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Establishment of an in vitro Differentiation and Dedifferentiation System of Rat Schwann Cells

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Abstract

In the peripheral nervous system, Schwann cells are the primary type of glia. This protocol describes an in vitro differentiation and dedifferentiation system for rat Schwann cells. These cultures and systems can be used to investigate the morphological and biochemical effects of pharmacological intervention or lentivirus-mediated gene transfer on the process of Schwann cell differentiation or dedifferentiation.

Keywords: Peripheral nerve, Schwann cells, Differentiation, Dedifferentiation, In vitro

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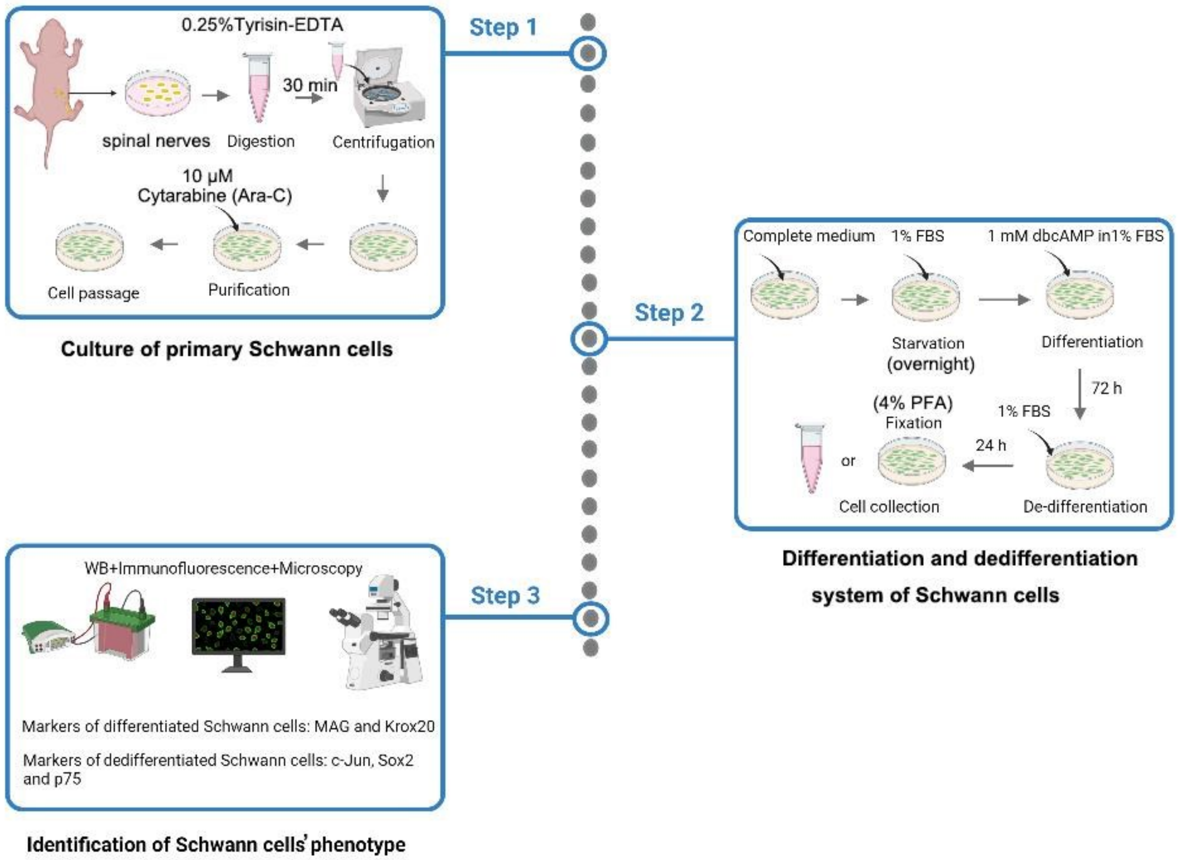
Graphical abstract



Background

Schwann cells are the primary glial cells of the peripheral nervous system. During axonal sorting and myelination in the peripheral nerves, Schwann cells originate from neural crest cells that differentiate into mature phenotypes (Cristobal and Lee, 2022). Moreover, Schwann cells' incredible plasticity is one of the most important characteristics following nerve damage or demyelination (Nocera and Jacob, 2020). Schwann cells undergo dedifferentiation after injury and redifferentiate to promote nerve regeneration and complete functional recovery (Jessen and Mirsky, 2019). Therefore, it is important to study Schwann cells' differentiation and dedifferentiation status to understand their role in nerve development and injury. As reported previously, dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP) induces Schwann cells to acquire a differentiated phenotype (Yamauchi et al., 2011). After stimulation with 1 mM dbcAMP, Schwann cells from rats change from bipolar or tripolar to flat within 24 h. By contrast, mouse Schwann cells retain their bipolar or tripolar morphology after dbcAMP treatment (Arthur-Farraj et al., 2011). And a previous study described in vitro assays of scalable differentiation and dedifferentiation of Schwann cell in the absence of neurons (Monje PV., 2018). ~~However, the details of how Schwann cells acquire a differentiated or dedifferentiated phenotype in vitro remain poorly understood. Herein~~Based on previous studies, we established a detailed and simplified system ~~of~~ for differentiation and dedifferentiation of Schwann cells. In order to clearly distinguish morphological changes, we performed the differentiation and dedifferentiation assay using rat Schwann cells. In brief, the first step was to obtain

Schwann cells from the spinal nerves of rats based on a previous study (Wen et al., 2017). During the second step, differentiation and dedifferentiation assays were conducted. Schwann cells were purified and passaged, and experiments were conducted on the third passage. Finally, Schwann cell status was determined by morphological examination, western blotting analysis, and immunofluorescence detection.

Materials and Reagents

1. Cell culture dish (3.5 and 6 cm) (JET BIOFILT, catalog numbers: CD000035, TCD-010-060)
2. 50 mL centrifuge tubes (Corning, catalog number: 430828)
3. 15 mL centrifuge tubes (Corning, catalog number: 430790)
4. 1.5 mL tube (JET BIOFILT, catalog number: CFT000015)
5. Polyvinylidene fluoride membrane (PVDF) (Bio-Rad, catalog number: 1620177)
6. Cell glass coverslips (diameter: 12 mm, thickness: 0.13–0.17 mm) (Fisherbrand, catalog number: FIS12-545-80)
7. Neonatal Sprague-Dawley (SD) rat [postnatal 1–2 days (P1–P2)]
8. Distilled water
9. 75% ethanol
10. Phosphate buffered saline (PBS) (Gibco, catalog number: 10010023)
11. Poly-L-lysine hydrobromide (PLL) (Sigma-Aldrich, catalog number: P1274)
12. 0.25% trypsin-EDTA (Gibco, catalog number: 25200072)
13. Fetal bovine serum (FBS) (Corning, catalog number: 35-076-CV)
14. DMEM/F12 (Gibco, catalog number: 11330057)
15. Cytosine arabinoside (Ara-C) (Sigma-Aldrich, catalog number: C1768)
16. Recombinant human heregulin β-1 (PeproTech, catalog number: 100-03)
17. Forskolin (Sigma-Aldrich, catalog number: F6886)
18. 4% paraformaldehyde (PFA) (Biosharp, catalog number: BL539A)
19. Dimethyl sulfoxide (DMSO), suitable for cell culture (Beyotime, catalog number: ST038)
20. Dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP) (Sigma-Aldrich, catalog number: D0627)
21. Triton X-100 (Sigma-Aldrich, catalog number: V900502)
22. Tween-20 (Sigma-Aldrich, catalog number: P1379)
23. Phalloidin (Abcam, catalog number: ab176759)
24. RIPA lysis buffer (FUDE Biological Technology, catalog number: FD009)
25. Rabbit monoclonal (EP1039Y) anti-p75 (Abcam, catalog number: ab52987)
26. Rabbit anti-Krox20 (Novus Biologicals, catalog number: 13491-1-AP)
27. Mouse monoclonal anti-c-Jun (BD Biosciences, catalog number: 610326)
28. Mouse monoclonal (9-9-3) anti-Sox2 (Abcam, catalog number: ab79351)
29. Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Thermo Fisher Scientific, catalog number: A11001)
30. Alexa Fluor® 568 goat anti-rabbit IgG (H+L) (Thermo Fisher Scientific, catalog number: A11011)
31. 4’,6-diamidino-2’-phenylindole (DAPI) (Sigma-Aldrich, catalog number: D8417)
32. Penicillin–streptomycin (Gibco, catalog number: 15140-122)
33. Omni-ECL™ Light Chemiluminescence kit (EpiZyme, catalog number: SQ201)
34. 10% dodecyl sulfate sodium salt-polyacrylamide gel (EpiZyme, catalog number: PG112)
35. 5% non-fat milk (Solarbio, catalog number: D8340)
36. Tris-buffered saline (Sigma-Aldrich, catalog number: 93318)
37. Horseradish peroxidase (HRP)-conjugated secondary antibody (Invitrogen, catalog numbers: 31430, 31460)
38. 1,000× Ara-C (10 mM in distilled water, see Recipes)
39. 1,000× dbcAMP (1,000 mM in DMSO, see Recipes)

40. 30 mM forskolin stock solution (see Recipes)
41. Complete growth medium of rat Schwann cells (see Recipes)
42. 10% FBS (see Recipes)
43. 3% FBS (see Recipes)
44. 1% FBS (see Recipes)
45. 1 mM dbcAMP (see Recipes)
46. 0.1% Triton X-100 (see Recipes)
47. 5% gelatin (see Recipes)
48. PBST (see Recipes)
49. Blocking buffer (see Recipes)

Equipment

1. Pipettes (Thermo Fisher Scientific, 10, 200, and 1,000 µL)
2. CO_2 incubator (Thermo Fisher Scientific, model: Heracell 150i)
3. Surgical scissors and forceps (Shenzhen RWD, catalog numbers: S14014 and F12029-09)
4. Spring scissors (Shenzhen RWD, catalog number: S11001)
5. Superfine forceps (Shenzhen RWD, catalog number: F13002)
6. Stereomicroscope (Jiangnan Novel, model: SZ6060)
7. Centrifuge (Eppendorf, model: Micro21)
8. Phase contrast microscope (Zeiss, model: Primovert)
9. Fluorescence microscope (Zeiss, model: Axio Imager A2)

Software

1. ImageJ (Version 1.8.0, https://imagej.en.softonic.com/)

Procedure

A. Preparation for primary Schwann cell cultures from neonatal rat

1. Prepare the culture plate: coat 3.5 cm cell culture dishes with 1 mL of PLL solution (0.1 mg/mL). Incubate overnight at 37 °C in a CO_2 incubator. This step is only used to increase cell adhesion to the culture dish. Therefore, the CO_2 content is not relevant at this point.

~~*Note: Percentage of CO_2 is not important at this time. This step is just to increase the adhesion of the cell dishes.*~~

2. The n~~N~~ext day, remove the PLL solution, ~~thoroughly~~ rinse the dish surface thoroughly with distilled water, and air dry before use. ~~Pipette~~ Add 1.5 mL of cold PBS ~~into~~ to each dish and place ~~them~~ on ice.

   Note: Do not use PBS to clean the dishes as this will cause salt crystallization after drying and disrupt cell adhesion.

3. Prepare culture media and solutions:
   a. Medium ~~A~~ for cell proliferation (culture medium to expand Schwann cells) (Medium I): DMEM/F12 containing 3% FBS, 3 µM forskolin, 10 ng/mL heregulin β-1, and 100 mg/mL penicillin–streptomycin.

b. Medium ~~B~~ for cell starvation (Medium II): DMEM/F12 containing 1% FBS and 100 mg/mL penicillin–streptomycin.

c. Medium ~~C~~ for Schwann cell differentiation (Medium III): DMEM/F12 containing 1% FBS, 100 mg/mL penicillin–streptomycin, and 1 mM dbcAMP.

d. Medium ~~D~~ for Schwann cell dedifferentiation (Medium IV): DMEM/F12 containing 1% FBS and 100 mg/mL penicillin–streptomycin.

~~*Note: Media should be made fresh just before use.*~~

B. Culture of primary Schwann cells from rat spinal nerves

Note: This part refers to a previous study (Wen et al., 2017).

1. Prepare surgical equipment (see **Figure 1**).

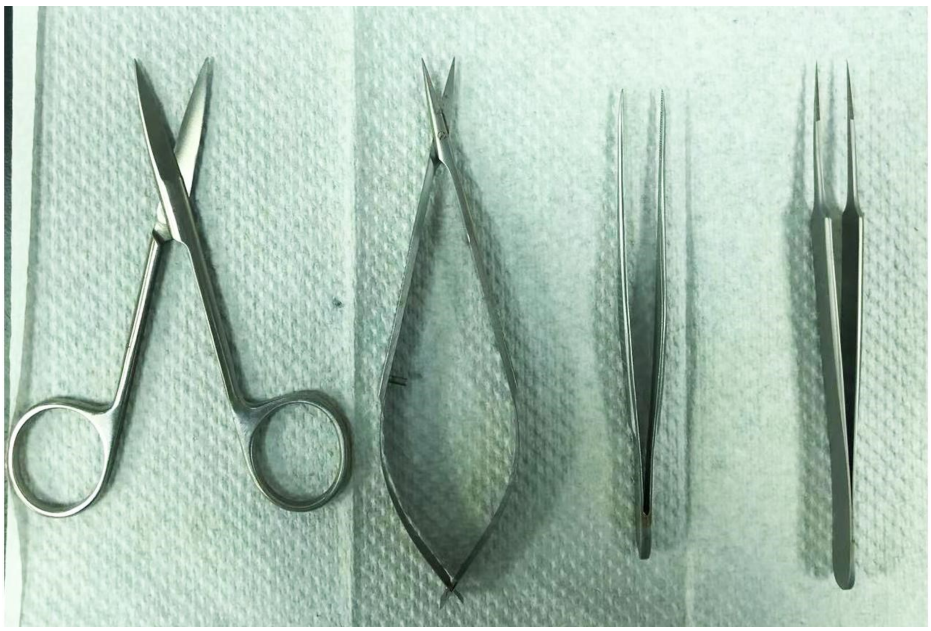


Figure 1. Surgical equipment used in this protocol

2. Tissue collection: anesthetize the neonatal rat by putting it on ice for 2–3 min, sterilize them with 75% ethanol, remove the head ~~decapitate~~ with surgical scissors and forceps, ~~put the head into a collection bag,~~ and collect the sciatic nerves and spinal nerves with superfine forceps.
3. Tissue digestion: prepare one 1.5 mL tube, ~~with~~ add 1 mL of PBS, and ~~keep chilled~~ cool on ice. Transfer these nerves to ~~the~~ a 1.5 mL tube and use spring scissors to cut them ~~with a spring scissor~~ into ~~small~~ 1-mm-long ~~pieces~~segments.~~;~~ T~~t~~hen, add 1 mL of pre-warmed 0.25% trypsin-EDTA ~~into~~ to the tube. Cap the tube and incubate at 37 °C in a CO_2 incubator for 30 min with ~~intermittent vibration~~ occasional shaking every 10 min.
4. Collect cells by centrifugation: stop digestion by adding 100 µL of FBS~~, to stop digestion and~~ then prepare a single cell suspension by gently ~~triturate~~ digesting the cell sample 30 times ~~using~~ with a 1 mL pipette ~~to make a single cell suspension~~. Then, centrifuge the cell suspension at 100× g for 5 min at room temperature and discard the supernatant. Resuspend the cell ~~pellet~~ sediment in 200 µL of ~~10% FBS in~~ DMEM/F12 containg 10% FBS, ~~plant~~ place the cell suspension in ~~the middle of the~~ a 3.5 cm PLL-coated ~~3.5 cm~~ culture dish, and culture

the cells in a CO_2 incubator for 1–2 h to ensure cell~~ attachment~~ adhesion. Add 1 mL of 10% FBS to the ~~dish~~ culture plated and culture the cells for 24 h.

*Note: At this time, t~~T~~he ~~cell~~cells suspension ~~at this point~~ is a mixture of Schwann cells and fibroblasts (see **Figure 2A**).*

5. Cell p~~P~~urification: replace the culture medium with DMEM/F12 containing 10% FBS and 10 μM Ara-C (add 1 μL of 10 mM Ara-C stock solution per 1 mL of medium) to eliminate the fibroblasts. After 48 h, replace the culture medium with Medium ~~A~~I of rat Schwann cells.

   *Note: After purification, approximately 1 × 10^4 Schwann cells per rat ~~should~~ can be obtained~~ from one rat~~. When 100% confluence~~t~~ is reached after expansion, the number of Schwann cells per dish ~~should~~ will be approximately 1 × 10^5–2 × 10^5 (see **Figure 2B**).*

6. Cell passage: when the culture reaches 90% confluenc~~y~~e, wash the cells with 1 mL of PBS, discard the PBS wash~~es~~, ~~and~~ add 1 mL of 0.25% trypsin-EDTA ~~into~~ to the cell dish~~,~~~~es~~ ~~for 2 min~~ and leave at room temperature for 2 min. Observe the cells under a phase contrast microscope ~~while~~ and gently ~~shaking~~ shake the ~~culture~~ dish. When the cells~~ start to drop from the dish~~ begin to detach, add 1 mL of 10% FBS to stop digestion, collect the cells in a 1.5 mL tube, centrifuge ~~for 5 min~~ at 100 × *g* for 5 min at room temperature, discard the supernatant, and gently resuspend the cells in 1 mL complete growth medium of rat Schwann cells.

   *Note: Schwann c~~C~~ells are passaged and expanded at a ratio of 1:3–1:4, ~~to expand~~ and ~~cells from~~ the third passage cells are used for further experiments (see **Figure 2C**).* At this time, cells are actively proliferating and fibroblasts are absent.

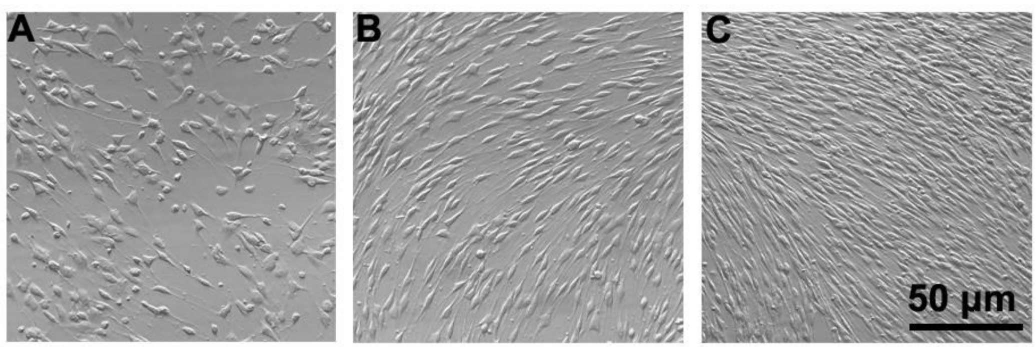


Figure 2. Purification and expansion of r~~R~~at Schwann cells~~' purification and expansion~~. A. ~~Cells~~ 24 h after cell dissection, ~~;~~ the culture~~s~~ should consist of Schwann cells and fibroblasts. Schwann cells have an elongated~~,~~ spindle-~~shaped~~like morphology, whereas fibroblasts are very flat. B. On day 3, purified rat Schwann cells ~~at~~ became confluent~~ce~~. C. After 2 days of growth ~~being expanded~~ in~~ complete growth medium~~ Medium I, ~~for two days and~~ the culture is routinely passaged to ~~the third~~ passage 3 in complete growth medium in the presence of forskolin and heregulin β-1, and the culture is ~~populated~~ filled with Schwann cells (>95% confluence)~~,~~ with ~~their characteristic~~ typical bipolar~~,~~ spindle~~-shaped~~ cell bodies.

C. Differentiation and dedifferentiation of Schwann cells (Figure 3)

1. Seed and starvation: seed 5,000 Schwann cells in a 3.5 cm dish (covered with glass coverslips, for immunofluorescence analysis) or seed 1 × 10^5 Schwann cells in a 6 cm dish (for western blotting analysis) in Medium ~~A~~I to allow a fast proliferation ~~expansion~~ of~~ the cell population~~ rat Schwann cells. After one day, remove the complete growth medium and ~~rapidly~~ immediately replace it with an equal volume of Medium ~~B~~II overnight to adapt to the lack of ~~mitogenic~~ mitotic stimulation.

Note: Complete growth medium (Medium I~~A~~) of rat Schwann cells containing forskolin ~~will~~ can induce Schwann cells to acquire a differentiated phenotype, and 10% FBS will induce Schwann cell~~s~~ ~~to proliferate~~ proliferation~~rather than differentiate~~ but not differentiation. Therefore, before inducing Schwann cell differentiation, we starved the cells by culturing them in Medium II at low serum concentrations and without forskolin.

2. Differentiation: remove the starvation medium (Medium II) from each dish, ~~by gentle aspiration,~~ replace ~~it~~ with an equal volume of Medium ~~C~~ III, and culture the cells in a CO_2 incubator for 72 h. Before the dedifferentiation process, o~~O~~bserve the cultures of dbcAMP-induced differentiated Schwann cells under ~~the~~ a phase contrast microscope to confirm the expected morphological changes. ~~prior to applying a dedifferentiating treatment.~~

   Note: After ~~approximately~~ about 6 h, ~~you can start to see~~ the rat Schwann cells began to show ~~the~~ morphological changes ~~in rat Schwann cells~~. ~~It is~~ We recommended ~~to include~~ starting with cultures ~~maintained~~ without forskolin and dbcAMP ~~from the outset to serve~~ as controls. To ~~maintain~~ extend the differentiation time of Schwann cells ~~differentiated for an extended period of time~~, simply replace the culture medium ~~carry out a medium replacement using new~~ with fresh differentiation conditioned~~al~~ medium (Medium III).

3. Dedifferentiation: after 72 h, ~~the~~ Schwann cells ~~have~~ differentiated ~~in response to~~ under the influence of dbcAMP ~~treatment~~. To induce dedifferentiation, simply ~~replace~~ change the culture medium ~~with~~ to Medium ~~D~~IV, and culture the cells in a CO_2 incubator for 24 h. ~~Proceed to collect~~ Continue collecting or ~~analyze~~ analyzing ~~the~~ cultures ~~on the second~~ the day after dbcAMP withdrawal, or ~~as needed according~~ as required ~~to~~by the experimental design.

   Note: dbcAMP-induced differentiated Schwann cells ~~cannot~~ does not dedifferentiate ~~when exposed~~ upon exposure to growth factors such as forskolin and heregulin β-1. Thus, ~~it is~~ we recommend~~ed to use~~ using a medium that does not contain forskolin and heregulin β-1 throughout the differentiation and dedifferentiation ~~assays~~ experiments. Approximately 6 h after removal of dbcAMP ~~removal~~, ~~nearly~~ almost half of the rat Schwann cells return to ~~a~~ typical bipolar or tripolar shape. You ~~may~~ can add ~~a~~ drugs or perform virus transfections before ~~the~~ dedifferentiation ~~assay~~ experiment.

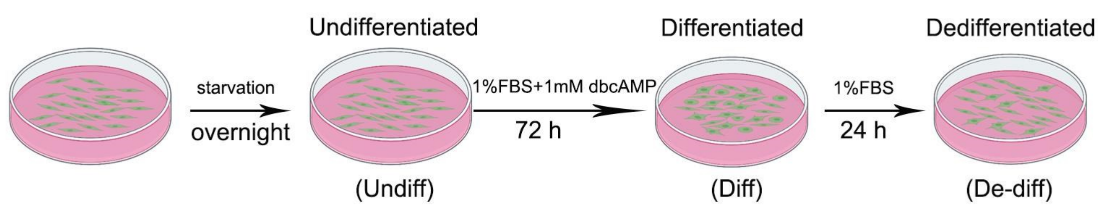


Figure 3. Differentiation and dedifferentiation system of rat Schwann cells. Schwann cells are ~~expanded~~ cultured in Medium ~~A~~ I for several days, ~~after which~~ then the third-passage cells are ~~re~~plated at an appropriate density and starved overnight in Medium ~~B~~ II. The next day, the Schwann cells are treated with 1 mM dbcAMP, which induces morphological change and upregulation of differentiation markers, ~~that can be~~ which could be examined by phase contrast microscope observation, immunofluorescence, or western blotting. After 72 h, ~~simply replace the culture medium with Medium D to induce~~ Schwann cell dedifferentiation can be induced by simply changing the culture medium to Medium IV. Undiff: Undifferentiated; Diff: Differentiated; De-diff: Dedifferentiated.

Data analysis

A. Phase contrast microscopy (Figure 4A)

1. To identify the phenotype of Schwann cells, observe their morphology under a phase contrast microscope. Schwann cells were treated with dbcAMP to obtain a differentiated phenotype with morphological transition from an elongated spindle-like shape to a flattened shape; simple dbcAMP removal can reverse the differentiated Schwann cells into an elongated bipolar morphology.

B. Immunofluorescence (Figure 4B)

1. Cell fixation: fix cells with 4% PFA for 30 min at room temperature.
2. Permeabilization and blocking: penetrate cells with 0.1% Triton X-100 (see Recipe 8) for 30 min and incubate with 5% gelatin (see Recipe 9) at room temperature for 1 h.
3. Primary antibodies: prepare a 1:200 dilution of mouse anti-c-Jun and a 1:100 solution of rabbit anti-Krox20 in 5% gelation solution. Incubate the cells with primary antibodies at 4 °C overnight.
4. Secondary antibodies: prepare a 1:400 dilution of Alexa Fluor® 488 goat anti-mouse IgG (H+L) or Alexa Fluor® 568 goat anti-rabbit IgG (H+L) in PBST (see Recipe 10) secondary antibodies. Incubate cells with the corresponding secondary antibodies at room temperature for 2 h, wash cells with PBST twice, and then incubate cells with 1 μg/mL DAPI for 5 min at room temperature to stain nuclei.

C. Western blotting (Figure 4C, 4D)

1. Cells lysis: add 100 μL of RIPA lysis buffer to the cell dish, blow, and collect lysates into a 1.5 mL tube.
2. Protein electrophoresis: prepare 10% dodecyl sulfate sodium salt-polyacrylamide gel, pipette 10 μg of protein into each well, and run the electrophoresis using the following parameters: 80 V and 300 mA for 1.5 h. When the electrophoresis is completed, remove the gel carefully and transfer proteins to a PVDF membrane.
3. Incubation of primary and secondary antibodies: after blocking with blocking buffer for 2 h at room temperature, prepare a 1:800 dilution of mouse anti-Sox2 and a 1:500 solution of rabbit anti-p75 in blocking buffer, incubate the membrane in primary antibody at 4 °C overnight, and incubate Horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h.
4. Visualization and calculation: visualize the membrane using Omni-ECL™ Light Chemiluminescence kit and calculate protein quantity using ImageJ.

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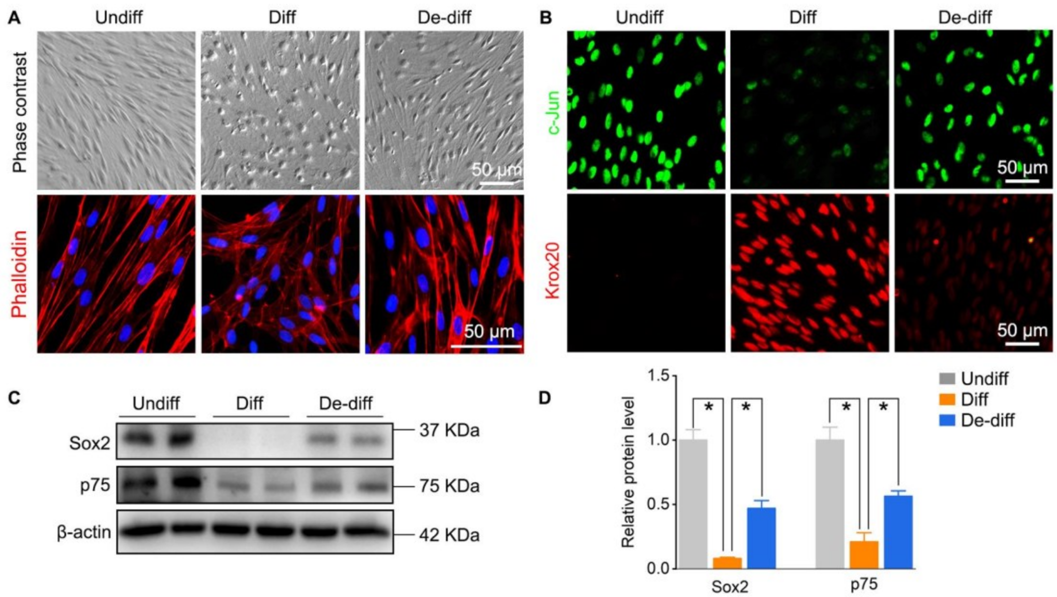

Figure 4. Identification of rat Schwann cells' phenotype. A. Cell morphology and cytoskeleton stained with phalloidin were observed under a microscope. B. Immunofluorescence of c-Jun (indicates immature or dedifferentiated Schwann cells) and Krox20 (indicates mature or differentiated Schwann cells). C, D. Western blotting analysis and data statistics of Sox2 and p75 (indicates immature or dedifferentiated Schwann cells). Undiff: Undifferentiated; Diff: Differentiated; De-diff: Dedifferentiated.

Recipes

1. **1,000× Ara-C**

   Dissolve 4.86 mg of Ara-C in 2 mL of distilled water to make a 1,000× stock solution of 10 mM and sterilize the solution by filtration. Store at -20 °C.

2. **1,000× dbcAMP**

   Dissolve 50 mg of dbcAMP in 101.756 μL of distilled DMSO to make a 1,000× stock solution of 1,000 mM and store at -20 °C.

3. **30 mM forskolin stock solution**

   Dissolve 10 mg of forskolin in 812 μL of distilled DMSO to prepare a 30 mM forskolin stock solution. Store at -20 °C.

4. **Complete growth medium of rat Schwann cells**

   48.5 mL of DMEM/F12 containing 1.5 mL of FBS, 3 μM forskolin, 10 ng/mL heregulin- β-1, and 100 mg/mL penicillin–streptomycin.

5. **10% FBS**

   45 mL of DMEM/F12 containing 5 mL of FBS supplemented with 1% penicillin–streptomycin.

6. **3% FBS**
   48.5 mL of DMEM/F12 containing 1.5 mL of FBS supplemented with 1% penicillin–streptomycin.

7. **1% FBS**
   49.5 mL of DMEM/F12 containing 0.5 mL of FBS supplemented with 1% penicillin–streptomycin.

8. **1 mM dbcAMP**
   Add 1 μL of 1,000× dbcAMP (1,000 mM) to 1 mL of 1% FBS.

9. **0.1% Triton X-100**
   Dilute 1 mL of Triton X-100 into 1,000 mL of PBS.

10. **5% gelatin**
    Dissolve 0.5 g of gelatin in 10 mL of PBS. Add 300 μL of Triton X-100 (0.3%) to the buffer.

11. **PBST**
    Add 1 mL of Tween-20 to 1,000 mL of PBS. Store at room temperature.

12. **Blocking buffer**
    5% non-fat milk in Tris-buffered saline containing 0.5% Tween-20

Acknowledgments

This protocol is adapted from the previous published papers (Zou et al., 2022), ~~and~~ (Wen et al., 2017) and (Monje PV., 2018). Thanks to Professor Jiasong Guo (Southern Medical University, Guangzhou, China).

Competing interests

There are no conflicts of interest or competing interests.

Ethics

All animal procedures were performed in accordance with the guidelines for the ethical treatment of animals.

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