a focal point in the study of peripheral nerve injuries (Kowalska and Sudol-Szopinska, 2012). Schwann cells and macrophages are particularly vital in peripheral nerve repair and are frequently utilized in *in vitro* research models (Gaudet et al., 2011; Stierli et al., 2019). Recent findings indicate that macrophage-induced angiogenesis can direct Schwann cell-mediated peripheral nerve regeneration (Cattin et al., 2015). Additionally, Zhou et al. demonstrated that endothelial cells can function as 'amateur phagocytes,' capable of engulfing myelin debris to support neuronal repair (Zhou et al., 2019). Pericytes, located perivascularly, are crucial in modulating endothelial cell proliferation, differentiation, stability, and permeability (Armulik et al., 2011; Geevarghese and Herman, 2014; Gerhardt and Betsholtz, 2003; Ribatti et al., 2011). Pericyte-derived extracellular vesicle-mimicking nanovesicles have also been shown to facilitate peripheral nerve repair (Yin et al., 2022b) and promote neurovascular regeneration in cavernous nerve injury models (Yin et al., 2020b). However, the angiogenic processes in sciatic nerve regeneration are still not fully characterized, and research is in preliminary stages. While Schwann cell *in vitro* culture models are well established and have been applied in sciatic nerve regeneration studies, further cell culture models incorporating endothelial cells and pericytes are necessary to enhance understanding of angiogenesis mechanisms during sciatic nerve repair.

To date, there are no reports on isolating endothelial cells and pericytes from sciatic nerves for use in studies on sciatic nerve regeneration. Therefore, the objective of this study was to isolate and culture endothelial cells and pericytes from injured sciatic nerves and establish an *in vitro* model suitable for investigating angiogenesis associated with sciatic nerve regeneration.

2. Materials and methods

2.1. Ethics statement and sciatic nerve transection model preparation

Eight-week-old male C57BL/6 mice (20–25 g; Orient Bio, Inc., Seongnam, Korea) were used in this study. All animal procedures were approved by the Institutional Animal Care and Use Committee of Inha University (Approval Number: INHA 221121–845) and performed in accordance with institutional guidelines. Mice were housed in a controlled environment with sterilized standard laboratory food and water available *ad libitum* and monitored daily for health and behavior (Ock et al., 2023). Anesthesia was induced via intraperitoneal injection of ketamine (100 mg/kg; Yuhan Corp., Seoul, Korea) and xylazine (5 mg/kg; Bayer Korea, Seoul, Korea). The sciatic nerve transection model was prepared following previously established methods (Yin et al., 2022b). In brief, under anesthesia, the sciatic nerves of both hind limbs were exposed and transected at the mid-thigh level. Five days post-transection, mice were euthanized via carbon dioxide exposure (100 % CO₂ at 10–30 % of container volume/min), with sciatic nerves collected post-mortem following cessation of heartbeat and respiration. Collected nerves were immediately transferred into Hank's Balanced Salt Solution (HBSS, GIBCO, Carlsbad, CA, USA) for subsequent primary cell culture.

2.2. Isolation and culture of mouse sciatic nerve endothelial cells

Mouse sciatic nerve endothelial cells were isolated from injured nerves using a modified method for endothelial cell isolation from penile tissue (Yin et al., 2012). Briefly, five days after sciatic nerve transection, mice were euthanized with 100 % CO₂, and segments of the transected sciatic nerve, including 2–3 mm of proximal and distal portions, were collected and placed in HBSS. Nerves were rinsed three times in PBS containing 1 % penicillin/streptomycin (GIBCO). The tissue was then sectioned into 2–3 pieces and positioned in a 60-mm culture dish. Ten microliters of Matrigel (Becton Dickinson, Mountain View, CA, USA) supplemented with recombinant human FGF basic 145 aa (R&D Systems Inc., Minneapolis, MN, USA) was added to cover the tissue, followed by polymerization at 37°C for 5 minutes. Subsequently, 3 mL of endothelial cell growth basal medium (EBM-2, Lonza, Houston, TX, USA) containing microvascular endothelial growth medium supplements and growth factors was added. Cultures were maintained at 37°C in a 5 % CO₂ atmosphere, with medium replacement every two days. Once the cells reached confluence and exhibited a spread across the dish bottom (approximately 1 week), only sprouting cells were used for subcultivation. These cells were subsequently seeded onto dishes coated with 0.2 % gelatin (Sigma-Aldrich, St. Louis, MO, USA). Cells from passages 3–5 were used in all subsequent experiments. HUVECs (Lonza, Cohasset, MN, USA) were used as positive control, cultured with the same medium described above according to guidelines and were all between passages 2 and 7 for this study.

2.3. Isolation and culture of mouse sciatic nerve pericytes

Mouse sciatic nerve pericytes were isolated from injured sciatic nerve tissue, following an adapted protocol originally developed for the isolation of pericytes from penile tissue (Neng et al., 2013; Yin et al., 2015, 2020a). Tissue collection and preservation protocols were consistent with those employed in sciatic nerve endothelial cell isolation. Sciatic nerves were washed three times in PBS containing 1 % penicillin/streptomycin (GIBCO) to ensure sterility. Tissues were then sectioned into fragments of approximately 1 mm and deposited by gravity into 35-mm cell culture dishes pre-coated with collagen I (Becton Dickinson). Following a 10-minute incubation at 37°C in 300 µL of Dulbecco's modified Eagle Medium (DMEM, GIBCO), supplemented with 10 % fetal bovine serum (FBS, GIBCO), 10 nM human pigment epithelium-derived factor (PEDF; Sigma-Aldrich) for the inhibition of endothelial cell proliferation and migration, and 1 % penicillin/streptomycin, an additional 900 µL of medium was added, and cultures were incubated at 37°C in a 5 % CO₂ atmosphere. Medium was refreshed every two days. Once cellular confluence was achieved (approximately one week after initiation), only sprouting cells were selected for subcultivation. Sprouting cells were transferred to dishes coated with 40 µL/mL collagen I (Advanced BioMatrix, San Diego, CA, USA), and cells from passages 3–5 were used in all subsequent experiments. Human pericytes from placenta (hPCs, PromoCell, Heidelberg, Germany) were used as positive control, cultured with the same medium described above according to guidelines and were all between passages 2 and 7 for this study.

2.4. Enzymatic isolation of endothelial cells and pericytes from injured sciatic nerves

The preparation and acquisition of sciatic nerve tissue refer to the isolation and culture of mouse sciatic nerve endothelial cells in the above section. The sciatic nerve tissue was digested with 1 mg/mL collagenase/dispase (Sigma-Aldrich) at 37°C, and the enzyme reaction was terminated by adding 10 % FBS (GIBCO) solution after 30 minutes. The undigested tissue was filtered out with a 40 µm cell strainer (Sigma-Aldrich) and centrifuged at 1500 rpm for 3 minutes. The cell pellet was suspended in 2 % bovine serum albumin (BSA) (Sigma-Aldrich) for

subsequent experiments.

2.5. Histological examinations

For immunofluorescence analysis, sciatic nerve tissues were fixed in 4 % paraformaldehyde at 4°C for 24 hours, while cultured cells were fixed in 4 % paraformaldehyde at room temperature for 15 minutes. Frozen tissue sections (12 μm thick) and cultured cells were blocked with 2 % bovine serum albumin (BSA) (Sigma-Aldrich) for 1 hour at room temperature before incubation with primary antibodies against PECAM-1 (1:50; Millipore, Temecula, CA, USA), PDGFR-β (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), S100 (1:50; Abcam, Cambridge, MA, USA), S100A4 (1:50; Abcam), neurofilament (1:50; Sigma-Aldrich), and NG2 chondroitin sulfate proteoglycan (1:50; Millipore) at 4°C overnight. Following multiple PBS washes with 0.3 % Triton, samples were treated with species-specific secondary antibodies conjugated to either tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) (1:100; Zymed Laboratories, South San Francisco, CA, USA) for 2 hours at room temperature. Samples were mounted in DAPI-containing solution (Vector Laboratories Inc., Burlingame, CA, USA) for nuclear staining and imaged using a confocal microscope (K1-Fluo; Nanoscope Systems, Inc., Daejeon, Korea). Quantitative histological analyses were conducted using an image analyzer (National Institutes of Health [NIH] 1.34; <https://imagej.net/ij/nih-image/>). For each immunofluorescence experiment, a minimum of four independent trials was performed.

2.6. Flow cytometry

Sciatic nerve endothelial cells and pericytes were harvested from culture flasks at passages 3–4 by enzymatic dissociation with 0.05 % trypsin-EDTA buffer (Gibco). Cells were resuspended in 300 μL of PBS containing 2 % BSA. To label specific cell populations, sciatic nerve endothelial cells or HUVECs were incubated with PE-conjugated anti-PECAM-1 antibody (1:100, Abcam), while sciatic nerve pericytes or hPCs were incubated with PE-conjugated anti-PDGFR-β antibody (1:100, Abcam). In addition, we performed flow cytometric analysis on cells extracted from enzymatically digested sciatic nerve tissue and analyzed the proportion of endothelial cells and pericytes. Incubation was conducted in the dark for 20–30 minutes at 4°C. Cells were subsequently washed twice with 2 % BSA by centrifugation at 500 g for 3 minutes and resuspended in 300 μL of cold 2 % BSA. Analysis and sorting of labeled cells (1×10^5 for analysis) were performed using a CytoFLEX LX Flow Cytometer (Beckman Coulter, Inc.) with CytExpert software (Beckman Coulter, Inc.).

2.7. In vitro tube formation assay

The angiogenic potential of cultured sciatic nerve endothelial cells and pericytes was assessed using a tube formation assay, as described previously (Yin et al., 2012). Briefly, 100 μL of growth factor-reduced Matrigel (Becton Dickinson) was dispensed into 48-well tissue culture plates on ice. Following gelation at 37°C for at least 10 minutes, HUVECs, hPCs, sciatic nerve endothelial cells and pericytes were seeded onto the gel at a density of 5×10^4 cells per well in 500 μL of starvation medium. The assay was conducted in a 5 % CO₂ atmosphere at 37°C for 18 hours. Phase-contrast microscopy (CKX41, Olympus, Tokyo, Japan) was used to capture images. For direct co-culture studies, endothelial cells and pericytes were labeled with CellTracker Blue CMF2HC Dye and CellTracker Red CMTPX Dye (Thermo Fisher Scientific, Fremont, CA, USA), respectively, and mixed at a 4:1 ratio. The mixed cells were then subjected to the tube formation assay in a medium comprising 50 % EBM-2 and 50 % DMEM starvation medium.

2.8. Sciatic nerve endothelial cell- or pericytes-derived extracellular vesicles (EV) isolation and characterization

Sciatic nerve endothelial cells (SNEC) or pericytes (SNPC) were cultured for 3 days in a T75 flask with 10 mL of medium containing EV-depleted FBS (Gibco). The medium was then collected and EVs from SNEC (SNEC-EVs) or SNPC (SNPC-EVs) were isolated using EXOCET EV isolation solution (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. SNEC-EVs and SNPC-EVs were resuspended in PBS, filtered through a 0.45-μm filter, and stored at –80°C until further use. SNEC-EVs and SNPC-EVs were quantified using an EXOCET EV quantitation assay kit (System Biosciences), and their concentration was adjusted to 1 μg/μL for subsequent experiments.

SNEC-EVs and SNPC-EVs were characterized by western blot analysis of positive and negative EV markers. Briefly, An equal amount of protein (50 μg/lane) from SNEC-EVs, SNPC-EVs, and whole SNEC or SNPC lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8–15 % gels and then transferred to polyvinylidene difluoride membranes. After blocking with 5 % nonfat dry milk for 1 hour at room temperature, membranes were probed with antibodies against the positive EV markers Alix (1:500; Novus Biologicals, Littleton, CO, USA), CD63 (1:500; Novus Biologicals, Littleton, CO, USA), and CD81 (1:500; Novus Biologicals), and the negative EV marker GM130 (BD Biosciences, San Jose, CA, USA) at 4°C overnight. The signals were visualized by using an ECL (Amersham Pharmacia Biotech, Piscataway, NJ, USA) detection system.

2.9. Ex vivo neurite sprouting assay

Mouse dorsal root ganglion (DRG) tissue was harvested and maintained as described previously (Yin et al., 2022a). DRG tissues from L3-L5 were isolated from 8-week-old C57BL/6 male mice under a dissecting microscope, transferred into sterile vials containing Hank's balanced salt solution (HBSS; Gibco), and then rinsed and washed twice with PBS. DRG tissues were cut into small pieces and plated on poly-D-lysine hydrobromide-coated, 8-well Nunc Lab-Tek Chamber Slides (Sigma-Aldrich), then entirely covered with matrigel and incubated for 10–15 minutes at 37°C in a 5 % CO₂ environment. Thereafter, 300 μL of complete neurobasal medium (Gibco) supplemented with 2 % serum-free B-27 (Gibco) and 0.5 nM GlutaMAX-I (Gibco) were added and DRG tissues were treated with PBS or different doses (1 μg/mL and 10 μg/mL) of SNEC-EVs and SNPC-EVs as indicated in the figures. Four days later, neurite outgrowth was assessed by fixing tissue segments in 4 % paraformaldehyde for at least 30 minutes and immunostaining with relative antibodies. Fluorescence signals were visualized using a K1-Fluo confocal microscope (Nanoscope Systems, Inc.). Quantitative analyses were performed using an image analyzer system (Image J 1.34; National Institutes of Health [NIH], <http://rsbweb.nih.gov/ij/>). For each immunofluorescence staining experiment, we evaluate at least four different samples or regions of interest.

2.10. Statistical analysis

The analysis was conducted with GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA). Results are expressed as the means ± standard errors of means (SEMs) of at least four independent experiments. The treated groups were compared with the PBS group using an unpaired t test. Statistical significance was accepted for P-values < 0.05.

3. Results

3.1. Identification of endothelial cells and pericytes in injured sciatic nerve

To investigate the presence of endothelial cells and pericytes in the

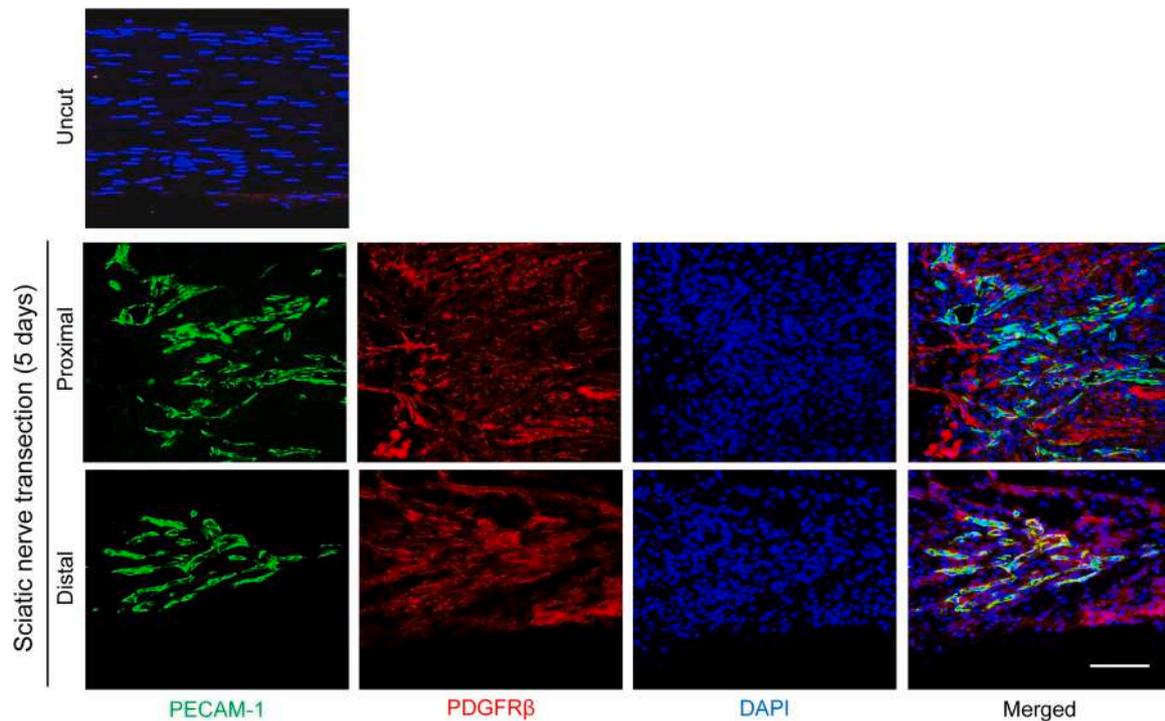


Fig. 1. Identification of endothelial cells and pericytes in injured sciatic nerve. Immunofluorescent double staining with PECAM1 (an endothelial cell marker, green) and PDGFR β (a pericyte marker, red) in uncut and transected sciatic nerve tissues (proximal and distal stumps) five days post-transection. Nuclei were labeled with DAPI (blue). Scale bar: 100 μ m. DAPI, 4',6-diamidino-2-phenylindole.

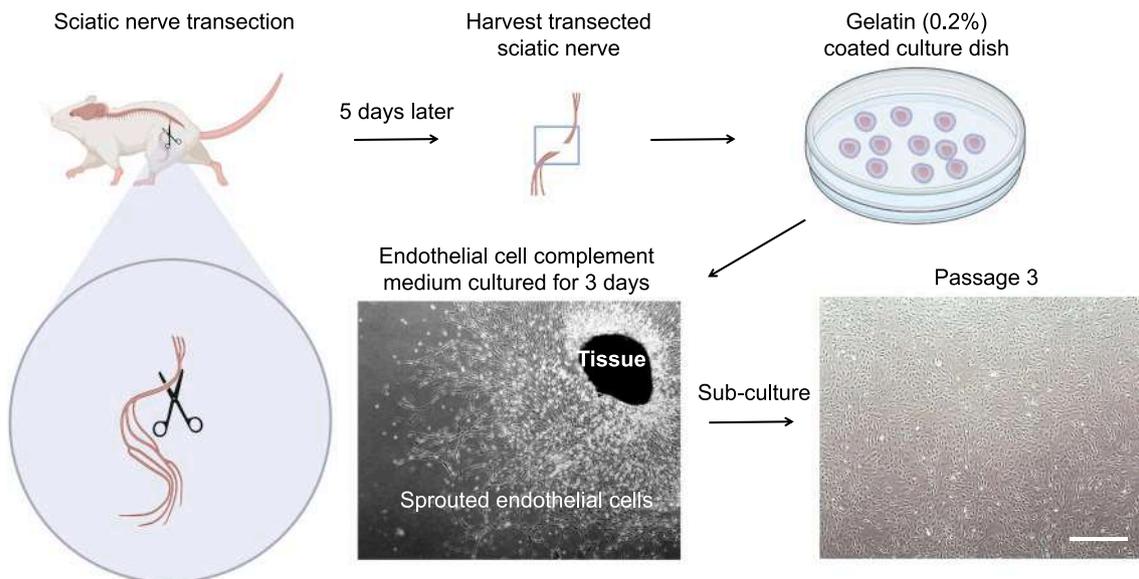


Fig. 2. Schematic diagram of the primary culture process of sciatic nerve endothelial cells. Five days after sciatic nerve transection, sciatic nerve tissues were placed in a 60-mm cell culture dish coated with 0.2 % gelatin and covered with Matrigel, then cultured for 1 week. After reaching confluence and spreading across the culture dish, only the sprouting cells were used for subcultivation. Scale bar: 100 μ m.

injured sciatic nerve, a sciatic nerve injury model was developed, and the injured sciatic nerve tissue was harvested after five days. Immunofluorescence staining was conducted using PECAM-1 (an endothelial cell marker, green) and PDGFR β (a pericyte marker, red). Immunofluorescence analysis revealed an absence of PECAM-1-positive endothelial cells and PDGFR β -positive pericytes in uninjured sciatic nerve tissue; however, numerous endothelial cells and pericytes were observed in the proximal and distal stumps of the injured nerve (Fig. 1). These findings establish the presence of endothelial cells and pericytes in injured sciatic

nerve tissue, supporting their subsequent isolation and analysis.

3.2. Isolation and characterization of endothelial cells from injured sciatic nerve

The isolation of sciatic nerve endothelial cells was adapted from established protocols for isolating endothelial cells from penile tissue, with modifications as described (Yin et al., 2020a, 2012). A schematic diagram of the isolation procedure is shown in Fig. 2. A total of 0.042 g

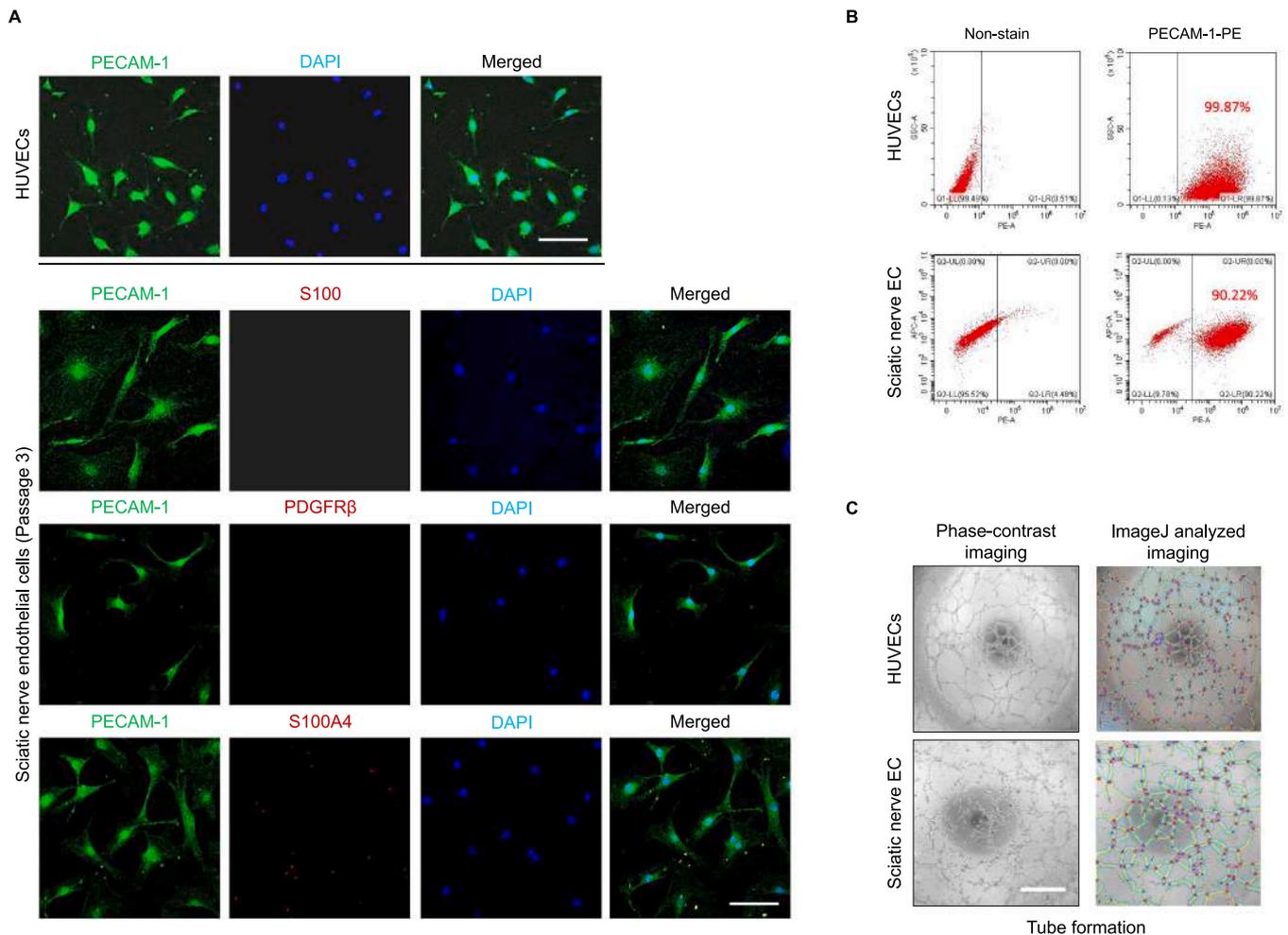


Fig. 3. Characterization of primary cultured sciatic nerve endothelial cells (EC). (A) Immunofluorescent staining with PECAM1 (an endothelial cell marker, green), S100 (a Schwann cell marker, red), PDGFR β (a pericyte marker, red), and S100A4 (a fibroblast marker, red) in HUVECs and sciatic nerve endothelial cells (passage 3). Nuclei were labeled with DAPI (blue). Scale bar: 50 μ m. (B) Flow cytometry analysis of cultured HUVECs and sciatic nerve endothelial cells (passage 3) with non-stain and PECAM-1-PE staining, showing 99.87 % (HUVECs) and 90.22 % (sciatic nerve endothelial cells) viability. (C) Tube formation assay in cultured HUVECs and sciatic nerve endothelial cells over 18 hours. Scale bar: 100 μ m. HUVECs, human umbilical vein endothelial cells.

of tissue was extracted from the injured sciatic nerves of 5 mice for cell culture. After three days of culture, sprouted cells were readily observable, and cells were sub-cultured after approximately one week. The yield of the passage1 of cultured cells was about 2.8×10^6 cells/mL. To minimize contamination from other cell types, cells were passaged to the third generation before use in subsequent experiments. Morphologically, the isolated cells exhibited an elongated, spindle-shaped appearance characteristic of endothelial cells (Fig. 2).

To verify the identity of these isolated cells, immunofluorescence staining was performed using PECAM-1 (endothelial cell marker), S100 (Schwann cell marker), PDGFR β (pericyte marker), and S100A4 (fibroblast marker). Similar to the positive control cell line (HUVECs), the isolated cells also demonstrated robust PECAM-1 positivity with minimal expression of markers associated with other cell types (Fig. 3A). Flow cytometry analysis further confirmed the identity and purity of HUVECs and isolated sciatic nerve endothelial cell. Compared with a purity of up to 99.87 % for HUVECs, the PECAM-1-PE-positive cells from isolated sciatic nerve endothelial cell accounting for 90.22 % of the total cell population (Fig. 3B). Finally, an *in vitro* angiogenesis assay on matrigel was conducted to assess the ability of these cells to form tube-like structures, a characteristic feature of endothelial cells. The sciatic nerve endothelial cells successfully formed well-organized capillary-like structures, similar to those observed in HUVECs (Fig. 3C). Collectively, these findings confirm that the isolated PECAM-1-positive cells are

indeed sciatic nerve endothelial cells.

3.3. Isolation and characterization of pericytes from injured sciatic nerve

The protocol for isolating sciatic nerve pericytes was adapted from methods used to isolate pericytes from penile tissue, with specific modifications (Neng et al., 2013; Yin et al., 2015, 2020a). Unlike the protocol employed for isolating sciatic nerve endothelial cells, Matrigel was not used to fix the tissue during pericyte isolation. Instead, the tissue fragments were allowed to settle by gravity into 35-mm cell culture dishes coated with type I collagen, with PEDF added to the culture medium. PEDF was utilized to promote pericyte proliferation while simultaneously inhibiting EC growth, thus minimizing EC contamination. A schematic diagram of the isolation procedure is provided in Fig. 4. A total of 0.031 g of tissue was extracted from the injured sciatic nerves of 5 mice for cell culture. After three days of culture, a substantial number of cells began to sprout from the tissue fragments, and these cells were subsequently sub-cultured after approximately one week. The yield of the passage1 of pericytes was about 2.0×10^6 cells/mL. To further reduce the likelihood of contamination by other cell types, the cells were passaged up to the third generation before initiating experimental procedures. Morphologically, the cells exhibited multidirectional processes characteristic of conventional pericytes (Fig. 4).

To confirm that the isolated cells were pericytes,

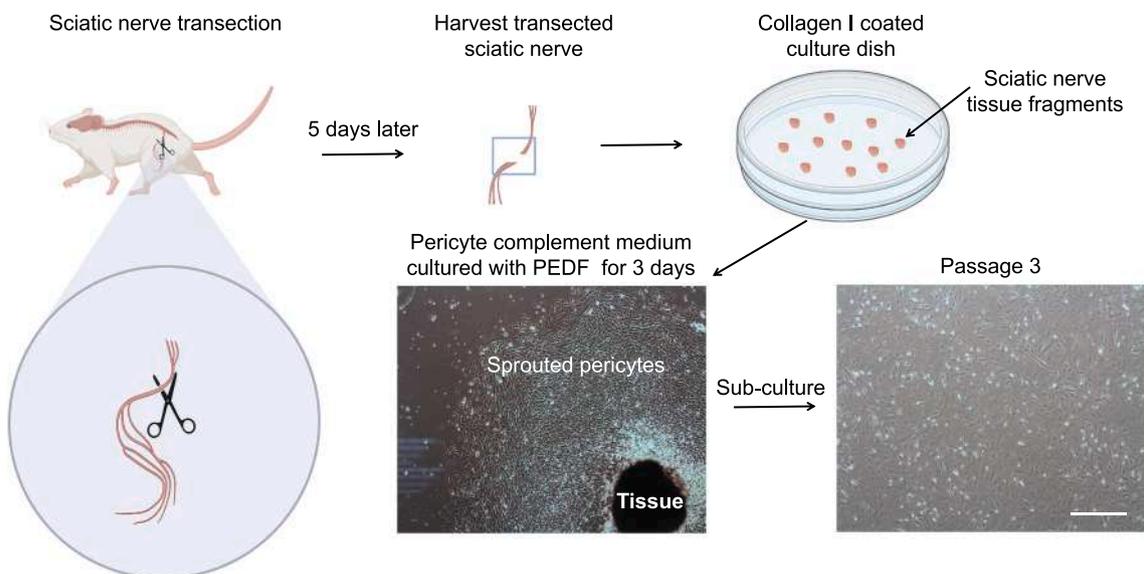


Fig. 4. Schematic diagram of the primary culture process of sciatic nerve pericytes. Five days post-transection, sciatic nerve tissues were placed in a 35-mm cell culture dish coated with collagen I and cultured for 1 week with PEDF. After reaching confluence, only the sprouting cells were used for subcultivation. Scale bar: 100 μ m. PEDF, pigment epithelium-derived factor.

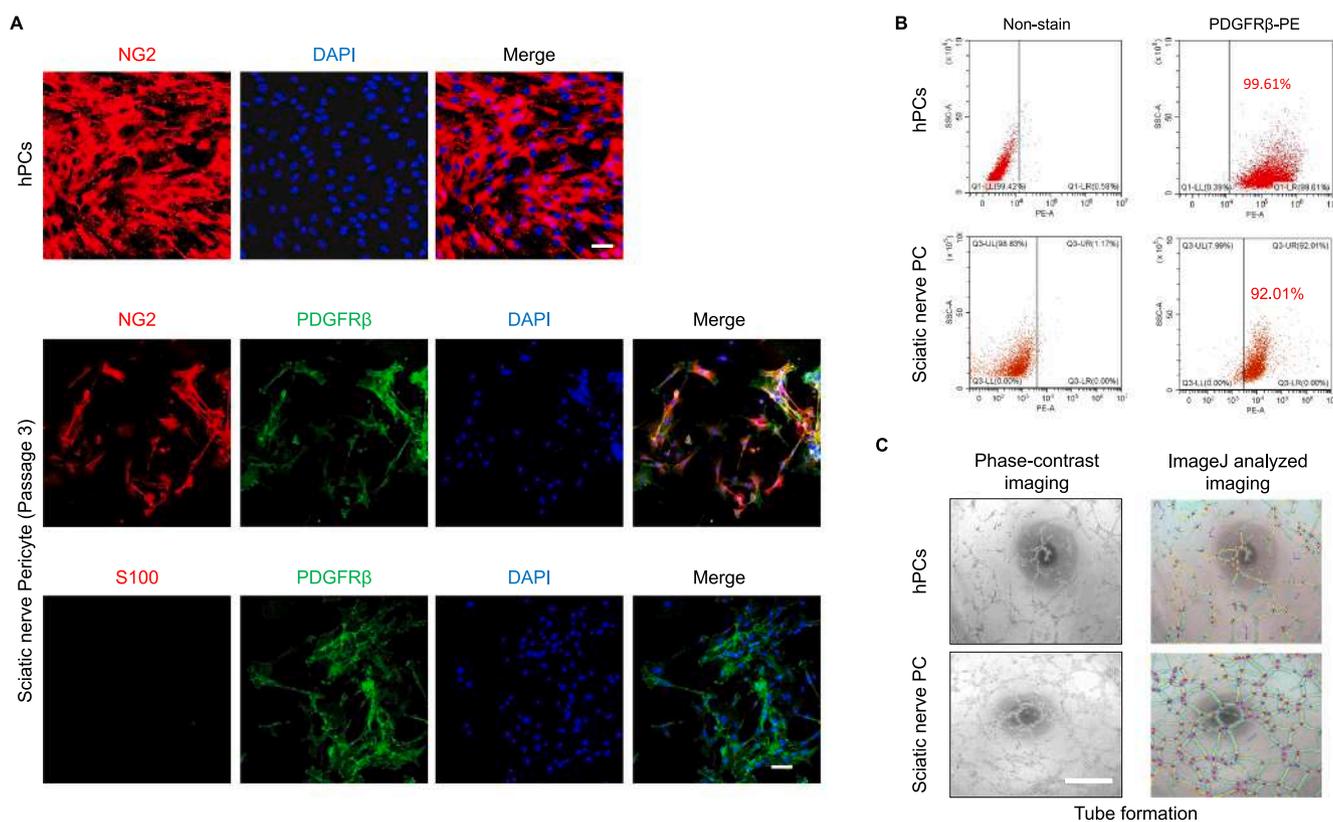


Fig. 5. Characterization of primary cultured sciatic nerve pericytes (PC). (A) Immunofluorescent staining with NG2 (a pericyte marker), PDGFR β (a pericyte marker), and S100 (a Schwann cell marker) in human pericytes from placenta (hPCs) and sciatic nerve pericytes (passage 3). Nuclei were labeled with DAPI (blue). Scale bar: 100 μ m. (B) Flow cytometry analysis of cultured hPCs and sciatic nerve pericytes (passage 3) with non-stain and PDGFR β -PE staining, showing 99.61 % (hPCs) and 92.01 % (sciatic nerve pericytes) viability. (C) Tube formation assay in cultured hPCs and sciatic nerve pericytes over 18 hours.

immunofluorescence staining was performed, targeting NG2 and PDGFR β (both pericyte markers) and S100 (a Schwann cell marker). Similar to the positive control cell line (human pericytes from placenta, hPCs), the isolated sciatic nerve pericytes also demonstrated high levels of NG2 and PDGFR β expression, with negligible S100 expression,

supporting the pericytes identity of the isolated cells (Fig. 5A). Additionally, flow cytometry analysis was conducted to assess the purity of the hPCs and cultured sciatic nerve pericytes. PDGFR β -PE positive pericytes accounted for 92.01 % of the cell population, indicating a high level of purity similar with hPCs (99.61 %) (Fig. 5B). Finally, the

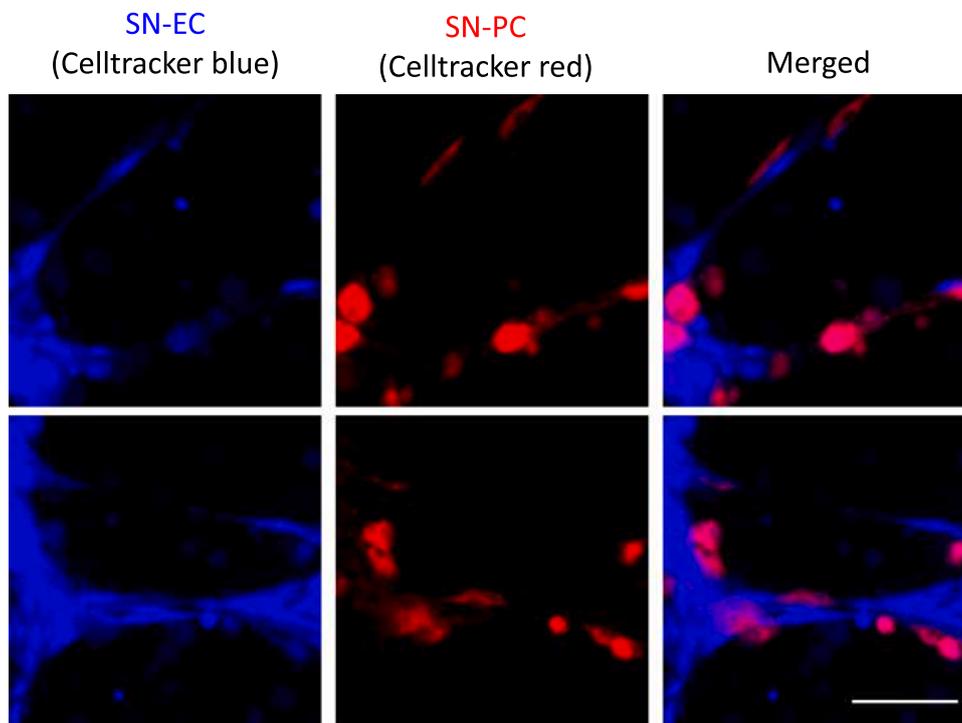


Fig. 6. Mixed co-culture tube formation analysis. Primary cultured sciatic nerve endothelial cells and pericytes were stained with Celltracker Blue CMF2HC Dye and Celltracker Red CMTPX Dye, respectively. The cells were co-cultured at a 1:4 ratio, and the tube formation assay on Matrigel was performed over 18 hours. Scale bar: 100 μ m.

functional capability of sciatic nerve pericytes to form tube-like structures and encapsulate endothelial cells, similar to hPCs, was evaluated using an *in vitro* angiogenesis assay on matrigel. Sciatic nerve pericytes successfully formed organized capillary-like structures (Fig. 5C). Furthermore, in a co-culture tube formation assay combining sciatic nerve endothelial cells and pericytes, the pericytes (labeled in red) were observed surrounding the endothelial cells (labeled in blue), mirroring the functional characteristics of conventional pericytes (Fig. 6). Collectively, these findings confirm that the PDGFR β -positive cells isolated are indeed sciatic nerve pericytes.

3.4. Isolated SNEC-EVs and SNPC-EVs contributes to nerve regeneration

It is well known that mouse schwann cell derived EVs promote axonal growth in peripheral nerve injury (Lopez-Leal and Court, 2016). SNEC-EVs and SNPC-EVs were isolated using EXOCET EV isolation solution and characterized by western blot analysis. EV positive markers (Alix, CD63, and CD81) were higher expressed in SNEC-EVs and SNPC-EVs than in the SNEC and SNPC lysates. In contrast, EV negative marker (GM130) was lower expressed in SNEC-EVs and SNPC-EVs than in the SNEC and SNPC lysates. Next, to investigate the effect of SNEC-EVs and SNPC-EVs on nerve regeneration, we conducted an *ex vivo* study using dorsal root ganglion (DRG) culture. The cultured DRG were treated with varying doses of SNEC-EVs and SNPC-EVs (1 μ g/mL and 10 μ g/mL) or PBS. After four days, immunofluorescence staining with neurofilament, a neuronal process marker, revealed significant induction in neurite sprouting in the SNEC-EVs and SNPC-EVs treated group compared to the PBS treated group with a dose dependent manner (Fig. 7B and C). These results highlight the potential and application value of our cultured SNEC and SNPC, and even the SNEC-EVs and SNPC-EVs secreted by these two cells, in promoting nerve regeneration.

4. Discussion

Peripheral nerve recovery is a multistep process that relies on the

intricate regulation of molecular and cellular signaling pathways (Klimovich et al., 2021). Critical to this process is the activation and proliferation of Schwann cells, which facilitate nerve regeneration by orchestrating cellular and biochemical responses in the peripheral nervous system following injury (Wei et al., 2024). Recent findings by Cattin et al. revealed that macrophage-derived VEGF-A stimulates the formation of a polarized vascular network within the injury bridge. These newly formed blood vessels provide tracks that guide Schwann cell migration across the wound surface, thereby supporting nerve regeneration (Cattin et al., 2015). However, the precise mechanisms through which blood vessels enhance nerve regeneration remain unclear. To elucidate the specific role of angiogenesis in nerve repair, this study capitalized on the robust vascular response following nerve injury by isolating and purifying vascular endothelial cells and pericytes from injured sciatic nerves for *in vitro* analysis.

We first confirmed through immunofluorescence staining that, in comparison to normal sciatic nerve tissue, injured sciatic nerve tissue contained a significantly higher number of endothelial cells and pericytes, suggesting an angiogenic response potentially involved in nerve regeneration (Fig. 1). Additionally, a review of the literature revealed that current *in vitro* models of peripheral nerve regeneration predominantly focus on macrophages and Schwann cells, with a notable absence of models incorporating sciatic nerve endothelial cells and pericytes. Following the non-enzymatic isolation method described by Yin et al. (2015, 2020a, 2012), we successfully separated endothelial cells and pericytes from injured sciatic nerve tissue. Traditional cell culture methods commonly employ enzymatic digestion using agents such as trypsin and collagenase to disassociate the extracellular matrix and obtain single cells (Piwocka et al., 2024). While effective, enzymatic methods are costly and may introduce cellular alterations, including reduced cell adhesion, impaired function, loss of viability, altered membrane permeability, and potential cellular rupture and deformation (Hu et al., 2023; Sriram et al., 2015). In fact, we have also used enzymatic digestion method to separate endothelial cells and pericytes from damaged sciatic nerve tissues. The results showed that the purity of

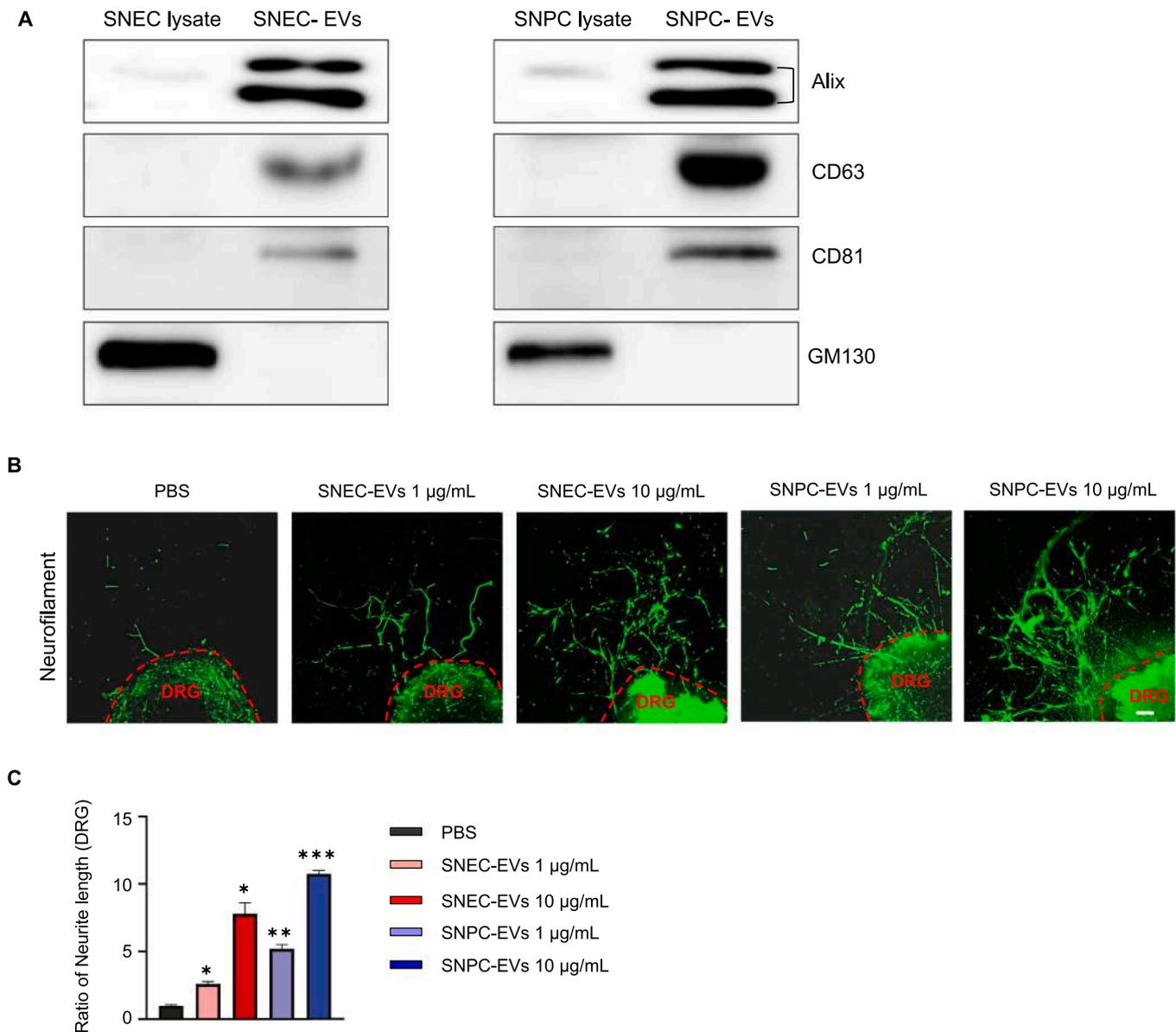


Fig. 7. Characterized SNEC-EVs and SNPC-EVs promotes nerve regeneration. (A) Representative western blots for EV positive markers (Alix, CD63 and CD81) or EV negative marker (GM130) in SNEC lysate, SNPC lysate, SNEC-EVs, and SNPC-EVs. (B) Neurofilament (green) immunofluorescence staining in mouse DRGs treated with PBS, SNEC-EVs (1 µg/mL and 10 µg/mL), and SNPC-EVs (1 µg/mL and 10 µg/mL) for four days. Scale bar, 100 µm. (C) Quantitative analysis of sprouted neurite length using an image analyzer. Results are presented as means ± SEM (n = 4; *P < 0.05, **P < 0.01, ***P < 0.001). The relative ratio of the PBS group was defined as 1. SNEC, sciatic nerve endothelial cells; SNPC, sciatic nerve pericytes; EVs, extracellular vesicles; DRG, dorsal root ganglion.

endothelial cells and pericytes extracted by this method was only 16.13 % (endothelial cells) and 14.94 % (pericytes) (Figure S1). In contrast, the non-enzymatic method minimizes cellular damage and maintains a high proliferation rate and cellular integrity. However, due to the low vascular content in normal sciatic nerve tissue, isolating a sufficient number of vessels for endothelial and pericyte culture remains challenging. Cattin et al. provided insight by demonstrating a marked increase in vasculature in the injured sciatic nerve area five days post-injury. Accordingly, we developed a sciatic nerve injury model and used immunofluorescence staining to confirm the near absence of vascular endothelial cells and pericytes in normal sciatic nerve tissue, while substantial vascularization was evident post-injury, aligning with prior findings (Cattin et al., 2015). This vascular proliferation facilitated the isolation of endothelial cells and pericytes from injured tissue, which were subsequently cultured and exhibited morphological characteristics consistent with typical endothelial cells and pericytes (Figs. 2 and 4).

We further confirmed that our cultured cells were indeed endothelial

cells and pericytes through positive and negative cell markers, and we assessed the purity of our cultured sciatic nerve endothelial cells and pericytes via flow cytometry, achieving a purity of approximately 90 % (endothelial cells) and 92 % (pericytes) (Figs. 3 and 5). Although this direct explant method circumvents the need for enzymatic digestion to isolate cells from tissue samples, it requires an extended culture period for cells to emerge from the explant tissue and may lead to the presence of non-target cells (Carrel and Burrows, 1911). We observed some cell contamination in early passages (passages 0 and 1) of cultured sciatic nerve endothelial cells and pericytes (Figure S2). Similarly, immunofluorescence staining revealed some cells negative for our cell markers, visible only with DAPI staining (indicated by arrows), suggesting the presence of unidentified cell types apart from endothelial cells, pericytes, fibroblasts, and neuronal cells (Figures S3 and S4). Although sub-culture significantly reduced the number of these unidentified cells, complete elimination was not feasible, representing a major limitation of this study and an area for further optimization.

Furthermore, our tube co-culture system demonstrated that cultured endothelial cells and pericytes can form well-organized capillary-like structures, with pericytes effectively enveloping endothelial cells, indicating the potential utility of these cultured cells in *in vitro* functional experiments. Additionally, these cells allow for testing of migration and proliferation under inflammatory conditions that mimic nerve injury. Here, we used SNEC-EVs and SNPC-EVs to indirectly demonstrate that SNEC and SNPC play a role in promoting nerve regeneration (Fig. 7). Future research may use exogenous SNEC-EVs and SNPC-EVs treatment or comprehensive analyses of gene expression profiles, including signal pathway exploration via antibody detection and single-cell RNA sequencing, to elucidate specific signaling pathways and downstream targets through which angiogenesis supports nerve regeneration following injury.

In summary, this study is the first to successfully isolate endothelial cells and pericytes from sciatic nerves, providing a reliable model for investigating neurovascular regeneration mechanisms *in vitro*. The use of endothelial cells and pericytes derived from injured sciatic nerves enhances the relevance of this model for studying targeted mechanisms in neurovascular regeneration, offering valuable insights into potential therapeutic strategies for promoting peripheral nerve repair.

CRedit authorship contribution statement

Huang Yan: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Jin Hai-Rong:** Methodology, Supervision, Writing – review & editing. **YIN GUO NAN:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Ryu Ji-Kan:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Vo Minh Nhat:** Methodology, Formal analysis. **Fridayana Fitri Rahma:** Methodology, Formal analysis, Data curation. **Liu Fang-Yuan:** Methodology, Formal analysis, Data curation.

Declaration of Competing Interest

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jneumeth.2025.110366](https://doi.org/10.1016/j.jneumeth.2025.110366).

Data availability

Data will be made available on request.

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