● RESEARCH ARTICLE

A novel primary culture method for high-purity satellite glial cells derived from rat dorsal root ganglion

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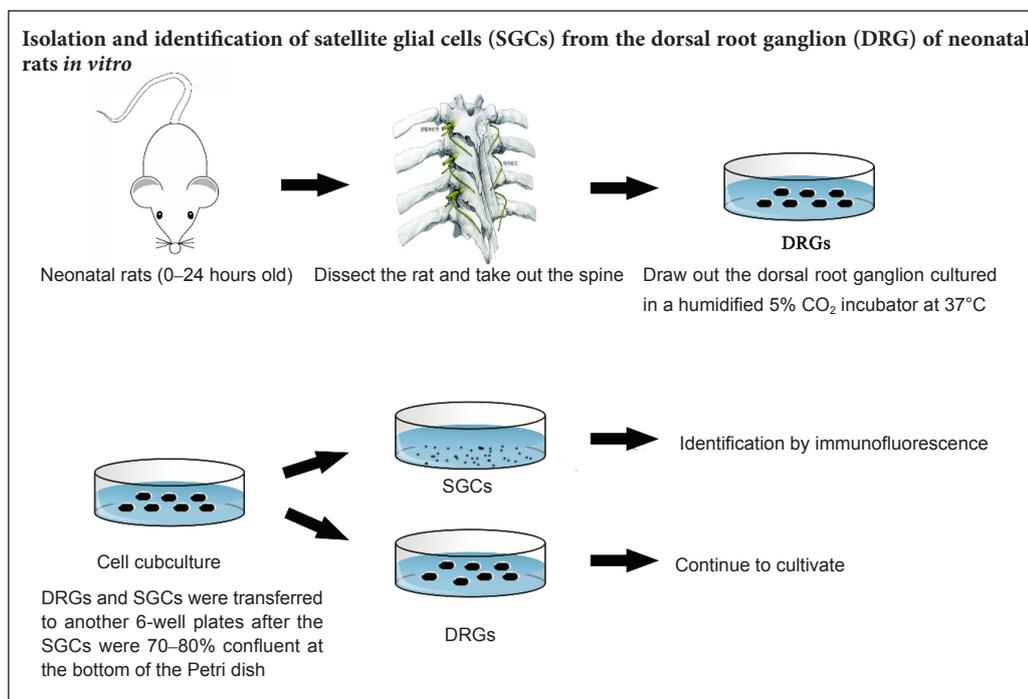
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Graphical Abstract

Isolation and identification of satellite glial cells (SGCs) from the dorsal root ganglion (DRG) of neonatal rats *in vitro*

Neonatal rats (0–24 hours old)   Dissect the rat and take out the spine   Draw out the dorsal root ganglion cultured in a humidified 5% CO_2 incubator at 37°C

DRGs

Cell cubculture

DRGs and SGCs were transferred to another 6-well plates after the SGCs were 70–80% confluent at the bottom of the Petri dish

SGCs → Identification by immunofluorescence

DRGs → Continue to cultivate

Abstract

Satellite glial cells surround neurons within dorsal root ganglia. Previous studies have focused on single-cell suspensions of cultured neurons derived from rat dorsal root ganglia. At present, the primary culture method for satellite glial cells derived from rat dorsal root ganglia requires no digestion skill. Hence, the aim of the present study was to establish a novel primary culture method for satellite glial cells derived from dorsal root ganglia. Neonatal rat spine was collected and an incision made to expose the transverse protrusion and remove dorsal root ganglia. Dorsal root ganglia were freed from nerve fibers, connective tissue, and capsule membranes, then rinsed and transferred to 6-well plates, and cultured in a humidified 5% CO_2 incubator at 37°C. After 3 days in culture, some cells had migrated from dorsal root ganglia. After subculture, cells were identified by immunofluorescence labeling for three satellite glial cell-specific markers: glutamine synthetase, glial fibrillary acidic protein, and S100β. Cultured cells expressed glutamine synthetase, glial fibrillary acidic protein, and S100β, suggesting they are satellite glial cells with a purity of > 95%. Thus, we have successfully established a novel primary culture method for obtaining high-purity satellite glial cells from rat dorsal root ganglia without digestion.

Key Words: *nerve regeneration; cell culture; dorsal root ganglia; immunofluorescence identification; satellite glial cells; neural regeneration*

Chinese Library Classification No. *R459.9; R361; Q2*

Introduction

Satellite glial cells (SGCs) are specialized cells that form a continuous layer around primary sensory neurons within dorsal root ganglia (DRG) and trigeminal ganglia (Fei et al., 2016; Liu et al., 2018). SGCs are believed to modulate the neuronal microenvironment and sensory transmission within sensory ganglia (Feldman-Goriachnik et al., 2018; Fornaro et al., 2018; Yamakita et al., 2018). These cells share many properties with astrocytes, including expression of glutamine synthetase (GS) and various neurotransmitter transporters (Takeda et al., 2009). SGCs are coupled by gap junctions and express purinergic receptors (Retamal et al., 2017; Spray and Hanani, 2017; Komiya et al., 2018). Further, they possess mechanisms for the release of cytokines, adenosine triphosphate, and possibly other chemical messengers (Takeda et al., 2009; Goto et al., 2017; Yi et al., 2018). Increasing evidence suggests that SGCs exhibit marked morphological and biochemical changes following peripheral nerve injury and inflammation (Hossain et al., 2017; Li et al., 2017; Liu et al., 2018). Moreover, when homeostasis is disrupted, as in peripheral nerve injury, SGCs become reactive and show a robust increase in glial fibrillary acidic protein (GFAP) expression (Liu et al., 2018). GS catalyzes adenosine triphosphate-dependent amidation of glutamate to glutamine, which is currently the best SGC marker (Zhu et al., 2018). Therefore, in this study, we used GFAP and GS to identify cultured SGCs that have migrated from DRG.

Recent studies have focused on cultured neurons from adult rat and other mammalian DRGs, and little attention has been paid to SGCs in DRG (Zemel and Muqeem, 2017). Thus, we focused on primary cultures of neonatal rat DRG SGCs as a research basis for experimental studies related to DRG SGCs.

Materials and Methods

Study approval and animals

All experiments were performed using neonatal rats (0–24 hours old) provided by the Animal Department of Kunming Medical University of China (license No. SCXK (Dian) 2015-0002). This study was performed in strict accordance with guidelines for the care and use of animals of Kunming Medical University (Kunming City, China). All animal experimental procedures were approved by the Committee on the Ethics of Animal Experiments at Kunming Medical University (approval No. KMMU2018018) on May 2, 2015.

DRG preparation and primary culture

Neonatal rats were sterilized with 75% alcohol (disinfection time > 3 minutes). Rats were decapitated and sacrificed. The skin, muscles, and ribs were cut to obtain the spine, which was cooled on ice in Dulbecco's modified Eagle's medium/nutrient mixture F12 Ham (DMEM/F12, 1:1 mixture) (C11330500BT; Gibco Life Technologies Inc., Grand Island, NY, USA). The spinal muscles were removed, and then both sides of spinous processes cut using ophthalmic scissors. The spinal cavity was exposed and emptied. Additionally, blood

vessels and nerve fibers in the spinal cavity were completely removed under a stereomicroscope. The DRG was then clamped under a stereomicroscope, followed by gentle removal of residual nerve fibers and capsules with fiber clamps (**Figure 1A**). Treated DRGs were collected in 35 mm Petri dishes on ice with DMEM/F12 (1:1) containing penicillin/streptomycin. A sufficient number of DRGs (approximately 54) were collected in 35 mm dishes and then seeded into 6-well plates containing SGC medium (500 μL per well) (**Table 1**). All operations were performed on ice. Cells were cultured in a humidified 5% CO_2 incubator at 37°C (**Figure 1B**).

Table 1 Optimum culture medium for dorsal root ganglion-satellite glial cells

Reagent	Concentration	Additive amount
DMEM/F12	–	47 mL
B27	–	1 mL
PSS	–	0.5 mL
GlutaMAX™-l	–	0.5 mL
BSA	30 mg/mL	0.5 mL
NRG1-β1	10 μg/mL	100 μL
Dexamethasone	25 μg/mL	76 μL
Insulin	5 mg/mL	58 μL
T3	10 μg/mL	50 μL
T4	400 μg/mL	50 μL
		Total solution: 50 mL

DMEM/F12: Dulbecco's modified Eagle's medium/nutrient mixture F-12; B27: B-27™ supplement; PSS: penicillin-streptomycin solution; BSA: bovine serum albumin; NRG1-β1: neuregulin 1-β1; T3: triiodothyronine 3; T4: triiodothyronine 4.

Cell subculture and purification

SGCs were obtained from DRG explants instead of established by digestion. DRGs were transferred to another 6-well plate coated with 25 μg/mL poly-D-lysine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA). After approximately 10 days, SGCs reached 70–80% confluency at the bottom of the Petri dish (**Figure 1C**). Next, 0.25% trypsin-ethylenediamine tetraacetic acid (Gibco Life Technologies Inc.) was used to digest SGCs, and terminated with an equal volume of 10% fetal bovine serum (Gibco Life Technologies Inc.). Cells were then mechanically dissociated to a homogenous solution by repeated pipetting, and centrifuged for 6 minutes at $1000 \times g$. The supernatant was removed and DRG-SGC medium added. The solution was then transferred from 6-well plates to 24-well plates for immunofluorescence or continued culture. Cultures were maintained in a humidified 5% CO_2 incubator at 37°C. The medium was replaced on the first day, and then continuously every second day. Cell morphology and growth were observed under an inverted microscope (EVOS™ XL Core Imaging System, Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence

Cultures were fixed in 4% paraformaldehyde (Aladdin, Shanghai, China) for 10 minutes at room temperature,

washed in phosphate buffered saline (PBS), and then incubated for 1 hour at room temperature in blocking solution containing 5% goat serum albumin and 0.3% Triton X-100 (Sigma-Aldrich) in PBS. The primary antibodies used were: SGC-specific marker, GS (anti-rabbit; Abcam, Cambridge, UK), astrocyte-specific marker, GFAP (anti-rabbit; Abcam), Schwann cell-specific markers, S100 beta (S100β) (anti-rabbit; Abcam) and SRY-box 10 (SOX10) (anti-rabbit; Abcam), neural stem cell-specific markers, p75 neurotrophin receptor (P75^{NTR}) (anti-rabbit; Abcam) and nestin (anti-mouse; Abcam), and sensory neuron-specific marker, calcitonin gene related peptide (anti-mouse; Abcam). Primary antibodies were added separately (at a dilution of 1:500) in 2% goat serum albumin in PBS with 0.3% Triton X-100 (0694; Sigma–Aldrich), and incubated overnight at 4°C. The following day, cultures were placed at room temperature for 30 minutes, and SGCs washed in PBS prior to incubation in secondary antibody Cy3-conjugated goat anti-mouse IgG (Abcam) and Alexa Fluor 488 goat anti-rabbit IgG (Abcam). Secondary antibodies were diluted separately (1:1000) in PBS containing 0.3% Triton X-100 for 2 hours at room temperature. Cells were washed in PBS and then incubated for 15 minutes with the nuclear dye, 4′,6-diamidino-2-phenylindole (DAPI) (A4099; Sigma-Aldrich) at a dilution of 1:1000 in 2% goat serum albumin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, cells were washed in PBS. Images were obtained using a Nikon microscope (Az100; Nikon, Tokyo, Japan) equipped with a fluorescent illuminator (L200/D; Prior Scientific, Rockland, MA, USA) and a digital camera (DS-Vi1; Nikon) connected to a computer. Images were used for further analysis.

Immunohistochemistry on SGC in DRG
To characterize SGCs *in vivo*, DRGs were dissected and sections cut using a cryostat. Sections were stained with primary antibodies to calcitonin gene-relatedpeptide and GS, as described previously. Primary antibodies were detected using appropriate fluorescently labeled secondary antibodies (Lagares et al., 2007).

Results
Morphological characteristics of primary cultured SGCs
DRGs were cultured in SGC medium. Cells migrated from surrounding DRGs within 3 days. Cells attached to 25 µg/mL poly-D-lysine hydrobromide-coated substrate and proliferated to form clusters after 7 days. The number of cells was sufficient for subculture by 14 days. By day 21, the cells completely covered the bottom of Petri dishes, and cell clusters were more evident. Cells adhered to the culture dish substrate and formed spherical clusters (**Figure 2**).

Morphological characteristics of SGCs after subculture
After the cells reached 70–80% confluency on the bottom of Petri dishes, DRGs were transferred to fresh 6-well plates coated with poly-D-lysine. Cells were transferred to 6-well plates for continued culture or 24-well plates for immunofluorescence. Three days after subculture, the cells were

elliptical with full bodies. Dipolar cells generated interconnecting protrusion. With prolonged culture time, the cells continued to proliferate (**Figure 3**).

Characterization of cultured SGCs by immunofluorescence
After subculture, the cells were cultured for 3 days, followed by labeling with three SGC-specific markers: GS, GFAP, and S100β. Fluorescence microscopy was performed as described previously. All cultured cells showed GS, GFAP, and S100β immunoreactivity (**Figure 4A–I**). Positive rates for SGCs expressing GS, GFAP, and S100β were 97.10%, 67.69%, and 91.66%, respectively (**Figure 4J**).

To confirm that the cultured cells are not Schwann cells, the Schwann cell-specific marker, SOX10, was also examined. No positive SOX10 expression was detected in cultured cells (**Figure 5**). Comparison of cell markers before and after DRG-SGC separation was also performed. Before DRG-SGC isolation, immunohistochemical staining of DRG tissue sections revealed a group of cells with positive GS expression around neurons (**Figure 6**).

To determine whether the cultured cells exhibit neural stem cell characteristics, cells were labeled with neural crest progenitor markers, namely nestin and P75^{NTR} (Li and Zhou, 2008; Piñero et al., 2018). Surprisingly, the cells were positive for nestin (**Figure 7A–D**). Some cells were also weakly positive for P75^{NTR} (**Figure 7E–H**).

Discussion
To establish a culture system of single-cells derived from DRG, most recent studies have focused on cultured neurons from rat DRG by obtaining a large number of DRGs followed by trypsin digestion into single-cell suspensions. In one study, neonatal rat DRGs were digested into single-cell suspensions using trypsin, with cytosine arabinoside added to purify the cells and remove all dividing cells. The cells were then cultured in DMEM/F12 medium with 10% fetal bovine serum and glial cell-derived neurotrophic factor (Hanani, 2010). In another study, neonatal rat DRGs were digested with trypsin and ethylenediamine tetraacetic acid, with the cells purified by alternating between DMEM/F12 medium and DMEM/F12 medium with cytosine arabinoside (Capuano et al., 2009; Gu et al., 2010). Additionally, DRGs from neonatal rats were digested in 2.5% trypsin for 20 minutes at 37°C. Fetal bovine serum was added to terminate digestion and single-cell suspensions incubated in DMEM/F12. Fibroblasts were removed by the differential velocity adherent method for 50 minutes, resulting in single-cell suspensions of rat DRG cells (Hanani, 2010). In this study, we established a novel method for culturing primary SGCs *in vitro* without digestion. SGCs successfully migrated from DRG *in vitro* with a SGC purification rate > 95%, which is sufficient for many cell tests.

After subculture, cells were cultured for 3 days, followed by labeling with three SGC-specific markers: GS, GFAP, and S100β. Our results show that all cultured cells exhibited GS, GFAP, and S100β immunoreactivity. Although studies have
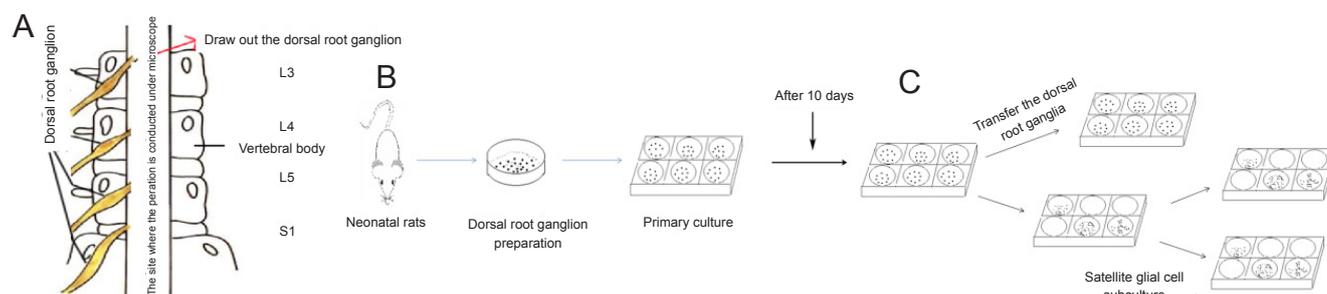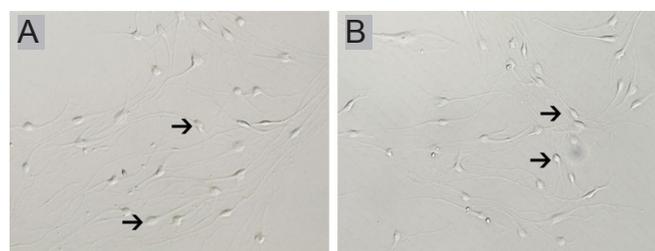
Figure 1 Isolation and primary culture of dorsal root ganglia.
(A) Location of the dorsal root ganglion, which can be removed along the direction of the red arrow. (B) Dorsal root ganglia were separated from nerve fibers, connective tissue, and capsule membranes, rinsed, and then transferred to 35-mm Petri dishes. This step reduces the probability of contamination. After sufficient dorsal root ganglia were collected, they were transferred to 6-well plates with satellite glial cell medium. (C) After culture for 7–9 days, satellite glial cells covered 70–80% of the bottom of Petri dishes. Dorsal root ganglia were then transferred to fresh 6-well plates, and the remaining satellite glial cells subcultured by 0.25% trypsin-ethylenediamine tetraacetic acid digestion. The optimal inoculation concentration of satellite glial cells was approximately 6×10^5 cells/mL.
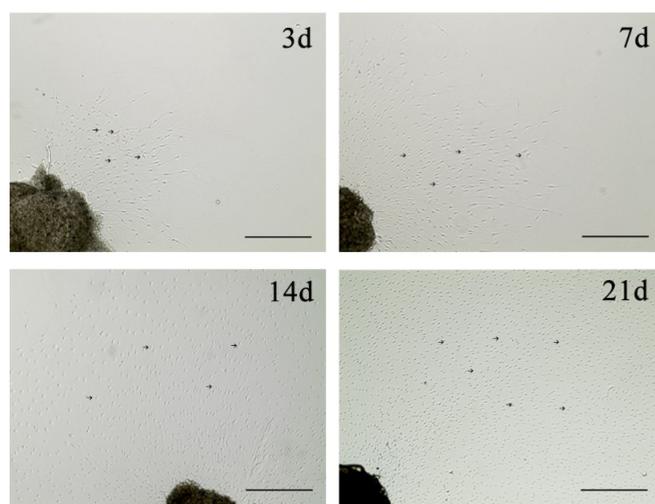
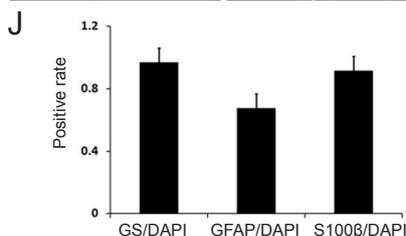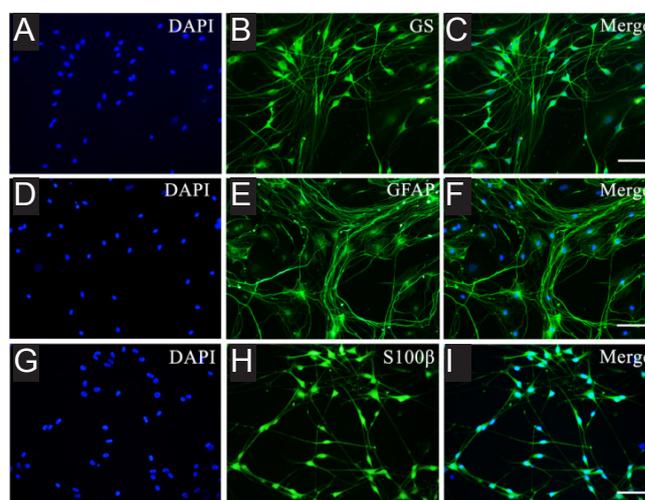**Figure 2 Morphology of migrated satellite glial cells from dorsal root ganglia.**
Phase contrast micrographs show satellite glial cells that have migrated from dorsal root ganglion explants at 3, 7, 14, and 21 days after *in vitro* culture. With prolonged culture time, the rate of migrated satellite glial cells increased. Scale bars: 500 μm. d: Days

Figure 4 Immunofluorescence characterization of satellite glial cells derived from neonatal rat dorsal root ganglia explants after 3 days of subculture.
(A–I) Immunofluorescence characterization of satellite glial cells derived from neonatal rat dorsal root ganglia explants after 3 days of subculture. Cytospun clusters collected from dorsal root ganglia explants were labeled for GS (green, A–C). The same cells were also labeled for GFAP (green, D–F). S100β (green) was also detected with nuclei counterstained with DAPI (blue: G–I). Scale bars: 100 μm. (J) Quantification of percentages for different marker combinations. GS: Glutamine synthetase; GFAP: glial fibrillary acidic protein; DAPI: 4′,6-diamidino-2-phenylindole.

Figure 3 Morphology of satellite glial cells after 3 days of subculture.
(A, B) Satellite glial cells are elliptical with full bodies. Dipole cells generate synapses with synaptic connections present between cells (arrows).

shown that Schwann cells also positively express S100β (Bierlein et al., 2017), S100β protein expression levels gradually increase during Schwann cell differentiation (Fujiwara et al., 2014). However, studies have also shown satellite cells in cardiac ganglia of newborns, which mainly express brain fatty acid binding protein. As they become more mature at postnatal day 21, these satellite cells show intense staining of both brain fatty acid binding protein and S100β (Fregoso and Hoover, 2012). In addition, both peripheral Schwann cells and satellite cells of DRG express S100β protein
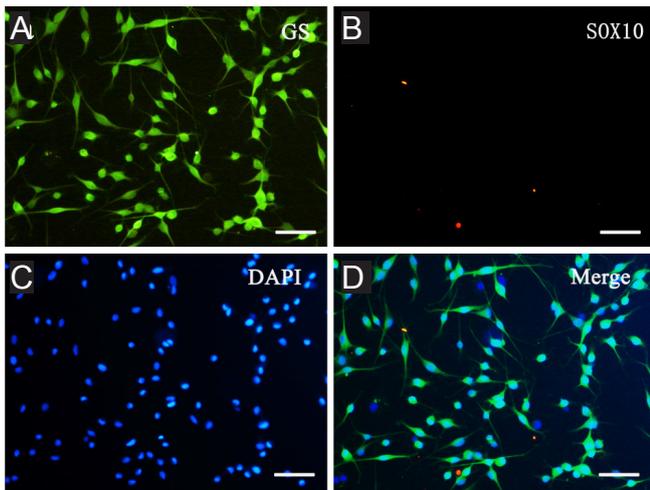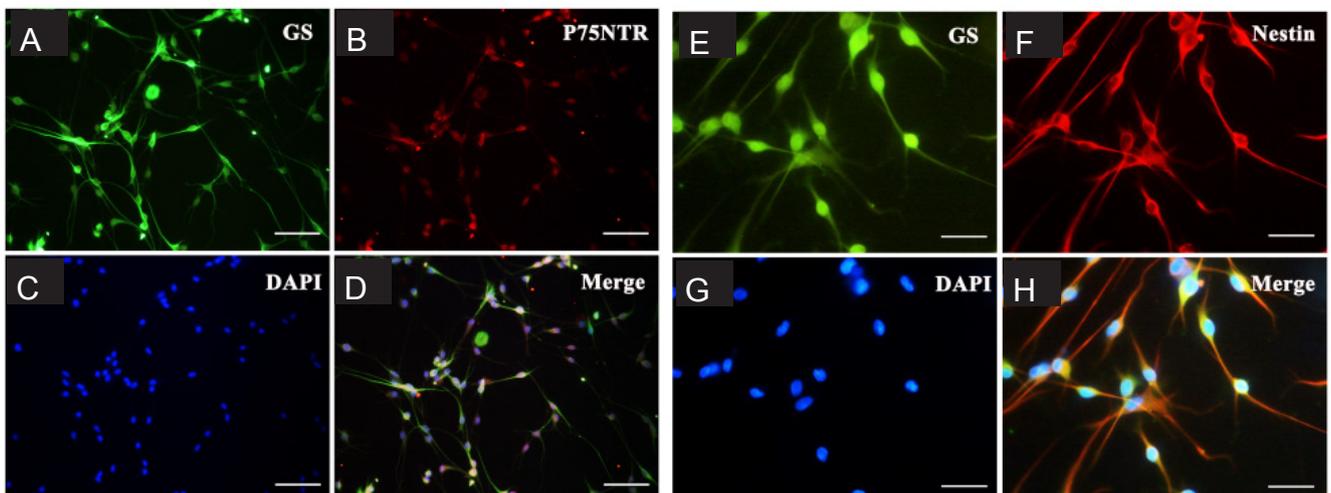
Figure 5 Immunofluorescence characterization of satellite glial cells derived from neonatal rat dorsal root ganglia explants after 3 days of subculture.

Cytospun clusters collected from dorsal root ganglia explants were labeled using a satellite glial cell-specific marker, GS (green, A). The same cells were also labeled using a Schwann cell-specific marker, SOX10 (red, B) and counterstained with DAPI (blue, C). Merged view of A, B, and C (D). Scale bars: 100 μm. GS: Glutamine synthetase; SOX10: SRY-box 10; DAPI: 4′,6-diamidino-2-phenylindole.
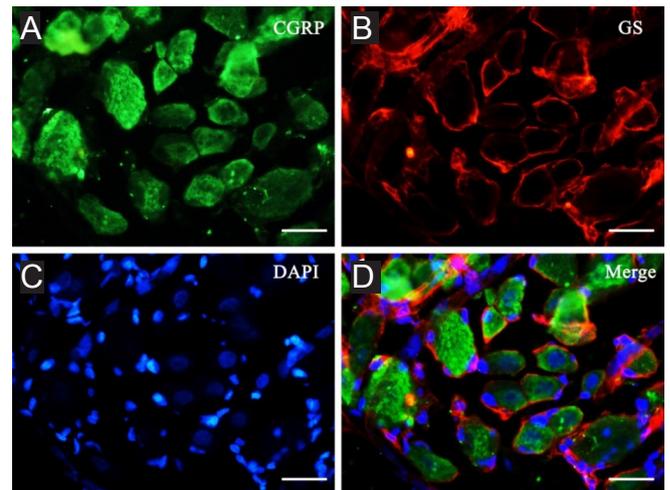
Figure 6 Immunofluorescence staining of a dorsal root ganglion before isolation.

Cytospun clusters collected from dorsal root ganglia explants were labeled for CGRP (green, A). The same cells were labeled with GS (red, B) and counterstained with DAPI (blue, C). Merged view of A–C (D). Scale bars: 50 μm. CGRP: Calcitonin gene-related peptide; GS: glutamine synthetase; DAPI: 4′,6-diamidino-2-phenylindole.

Figure 7 Immunofluorescence characterization of satellite glial cells derived from neonatal rat dorsal root ganglia explants after 3 days of subculture.

(A–D) Determining neural stem cell characteristics of satellite glial cells. Cytospun clusters collected from dorsal root ganglia explants were labeled for GS (green, A). The same cells were also labeled for P75NTR (red, B) and counterstained with DAPI (blue, C). Merged view of A, B, and C (D). Scale bars: 100 μm. (E–H) Cytospun clusters collected from dorsal root ganglia explants were labeled for GS (green, E). The same cells also were labeled for nestin (red, F) and counterstained DAPI (blue, G). Merged view of E, F, and G (H). Scale bars: 50 μm. GS: Glutamine synthetase; P75NTR: p75 neurotrophin receptor; DAPI: 4′,6-diamidino-2-phenylindole.

(Duobles et al., 2008). Therefore, SGCs are strongly labeled with markers including GS, GFAP, and S100β (Fei et al., 2016). Nevertheless, no previous report has demonstrated GS-positive Schwann cells in DRG. Moreover, no positive expression of the Schwann cell-specific marker, SOX10, has previously been reported in cultured cells. Comparison of cell markers was performed before and after DRG-SGC separation. Before DRG-SGC isolation, immunohistochemical staining of DRG tissue sections revealed a group of cells with positive GS expression around neurons. Thus, the cultured cells were confirmed as SGCs.

To determine whether the cultured cells exhibit neural stem cell characteristics, we labeled them with markers for neural crest progenitors, namely nestin and P75[NTR] (Li and Zhou, 2008, Piñero et al., 2018). Surprisingly, the cells were positive for nestin, with some also weakly positive for

P75[NTR]. This shows that our cultured SGCs exhibit the characteristics of neural stem cells. Previous studies have shown that some DRG cells express nestin and P75[NTR], thereby exhibiting neural stem cell characteristics (Li et al., 2007; Tongtako et al., 2017). Despite this, the cell type in this study was not clear. In this study, we have identified the cells as SGCs with neural stem cell characteristics.

Recent developments in DRG-SGC culture systems and their applications

DRGs form the somatovisceral primary sensorium, which is rich in sensory neurons and widely used to observe and study cell death, synaptic growth, growth factor signaling pathways, and hyperalgesia. Following peripheral nerve injury, SGCs undergo changes in cell number, structure, and function (Liu et al., 2018). Peripheral nerve transection increases gap junctions and intercellular coupling of SGCs, and also decreases membrane resistance (Pannese et al., 2003). SGCs upregulate production of proinflammatory cytokines, such as tumor necrosis factor-α, for development and maintenance of neuropathic pain (Bai et al., 2016). Together, these examples illustrate the importance of SGC culture systems in understanding regulatory mechanisms.

Factors affecting survival and proliferation of SGCs *in vitro*

At present, the animals used for DRG cultures include chicken, cat, and adult rats, while human DRG are seldom used owing to medical ethics and limited resources. In our experiment, SGC cultures were derived from DRG of neonatal rats. The age of the experimental animals directly affected the culture results. Cultured ganglion from different ages can survive various culture conditions and methods. DRG from neonatal rats exhibit a high survival rate and faster cell migration than cells from adult rat cultured *in vitro*. During DRG isolation, care must be taken when removing tissue around the spinal cord. Indeed, isolation is difficult due to its location and small volume. Additionally, DRG sampling time is a key factor affecting the success of SGC cultures: a short sampling time guarantees activity of cultured cells, allowing for further experimental studies. Furthermore, it is important to fully remove the surrounding surface envelope and neurofilaments after isolating DRG. This is conducive for DRG growth and also a key step for removing fibroblasts and Schwann cells to obtain high-purity of SGCs. SGCs grew and attached to plastic Petri dishes. Appropriately, rapid adhesion to a growth matrix is a factor that affects *in vitro* survival of SGCs. Poly-D-lysine hydrobromide is a cationic polymer that is often used for attaching and immobilizing cells (Fang et al., 2012). In this study, poly-D-lysine hydrobromide (molecular weight > 300,000) was superior to poly-L-lycine (molecular weight < 300,000) for DRG-SGC-attached growth in plastic Petri dishes.

Purification and culture of SGCs

Obtaining a high-purity SGC culture system facilitates study of biological characteristics and functions of specific cells.

The DRG contains a rich number of neurons, Schwann cells, fibroblasts, and SGCs. Consequently, isolation of SGCs remains one of the difficulties in DRG isolation and culture (Li et al., 2007). There is no rapid and effective method for purifying SGCs. Therefore, to obtain pure and homogeneous SGCs during culture, the most direct method involves addition of glutamine to the medium to inhibit growth of other cells, such as neurons. Glutamine can induce death of mature neurons as a result of excitotoxicity, but has no effect on glial cells. In this study, serum-free medium combined with B27 additive inhibited division and proliferation of fibroblasts and Schwann cells and selectively promoted survival of SGCs, resulting in a SGC purification rate of approximately 95%. Adherence rate of DRG-SGCs depended on different culture media and time. Isolation and purification of SGCs can be achieved by the differential velocity adherent culture method and addition of a cytostatic compound (Owen and Egerton, 2012).

Conclusion

In this study, we have successfully established a novel SGC primary culture method that uses rat DRG without digestion. The resulting SGC purification rate (> 95%) is sufficient for a variety of cell tests. However, the SGC culture method has difficulties, with skill required in obtaining DRG to ensure purity of the cells obtained. Further, final acquisition of high-purity SGCs takes a long time, approximately 10 days. Nonetheless, DRG obtained by this method can be repeatedly cultured, with the cells of high-purity.

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Additional file: *Open peer review reports 1 and 2.*

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