

# Growth and Survival of Satellite Glial Cells in Different Culture Supplementations

**Na Wei**

Tangdu Hospital, Fourth Military Medical University

**Ya-Ping Liu**

Tangdu Hospital, Fourth Military Medical University

**Rui-Rui Wang**

Tangdu Hospital, Fourth Military Medical University

**Xiao-Liang Wang**

Tangdu Hospital, Fourth Military Medical University

**Yan Yang**

Tangdu Hospital, Fourth Military Medical University

**Ting He**

Tangdu Hospital, Fourth Military Medical University

**Huan Wang**

Tangdu Hospital, Fourth Military Medical University

**Yao-Qing Yu (✉ [yyq7803@163.com](mailto:yyq7803@163.com))**

Tangdu Hospital, Fourth Military Medical University

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## Research Article

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## **Growth and survival of satellite glial cells in different culture supplementations**

Na Wei<sup>1,3</sup>, Ya-Ping Liu<sup>1,3</sup>, Rui-Rui Wang<sup>1,3</sup>, Xiao-Liang Wang<sup>1</sup>, Yan Yang<sup>1</sup>, Ting He<sup>1</sup>,

Huan Wang<sup>2\*</sup>, Yao-Qing Yu<sup>1\*</sup>

<sup>1</sup>Institute for Biomedical Sciences of Pain, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710038, China

<sup>2</sup>Department of Dermatology, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, P. R. China

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<sup>3</sup>Na Wei, Ya-Ping Liu and Rui-Rui Wang contributed equally to this study.

\*Corresponding authors:

If accepted, the page proofs and reprint request should be addressed to:

Yao-Qing Yu, MD, PhD

Institute for Biomedical Sciences of Pain, Tangdu Hospital,

The Fourth Military Medical University,

569 Xinsi Road, Baqiao, Xi'an, 710038, PR China

E-mail: yyq7803@163.com

Or

Huan Wang, MD

Department of Dermatology, Tangdu Hospital,

The Fourth Military Medical University,

569 Xinsi Road, Baqiao, Xi'an, 710038, PR China

Email: pfkwhuan@163.com

**Keywords:** satellite glial cell; proliferation; apoptosis; cell culture; glutamine; glial fibrillary acidic protein, glutamine synthetase

**Abbreviations:**

Bax, Bcl-2 Associated protein X; DAPI, 4',6-Diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's Modified Eagle Medium; HG, High glucose; DRG, dorsal root ganglion; GS, glutamine synthetase; GFAP, glial fibrillary acidic protein; IB4, isolectin B4; NB, Neurobasal-A; NF200, neurofilament 200; PI, propidium iodide; SGC, satellite glial cell

## **Abstract**

**Background:** In dorsal root ganglion (DRG), satellite glial cells (SGCs) tightly surrounded neurons and modulated microenvironment and sensory transmission. However, the biological properties of primary SGCs in culture were not fully understood. In the present study, we provided a method to harvest abundant and high-purity SGCs from neonatal rats. Three supplementations containing Dulbecco's Modified Eagle Medium (DMEM)/F12, DMEM high glucose (HG) and Neurobasal-A (NB) were used to evaluate SGCs growth and survival in culture.

**Results:** CCK-8 proliferation assay showed the increased proliferation in DMEM/F12 and DMEM/HG, but not in NB medium. NB medium caused cell death indicated by Bax, AnnexinV and PI staining. Glutamine was the major nutrition source for SGCs in culture and its exogenous application improved the poor proliferation and severe cell death in NB medium. SGCs markers GS and GFAP were similar in three supplementations and intensively expressed in culture. Differently, GS but not GFAP was remarkable in the intact DRG under normal condition.

**Conclusions:** These results suggested that SGCs growth in culture depended on time and culture supplementation and DMEM/F12 medium was recommended to get high-purity SGCs. Glutamine was the major nutrition and the key molecule to maintain cell growth and survival in culture. Our study shed a new light on understanding the biological property and modulation of glial cells in the peripheral sensory ganglia.

## **Introduction**

In dorsal root ganglion (DRG), satellite glial cells (SGCs) were interconnected by gap junctions and tightly surrounded neurons [1,2]. SGCs modulated the neuronal microenvironment and sensory transmission [3,4]. Generally, SGCs in the peripheral nervous system were roughly equivalent in function to astrocytes in the central nervous system and contribute to pain [5]. It was widely reported that peripheral nerve injury and inflammation would cause SGCs changes in cell number and inflammatory mediators such as glutamate, adenosine triphosphate (ATP), gamma-amino-butyric acid (GABA), substance P, CGRP [6,7,8,9].

Till now, it was still a challenge to harvest high-purity SGCs. The primary DRG cell cultures mostly consisted of neurons, SGCs and a moderate number of macrophages [10,11,12]. Even in the neuronal culture, DRG neurons were contacted a coterie of adherent satellite cells not removed during the harvesting procedure and neurons without satellite cells were uncommon [13]. To purify glia in the primary culture, DMEM/F12 medium and cytosine arabinoside were used [14, 15]. Sometimes, cytosine arabinoside was added to remove dividing cells after getting single-cell suspension and glial cell-derived neurotrophic factor were followed to harvest SGCs [16]. Recently, a novel primary culture method for obtaining high-purity satellite glial cells migrated from DRG explants was reported [17]. In this method, glia started to migrate from DRGs in 3 days, formed clusters after 7 days, and was to be sufficient for subculture after 14 days. Although enzymatic digestion procedure was not necessary in the migration method, it really took a very long time (more than 14 days)

to get high-purity SGCs. The major concerns were how to trigger, control and promote the efficiency of SGCs migration from ganglia in the early stage. It also should be noted that SGCs might be undergoing phenotypic changes over time in culture [18]. Contrast to the well-established primary neuronal culture, it was still an interesting question on how to efficiently get high-purity SGCs from the peripheral sensory ganglia.

SGCs expressed various neurotransmitters and glial markers, such as glial fibrillary acidic protein (GFAP), glutamine synthetase (GS) and S100 [17,19]. GFAP expression was increased in SGCs after nerve injury, including nerve transection [20], partial nerve ligation [21], spared nerve injury and spinal nerve ligation (SNL) [22,23]. Local tissue inflammation and chemotherapeutic agent also caused SGCs activation [24,25]. GFAP was recently assessed to evaluate SGCs differentiation in cell culture condition [26]. Collectively, GFAP was widely known as a marker of SGCs activation [27]. GS could catalyze adenosine triphosphate-dependent amidation of glutamate to glutamine and was known as a better marker for SGCs [28]. Tongtako et al found that SGCs in DRG were immunopositive for GFAP in dogs and monkeys, whereas GFAP in murine SGCs was absence. GS represented a good SGC marker in murine and simian but not in canine. The authors further showed an intermediate glial cell population with phenotypical characteristics of oligodendrocytes and astrocytes in SGCs [26]. These results strongly suggested that the expressions of SGCs markers were changeable. There was very limited data to compare the expression of SGCs markers in vitro and in vivo.

In the present study, we provided a method to obtain high-purity SGCs culture from DRG of neonatal rats. We compared the biological properties of SGCs in DMEM/F12, DMEM/HG and NB supplementations and found that DMEM/F12 medium was valuable for SGC growth and survival. The poor proliferation and severe cell death in NB medium were improved by exogenous glutamine application. SGCs intensively expressed GFAP and GS in culture condition, whereas GS was a better SGC marker in the intact DRG under normal condition. Our study shed a new light on understanding the biological property and modulation of glial cells in peripheral sensory ganglia.

## **Materials and Methods**

### *Animals and reagents*

Sprague-Dawley albino rats were prepared from Laboratory Animal Center of Fourth Military Medical University (FMMU). The animals had access to water and food ad libitum, and were maintained at room temperature (22-26 °C) with a light/dark cycle of 12 h. The experimental procedures were approved by the Institutional Animal Care and Use Committee of FMMU. The number of animals used and their sufferings were minimized.

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich. Antibodies, reagents and Kits were listed in Table S1 for details.

### *Isolation and culture of satellite glial cells*

DRGs were enzymatically digested, dissociated as previously described [12]. Postnatal day 1-3 rats were killed by cervical dislocation and the spinal column was opened. Up to 10 DRGs along the vertebral column were removed under aseptic

conditions and put into Petri dishes filled with cold, oxygenated Dulbecco's modified Eagle's medium (DMEM) (HyClone). The epineurium and nerve roots were removed and the DRGs were transferred to 5 mg/ml collagenase type II (HyClone). The isolated DRGs were incubated with collagenase at 37°C for 20 min. Then DRGs were treated with 0.125% trypsin (HyClone) and 10 mg/ml DNase I (Roche) for 10 min at 37°C. The solution was spun at 1500 rpm for 5 min and enzyme was removed. After washing with fresh culture solution, pipetting ten times, DRGs were naturally precipitated for 5 minutes. Collect the supernatant, and centrifuged at 1500 rpm. Cells were resuspended with 10% fetal bovine serum and 1% penicillin/streptomycin and seeded in culture dish coated by 200 mg/ml Poly-L-Lysine (Gibco). After 30 min seeding, the upper single cell suspension of neurons and a small number of SGCs were gently removed. The culture medium was changed every 3 days for further experiments. The components of cell culture supplementations containing Dulbecco's Modified Eagle Medium (DMEM)/F12, DMEM/HG and Neurobasal-A (NB) were listed in Table 1.

#### *Immunocytochemistry and immunohistochemistry staining*

Immunocytochemistry was performed as previously described [29]. SGC cells attached to coverslips were fixed, permeabilized and blocked. Then coverslips were incubated with primary antibodies overnight at 4 °C. Then coverslips were incubated with the secondary antibodies for 2 h at room temperature. Immunohistochemistry staining DRG sections was performed as previously described [30,31]. The rats were anesthetized with i.p. pentobarbital (50 mg/kg) and fixed with 4% paraformaldehyde.

Tissues were dissected, postfixed for 8 h, and cryoprotected in 20% sucrose in PBS overnight at 4 °C. Transverse frozen sections (20 µm thick) were cut on CM1900 freezing microtome (Leica, Germany), incubated for 4 h in 0.05% Triton X-100 and 10% goat serum in phosphate buffered saline (PBS) at room temperature, followed by incubation with primary antibodies at 4 °C overnight with agitation. After three washes with PBS, the sections were incubated with secondary antibodies for 2 h at room temperature. In the present study, we used the following primary antibodies (see Table S1 for details): mouse anti-Neurofilament 200 (Sigma), rabbit-anti GS (GeneTex), rabbit-anti GFAP (Millipore). Secondary antibodies were Cy3-conjugated sheep anti-rabbit IgG (1:400, Santa Cruz), FITC-conjugated goat anti-rabbit IgG (Chemicon), FITC-conjugated bovine anti-mouse IgG (Santa Cruz), Alexa Fluor 350 donkey anti-mouse IgG (Invitrogen), Alexa Fluor 594 conjugated isolectin GS-IB4 (Thermo). 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) was used for cell counter staining. Photomicrographic images were obtained under a laser scan confocal fluorescent microscope (Olympus FV1000, Japan). Cells were counted by Image-Pro Plus digitizing software (Olympus, Japan) based on the optical density and the size of the object and only that were clearly positive under the microscope were analyzed.

#### *Cell proliferation assay*

The proliferation rate of SGCs supplemented with DMEM/F12, DMEM/HG, and NB was examined by CCK-8 as previously report [32]. Briefly, SGCs were seeded in to 96-well culture plate (Corning) and maintained for different time points including 1, 2,

3, 5, 8, 10 days. Cell counting Kit-8 (CCK-8) solution (10 ml per well) was added at 37 °C for 1 h. Then, the optical density (OD) was measured at 450 nm wavelength using a microplate reader. Additionally, SGCs were imaged and counted by live cell imaging system (Cell-R, Olympus, Japan).

#### *Cell death assay*

In the present study, to test the death level of SGCs, cell death promotor Bax was detected by immunocytochemistry staining of Bax antibody (Santa Cruz). The method was performed as mentioned above. SGCs were seeded in a 24 well plate and cultured in DMEM/F12, DMEM/HG and NB for 8 days. DAPI nuclear counterstain was performed to reveal the percentage of Bax-positive cells.

#### *Cell apoptosis assay*

Apoptosis was measured by Annexin V-PI Apoptosis Detection Kit (BestBio) according to the manufacturer's instructions. Briefly, SGCs were incubated with distinct culture supplementations for 8 days. Cells were then incubated with Annexin V and propidium iodide (PI) buffer for 15 min at room temperature. Apoptotic cells were observed after staining with annexin V-FITC and PI and further imaged by fluorescence microscopy (Olympus). The percentages of Annexin V- and PI-positive cells were measured for quantitation.

#### *Statistical analysis*

All data were expressed as means  $\pm$  SEM. Statistical analyses were performed using Prism 6 (v6.0e, GraphPad). For comparison between two groups, unpaired two-tailed t test was used. For the comparison between three or more groups, one-way ANOVA

with Tukey post hoc analysis was performed. Normality and equal variance tests were performed for all statistical analyses.  $P < 0.05$  was considered statistically significant.

## **Results**

### **SGCs growth depended on time and culture supplementation and NB medium impaired cell proliferation**

To obtain high-purify satellite glial cells (SGCs), following procedures were performed (Fig. S1). Neonatal rats (P1-P3) were euthanized by cervical dislocation and DRGs along the vertebral column were removed under aseptic conditions. DRG were separated from nerve fibers, connective tissue, and capsule membranes in cell culture solution. Single cell suspension was enzymatically prepared with 5 mg/ml collagenase II for 20 min. Then 0.125% trypsin and 10 mg/ml DNase I were applied for 10 min. Individual cells containing SGCs were plated in culture dishes. After 30 min natural precipitate, the upper single cell suspension was gently removed to exclude the majority of DRG neurons. SGCs were further cultured in the different supplementations for experiments.

To test the effect of culture medium on the growth of SGCs, we used three supplementations containing DMEM/F12, DMEM/HG and Neurobasal-A (NB). Firstly, we counted the number of SGCs under 100 times magnification. In DMEM/F12, the number of SGCs gradually increased with the prolonged culture time from 4 days to 8 days. Differently, the numbers of SGCs were not significantly changed in DMEM/HG and NB at 2 and 4 days (Fig. 1a). These results suggested that SGCs growth correlated with cell culture supplementation. Then, SGCs proliferation

was evaluated by CCK-8 assay at a series of time points at 1, 2, 3, 5, 8 and 10 days after seeding. In DMEM/F12, optical density (OD) value dramatically increased after 5 days ( $0.3\pm 0.01$ , n=5 reduplicates) and peaked at 10 days ( $0.9\pm 0.04$ , n=5 reduplicates). DMEM/HG increased OD value at 10 days after seeding ( $0.6\pm 0.07$ , n=5 reduplicates). However, OD value was not changed by NB medium even at 10 days after seeding ( $0.1\pm 0.08$ , n=5 reduplicates) (Fig. 1b). We further observed the morphological characteristics of primary SGCs culture in DMEM/F12 medium under phase-contrast micrographs at 1, 2, 3, 5, 8 and 10 days. Except for the gradually increased number of cells, SGCs exhibited remarkable morphological changes from the early elliptical cells with full bodies to the late dipolar cells with protrusion (Fig. 1c). These results showed that SGCs growth depended on time and culture media. DMEM/F12 was highly recommended to get abundant SGCs in several days, whereas NB medium impaired SGCs proliferation in culture.

### **SGC markers were not affected by the different culture supplementations**

Then we assessed the effects of different supplementations on SGCs markers by immunofluorescent staining at 10 days. Both GFAP and GS were intensively expressed in the majority of SGCs in the different culture supplementations (Fig. 2a). Statistically, the percentages of GFAP- and GS-positive cells in DMEM/F12 were  $99.7\pm 0.05\%$  and  $99.7\pm 0.12\%$ , respectively (Fig. 2b, n=5,  $p>0.05$ ). The percentages of GFAP- and GS-positive cells in DMEM/HG were  $98.6\pm 0.34\%$  and  $98.6\pm 0.49\%$ , respectively (Fig. 2c, n=5,  $p>0.05$ ). The percentage of GFAP and GS-positive cells in NB were  $84.4\pm 4.58\%$  and  $86.6\pm 6.67\%$ , respectively (Fig. 2d, n=4,  $p>0.05$ ).

Expressions of SGCs markers in the majority of cells supported that our protocol was valuable to obtain high-purity SGCs. Additionally, our results suggested that SGC markers GFAP and GS were not significantly affected by culture supplementations.

### **NB medium caused severe cell death and apoptosis in SGCs**

To characterize the influence of culture medium on SGC survival, cell death promotor Bax (Bcl-2 Associated X protein) was detected at 8 days. In DMEM/F12 and DMEM/HG medium, Bax expressions were rarely observed. In contrast, the majority of SGCs in NB medium were Bax-positive (Fig. 3a). Statistically, the percentage of Bax-positive cells was  $4.0\pm 1.17\%$ ,  $3.4\pm 1.11\%$ ,  $65.9\pm 12.69\%$  in DMEM/F12, DMEM/HG and NB, respectively (Fig. 3b,  $n=7-10$ ,  $p<0.001$ ). Bax antibody staining results indicated that NB medium caused more significant cell death than DMEM/F12 and DMEM/HG.

Apoptosis or programmed cell death produced a series of changes in morphological and biochemical properties. We further assessed the effect of culture supplementation on SGCs apoptosis. The early apoptosis marker Annexin V and late apoptosis marker propidium iodide (PI) were detected at 8 days (Fig. 4a). Statistically, the percentage of Annexin V-positive cells was  $5.8\pm 0.78\%$ ,  $14.5\pm 1.93\%$ ,  $71.0\pm 5.80\%$  in DMEM/F12, DMEM/HG and NB, respectively (Fig. 4b,  $n=6-8$ ,  $p<0.001$ ). The percentage of PI-positive cells was  $1.4\pm 0.31\%$ ,  $7.4\pm 2.07\%$ ,  $22.6\pm 3.84\%$  in DMEM/F12, DMEM/HG and NB, respectively (Fig. 4c,  $n=6-8$ ,  $p<0.05$  or  $p<0.001$ ). These results confirmed that NB supplementation caused the expressions of apoptosis markers and triggered cell death. Interestingly, Annexin V, PI and Bax could be shown in different

SGCs, further supporting the different stages of cell death (Fig. S2). Collectively, these results suggested that NB medium caused severe cell death in culture condition.

### **Glutamine was the major nutrition source for SGCs in culture and its exogenous application improved cell growth and survival**

To reveal the key regulator determining SGCs growth and survival in culture, we further checked the components of culture medium (Table 1). DMEM/F12, DMEM/HG and NB supplementations shared the similar components in majority of compounds and chemicals, including the nutrition source glucose (17-25 mM). Interestingly, we found that the other major nutrition source L-glutamine was over 2.5 mM in DMEM/F12 and DMEM/FHG, whereas it was absence in NB medium.

GlutaMAX (L-alanyl-L-glutamine) was a dipeptide substitute for L-glutamine, maintained a fresh supply of L-glutamine during long-term culture and could not spontaneously break down to form ammonia. Therefore, we firstly used exogenous GlutaMAX into NB medium to test the roles of glutamine on SGCs growth (Fig. 5a). At 6 days, the number of SGCs in control, 0.025, 0.25, 2.5 mM GlutaMAX was  $63\pm 3.77$ ,  $73\pm 4.92$ ,  $212\pm 218.90$  and  $396\pm 30.98$ , respectively. At 8 days, the number of SGCs in the four groups were  $47\pm 2.42$ ,  $52\pm 2.75$ ,  $394\pm 9.87$  and  $665\pm 18.18$ , respectively (Fig. 5b,  $n=6$ ,  $p<0.001$ ). These data demonstrated that exogenous glutamine application in NB medium increased SGCs proliferation in a dose-related manner.

We further used exogenous GlutaMAX into NB medium to test the roles of glutamine on SGCs apoptosis (Fig. 6a). In the four groups of control, 0.025, 0.25 and

2.5 mM GlutaMAX, the percentage of Annexin-positive cells was  $70.0\pm 4.79\%$ ,  $32.7\pm 3.03\%$ ,  $27.1\pm 3.10\%$  and  $32.0\pm 2.00\%$ , respectively. The percentage of PI-positive cells was  $22.7\pm 2.99\%$ ,  $11.4\pm 3.21\%$ ,  $3.5\pm 0.77\%$  and  $5.6\pm 1.31\%$ , respectively (Fig. 6b,  $n=12$ ,  $p<0.001$ ). Collectively, these results indicated that glutamine, but not glucose, was the major nutrition source in SGCs culture and its deprivation would cause poor proliferation and severe cell death. Exogenous glutamine application improved the SGCs growth and survival.

### **GS, but not GFAP, was good SGC marker in vivo and in vitro under normal condition**

Finally, we observed the expression profiles of SGC markers in the intact DRG (Fig. S3). Immunofluorescent labeling showed that GS was detectable whereas GFAP was nearly undetectable in normal DRG at postnatal day 2 (P2). Similarly, in normal intact DRG of adult rats (P90), GS was widely distributed whereas GFAP was in very low level. Further immunostaining results showed that GS-positive SGCs tightly surrounded medium- and large-sized DRG neurons indicated by neurofilament 200 (NF200). Differently, isolectin B4 (IB4) positive small-sized DRG neurons were not directly connected with SGC. These results indicated that GFAP were differently expressed in cell culture and intact DRG. Furthermore, SGCs tightly surrounded medium- and large-sized DRG neurons under normal condition.

### **Discussion**

In the present study, three cell culture supplementations with DMEM/F12, DMEM/HG and NB were evaluated for SGCs growth and survival. We found that

DMEM/F12 promoted SGCs growth whereas NB medium caused cell death indicated by Bax, AnnexinV and PI staining. As the major nutrition source for SGCs in culture, glutamine exogenous application improved the poor proliferation and severe cell death in NB medium. Our findings indicated that DMEM/F12 medium was recommended to get high-purity SGCs and glutamine will inhibit cell death and is the key molecule to maintain cell growth and survival in culture.

In the present study, we found the difference on the expression profiles of SGCs marker GFAP in culture condition and in the intact DRG. GFAP- and GS-positive SGCs were over 99%, 98% and 84% in DMEM/F12, high glucose and NB medium. In the intact DRGs (P2 and P90), GFAP expression was nearly undetectable and GS-positive SGCs were tightly surrounded medium- and large-sized DRG neurons. Our observations were consistent with the previous reports on the low expression of GFAP in normal DRGs [23,33,34]. GFAP was widely known as a marker of SGCs activation [27] and the strong GFAP staining in normal SGCs only reported in 4-month-old rodent [35, 36]. The reason for the different expression profiles of GFAP in vitro and in vivo remains unknown. SGCs markers might be altered under in vitro condition of long term cultures. Tongtako et al reported that GFAP could be used to evaluate SGCs differentiation in the culture. Astrocytic differentiation medium increased the percentage of GFAP+ cells, whereas oligodendrocytic differentiation medium (B104-conditioned DMEM with RA) reduced GFAP expressions compared to the control medium [26]. It was also reported that the expression of GS decreased while purinergic receptor P2X7 was maintained over time in culture, indicating that

SGCs might be undergoing phenotypic changes [18]. In our study, we found that GFAP and GS were similar in DMEM/F12, DMEM/HG or NB supplementation. These results suggested that the expressions of SGC markers GFAP and GS were not significantly affected by culture supplementations. Nevertheless, it was still an interesting to investigate the phenotype switch of SGC markers, if true, in long-term culture condition and even in vivo.

The balance of cell death promotor Bax and repressor Bcl-2 contributed to cell survival [37,38]. In the peripheral sensory system, cell death was widely investigated in neurons and multiple chemicals could trigger the process. For example, cisplatin initiated the mitochondrial stress pathway in DRG neurons and NGF blocked death upstream of Bax activation [39]. Halothane increased neuronal cell death vulnerability by downregulating miR-214 and upregulating Bax [40]. Tunicamycin induced apoptosis through caspase activation and mitochondrial dysfunction in DRG neurons [41]. It was shown that Bax was required for neuronal apoptosis and permitted DRG neurons to survive in the absence of neurotrophin signaling. Bax knockout rescued programmed cell death and produced a 50% increase in the number DRG neurons [42,43,44]. Apoptosis or programmed cell death involved in a series of changes in morphological and biochemical properties. Annexin V, a 36-kDa calcium-binding protein associated with phosphatidylserine, was detectable in apoptotic cells and necrotic cells. Propidium iodide (PI) does not stain live or early apoptotic cells due to the presence of an intact plasma membrane. In late apoptotic and necrotic cells, the integrity of the plasma and nuclear membranes decreases and PI passed through the

membranes, intercalated into nucleic acids, and displayed red fluorescence. Therefore, the Annexin V/PI approach indicated the different stages of cell death [45,46].

To our knowledge, the mechanism of SGCs death was quite limited. Using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), it was shown that sciatic nerve injury induced apoptosis in SGCs, but not DRG neurons 30 days after injury [47]. NO-cGMP pathway also involved in cell death in DRG and blockade of NO or the cGMP synthesizing enzyme soluble guanylate cyclase, could result in apoptosis of neurons and Schwann cells [48]. Recently, it was shown that the number of caspase-3+ SGCs in culture was significantly reduced by fibroblast growth factor 2 (FGF-2), epidermal growth factor, (EGF), ciliary neurotrophic factor (CNTF), heregulin 1 $\beta$  (HRG), or forskolin (Fors) supplementation [26]. These results suggested that multiple regulators contribute to the process of SGC apoptosis. In the present study, SGCs survival was evaluated by apoptosis makers including Bax, AnnexinV and propidium iodide (PI) in the different culture supplementations. We found that NB medium caused the dramatic increase in these apoptosis makers. Interestingly, Bax, AnnexinV and PI could be detected in different cells, suggesting the different stages of SGCs apoptosis in culture.

Bax-related apoptosis prevention was verified in some reports on neuronal rescue strategies. For example, Isoquercetin ameliorated tunicamycin-induced apoptosis in rat DRG neurons via suppressing ROS-dependent endoplasmic reticulum stress [41]. Electro-acupuncture treatment resulted in a significant downregulation of Bax protein and its mRNA, but an upregulation of Bcl-2 after SCI [49]. Liu et al showed that

Electro-acupuncture-modulated miR-214 prevented neuronal apoptosis by targeting Bax in rats after spinal cord injury [50]. In the present study, we firstly reported that exogenous glutamine improved the poor proliferation, reversed Bax, AnnexinV and PI expressions, and rescued the severe cell death in SGCs culture. Therefore, glutamine might become an attractive molecule to regulate Bax-related growth and survival in SGCs.

Glutamine and glucose are two major nutrition sources contributed to multiple biosynthetic pathways and cellular functions. Glutamine metabolism involved a glucose-independent tricarboxylic acid (TCA) cycle and supported cell survival and proliferation under hypoxia and glucose deficiency in cancer [51,52]. The entry of glutamine, after its conversion to glutamate and then to  $\alpha$ -ketoglutarate, into the TCA and oxidation to succinate, fumarate, and malate were highly facilitated by MYC expression [53]. Glutamine also played important roles in immune system as an essential nutrient for lymphocyte proliferation and cytokine production, macrophage phagocytic plus secretory activities, and neutrophil bacterial killing [54,55]. Today, glutamine's metabolic and non-metabolic functions in cancer cell were recognized<sup>51</sup>. In the present study, we found the poor proliferation and severe SGCs death in NB medium without glutamine. We also showed the protective effects of glutamine, but not glucose, on SGCs growth and survival. Our study supported that glutamine, but not glucose, was the key nutrition source for SGCs in culture. Glutamine was a multifaceted amino acid and promoted protein synthesis not only by delivering substrate and energy but also by stimulating transcription of genes in the promoter

region [56]. It will be interesting to reveal the glutamine-activated signaling pathway in SGCs growth and survival in future.

## **Conclusions**

In summary, we demonstrated that SGCs growth in culture depended on time and culture supplementation and DMEM/F12 medium was recommended to get high-purity SGCs. Glutamine was the major nutrition source and its exogenous application improved growth and survival of SGCs in culture. The high-purity SGCs will be valuable to investigate the function and modulation of glia in the peripheral sensory nervous system.

**Conflict of interest**

The authors declare no competing financial interests.

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Not applicable.

**Authors' contributions**

Author YQY conceived and designed the work. NW, YPL and RRW conducted experiments, XLW and YY managed cell culture, HW and TH performed data analysis, YQY and HW wrote the manuscript. All authors have read and approved the manuscript.

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**Availability of data and materials**

All data generated or analysed during this study are included in this published article.

**Declarations****Ethics approval and consent to participate**

All animal experiments were performed in accordance with ARRIVE guidelines and approved by the Institutional Animal Care and Use Committee of FMMU. The number of animals used and their sufferings were minimized.

**Consent for publication**

Not applicable.

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## Figure Legends

**FIGURE 1.** The effect of cell culture supplementation on SGCs growth. **a** Statistic analysis of the numbers of SGCs supplemented with DMEM/F12, DMEM/HG, and NB in the different days. **b** CCK-8 assay on the proliferation of SGCs supplemented with DMEM/F12, DMEM/HG, and NB in the different days. **b** Morphological characteristics of SGCs supplemented with DMEM/F12 were demonstrated under phase-contrast micrograph at the different days. With the prolonged culture time, SGC increased in the number of cells and exhibit marked morphological changes from the early elliptical cells with full bodies to the late dipolar cells with protrusion. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu\text{m}$ . Data are means  $\pm$  SEM.

**FIGURE 2.** The effect of cell culture supplementation on SGCs markers. **a** Immunofluorescence of SGC markers GFAP (green) and GS (red) in the different supplementations. DAPI (blue) was the nuclear counterstain. **b** Statistic analysis of the percentage of GS and GFAP-positive cells in DMEM/F12 medium. **c** Statistic analysis of the percentage of GS- and GFAP-positive cells in DMEM/HG medium. **d** Statistic analysis of the percentage of GS- and GFAP-positive cells in NB supplement. Scale bars, 50  $\mu\text{m}$ . Data are means  $\pm$  SEM.

**FIGURE 3.** The effect of cell culture supplementation on SGCs death. **a** Bax (red) antibody staining of SGCs in DMEM/F12, DMEM/HG, and NB in 8 days after cell culture. DAPI (blue) was the nuclear counterstain. **b** Statistic analysis of the percentage of Bax-positive SGCs in the different culture supplementations. \*\*\*,  $P <$

0.001. Scale bars, 50  $\mu$ m. Data are means  $\pm$  SEM.

**FIGURE 4.** The effect of cell culture supplementation on SGCs apoptosis. **a** Early stage of apoptosis marker Annexin V (green) and late stage of apoptosis marker propidium iodide (PI, red) staining in SGCs in DMEM/F12, DMEM/HG, and NB at 8 days after cell culture. **b** Statistic analysis of the percentage Annexin V-positive SGCs in the different culture supplementations. **c** Statistic analysis of the percentage PI-positive SGCs in the different culture supplementations. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu$ m. Data are means  $\pm$  SEM.

**FIGURE 5.** The effects of exogenous glutamine on SGCs growth in NB supplementation. **a** The growth of SGCs in NB medium with the different concentration of GlutaMAX (0.025, 0.25, and 2.5 mM). **b** Statistic analysis of the numbers of SGCs supplemented with the different concentration of GlutaMAX. \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu$ m. Data are means  $\pm$  SEM.

**FIGURE 6.** The effects of exogenous GlutaMAX on SGCs apoptosis in NB supplementation. **a** Expressions of Annexin V (green) and PI (red) in SGCs supplemented with NB medium and the different concentration of GlutaMAX (0.025, 0.25, and 2.5 mM) at 8 days. **b** Statistic analysis of the percentage Annexin V-positive SGCs in the different concentration of GlutaMAX. **c** Statistic analysis of the percentage PI-positive SGCs in the different concentration of GlutaMAX. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu$ m. Data are means  $\pm$  SEM.

**FIGURE S1.** Procedures for satellite glial cell (SGC) isolation and culture from dorsal root ganglia (DRG). Neonatal rats (P1-P3) were euthanized by cervical dislocation, laminectomy was performed to harvest lumbar and thoracic DRGs. DRGs were separated from nerve fibers, connective tissue, and capsule membranes. Single cell suspension were enzymatically prepared with collagenase II, trypsin and DNase I and triturated by pipette. Individual cells containing SGC were plated in dish and the upper single cell suspension was removed to exclude the majority of neurons and a small number of SGCs. The different supplementations were used for culture and experiment.

**FIGURE S2.** Triple staining of Annexin V, PI, and Bax in the different cell culture supplementations. Annexin V-FITC (green), PI (red), and Bax (blue) immunostaining of SGCs in DMEM/F12, DMEM/HG, and NB at 8 days in culture. Note the early, late and final stages of cell death in the different SGCs. Scale bars, 50  $\mu$ m.

**FIGURE S3.** Expression profiles of SGC markers in intact DRG. Immunofluorescent labeling showed that GS (red) was detectable whereas GFAP (green) was undetectable in normal DRG of mice at postnatal day 2 (P2). Similarly, GS (red) was widely distributed whereas GFAP (green) was in low level in normal DRG of adult mice (P90). In normal condition, GS-positive SGCs (green) tightly surrounded medium- and large-sized neurons indicated by NF200 (red). Differently, IB4 positive small-sized DRG neurons (red) were not directly connected with SGCs. DAPI (blue) was the nuclear counterstain. Scale bars, 50  $\mu$ m.

# Figures

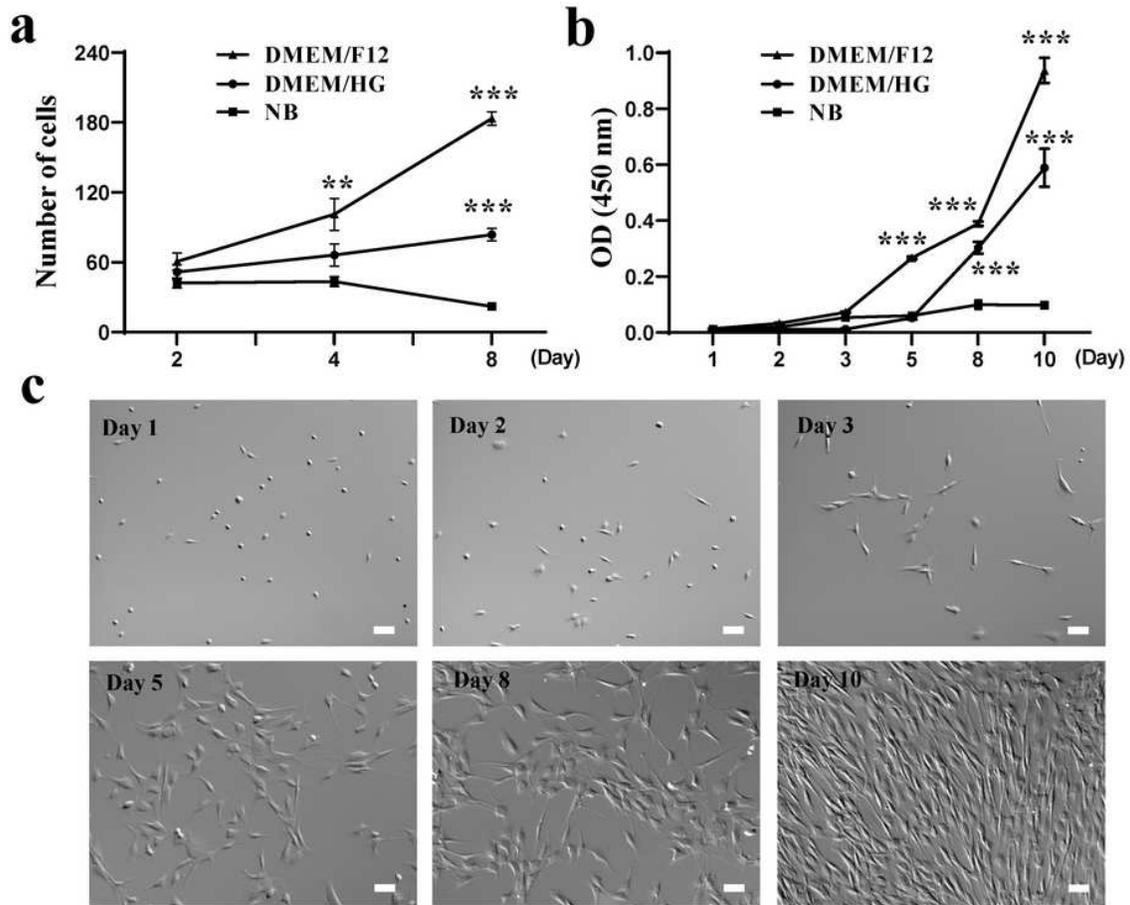


Figure 1

## Figure 1

The effect of cell culture supplementation on SGCs growth. a Statistic analysis of the numbers of SGCs supplemented with DMEM/F12, DMEM/HG, and NB in the different days. b CCK-8 assay on the proliferation of SGCs supplemented with DMEM/F12, DMEM/HG, and NB in the different days. b Morphological characteristics of SGCs supplemented with DMEM/F12 were demonstrated under phase-contrast micrograph at the different days. With the prolonged culture time, SGC increased in the number of cells and exhibit marked morphological changes from the early elliptical cells with full bodies to the late dipolar cells with protrusion. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu\text{m}$ . Data are means  $\pm$  SEM.

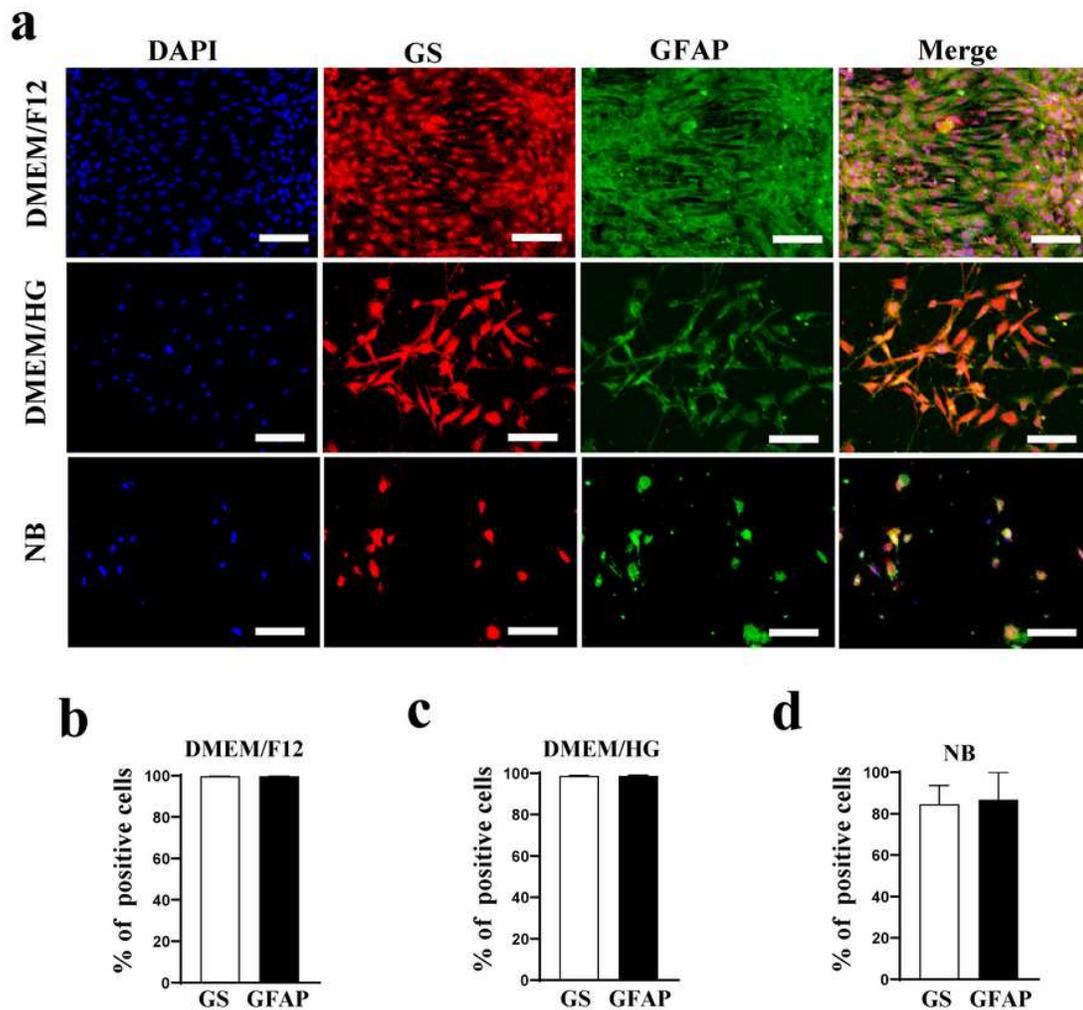


Figure 2

## Figure 2

The effect of cell culture supplementation on SGCs markers. a Immunofluorescence of SGC markers GFAP (green) and GS (red) in the different supplementations. DAPI (blue) was the nuclear counterstain. b Statistic analysis of the percentage of GS and GFAP-positive cells in DMEM/F12 medium. c Statistic analysis of the percentage of GS- and GFAP-positive cells in DMEM/HG medium. d Statistic analysis of the percentage of GS- and GFAP-positive cells in NB supplement. Scale bars, 50  $\mu$ m. Data are means  $\pm$  SEM.

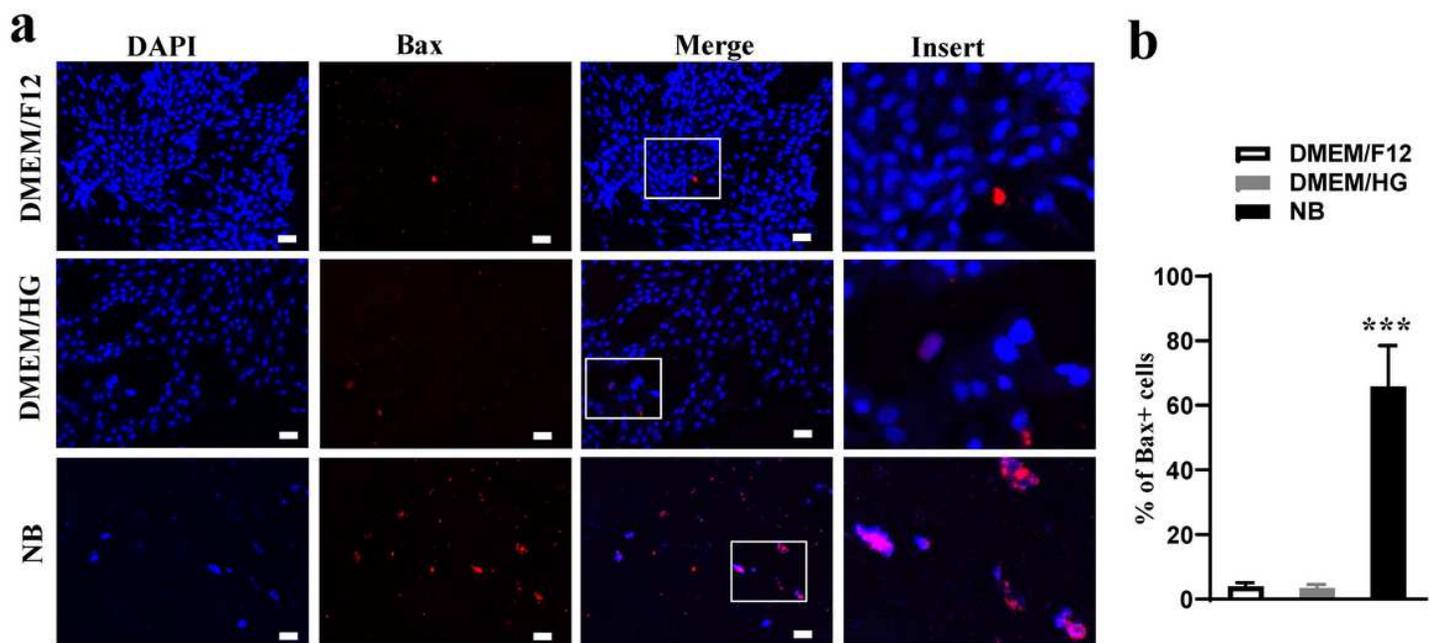


Figure 3

### Figure 3

The effect of cell culture supplementation on SGCs death. a Bax (red) antibody staining of SGCs in DMEM/F12, DMEM/HG, and NB in 8 days after cell culture. DAPI (blue) was the nuclear counterstain. b Statistic analysis of the percentage of Bax-positive SGCs in the different culture supplementations. \*\*\*,  $P < 33$  0.001. Scale bars, 50  $\mu$ m. Data are means  $\pm$  SEM.

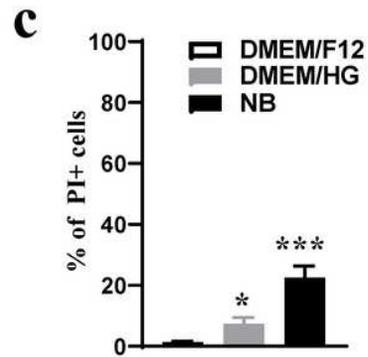
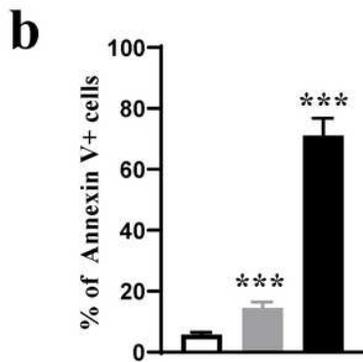
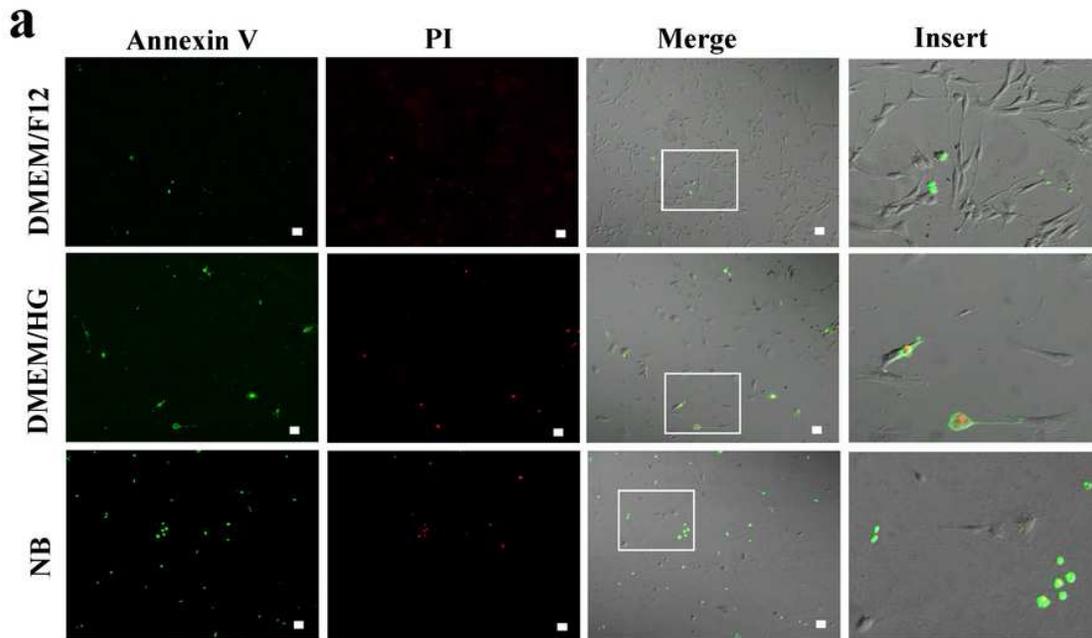


Figure 4

## Figure 4

The effect of cell culture supplementation on SGCs apoptosis. a Early stage of apoptosis marker Annexin V (green) and late stage of apoptosis marker propidium iodide (PI, red) staining in SGCs in DMEM/F12, DMEM/HG, and NB at 8 days after cell culture. b Statistic analysis of the percentage Annexin V-positive SGCs in the different culture supplementations. c Statistic analysis of the percentage PI-positive SGCs in the different culture supplementations. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu\text{m}$ . Data are means  $\pm$  SEM.

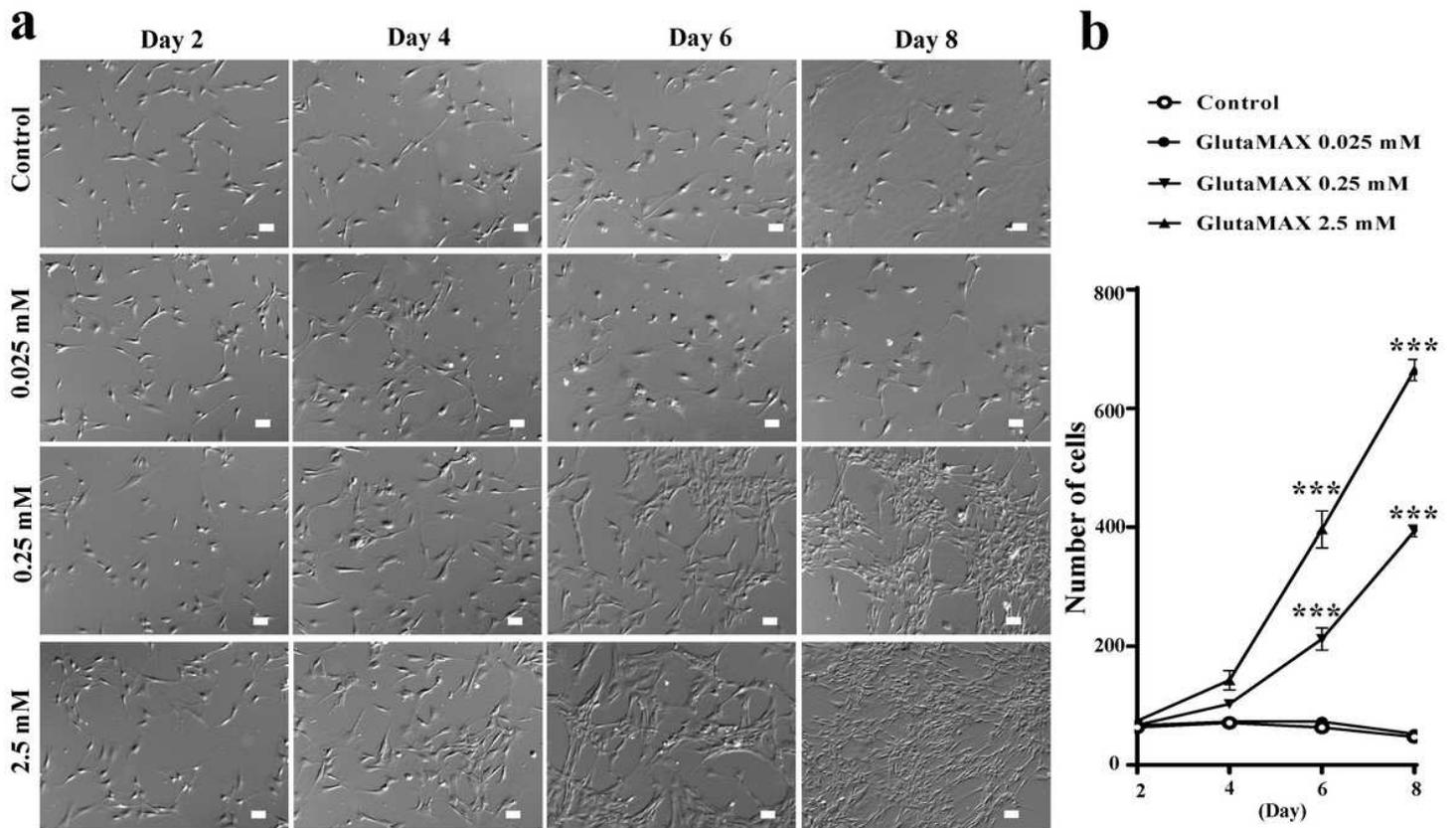


Figure 5

### Figure 5

The effects of exogenous glutamine on SGCs growth in NB supplementation. a The growth of SGCs in NB medium with the different concentration of GlutaMAX (0.025, 0.25, and 2.5 mM). b Statistic analysis of the numbers of SGCs supplemented with the different concentration of GlutaMAX. \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu$ m. Data are means  $\pm$  SEM.

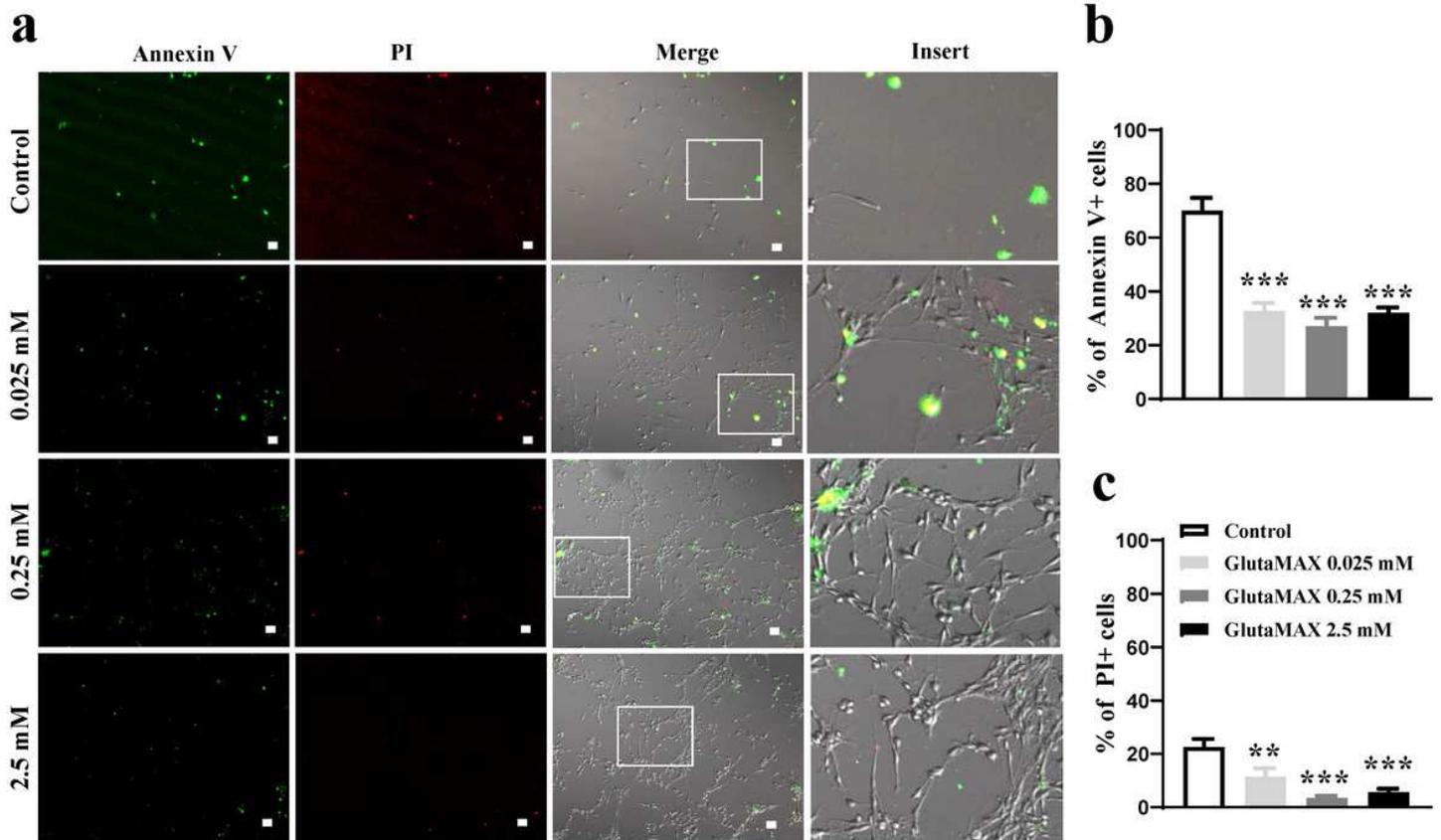


Figure 6

## Figure 6

The effects of exogenous GlutaMAX on SGCs apoptosis in NB supplementation. a Expressions of Annexin V (green) and PI (red) in SGCs supplemented with NB medium and the different concentration of GlutaMAX (0.025, 0.25, and 2.5 mM) at 8 days. b Statistic analysis of the percentage Annexin V-positive SGCs in the different concentration of GlutaMAX. c Statistic analysis of the percentage PI-positive SGCs in the different concentration of GlutaMAX. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu$ m. Data are means  $\pm$  SEM.

## Supplementary Files

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