Isolation and Characterization of Neural Crest Progenitors from Adult Dorsal Root Ganglia

Hong-Yun Li,a,b Evonne Hwee Min Say,a Xin-Fu Zhoua

aDepartment of Human Physiology, Flinders University, Adelaide, South Australia, Australia; bDepartment of CNS Trauma and Rehabilitation, Research Institute of Surgery, Daping Hospital, Chongqing, People’s Republic of China

Key Words: Neural crest progenitors • Dorsal root ganglia • Neurogenesis • Explant culture • Satellite glial cells

ABSTRACT

After peripheral nerve injury, the number of sensory neurons in the adult dorsal root ganglia (DRG) is initially reduced but recovers to a normal level several months later. The mechanisms underlying the neuronal recovery after injury are not clear. Here, we showed that in the DRG explant culture, a subpopulation of cells that emigrated out from adult rat DRG expressed nestin and p75 neurotrophin receptor and formed clusters and spheres. They differentiated into neurons, glia, and smooth muscle cells in the presence or absence of serum and formed secondary and tertiary neurospheres in cloning assays. Molecular expression analysis demonstrated the characteristics of neural crest progenitors and their potential for neuronal differentiation by expressing a set of well-defined genes related to adult stem cells niches and neuronal fate decision. Under the influence of neurotrophic factors, some of these progenitors gave rise to neuroepitide-expressing cells and protein zero-expressing Schwann cells. In a 5-bromo-2’-deoxyuridine chasing study, we showed that these progenitors likely originate from satellite glial cells. Our study suggests that a subpopulation of glia in adult DRG is likely to be progenitors for neurons and glia and may play a role in neurogenesis after nerve injury.

STEM CELLS 2007;25:2053–2065

INTRODUCTION

NSC exist in the central nervous system (CNS) in adult mammals and are important in neurogenesis and plasticity in physiological and pathological conditions [1, 2]. Significant progress has also been made recently in the identification of neurogenic cells from various adult non-neural peripheral tissues such as gut [3], skin [4, 5], connective tissue [6], and even adult heart [7]. Although neural stem/progenitor cells have been isolated from postnatal dorsal root ganglia (DRG) [8], adult enteric nervous system [3, 9, 10], and adult otic placode-derived spiral ganglion [11], their in vivo cell identity has not been characterized. Whether adult DRG, like the brain, contain an NSC niche and have the potential for neurogenesis in mammals is an intriguing question. The resolution of this question is important not only for understanding neurogenesis in the peripheral nerve system (PNS) but also for harnessing the potential application of peripheral NSCs for the treatment of diseases in both CNS and PNS.

A subpopulation of sensory neurons in the DRG undergoes apoptosis after peripheral nerve injury in the adult, resulting in a loss of 20%–30% of DRG neurons in the first 2–3 months [12]. However, the total number of neurons recovers to normal levels several months later [13–16]. Despite several investigations into neurogenesis in the adult mammalian DRG, the result has been equivocal. Using stereological and profile-counting methodologies, several studies failed to show any evidence of neuron addition in adults [17–20]. Other groups, however, suggested the possibility of neurogenesis in the adult DRG, as the number of neurons in adult rats was remarkably higher than those in neonates [21–24]. Rigorous searches, however, failed to identify NSCs in the DRG [13, 24]. Critical evidence supporting for the neurogenesis hypothesis in adult DRG requires isolation and characterization of neural stem cells. We hypothesize that adult DRGs contain neural progenitors that may be involved in the neurogenesis of adult DRGs. In the present study, we used adult rat DRGs as a model to test our hypothesis. We have demonstrated, by in vitro explant culture, which progenitor cells exist in the adult DRG and that these progenitors may originate from glial cells in vivo.

MATERIALS AND METHODS

Animals and Reagents

Male and female Sprague-Dawley rats, 8–12 weeks old, were used. All procedures were approved by Animal Welfare Committee of Flinders University. Unless otherwise specified, all reagents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com).

Explant Culture

Animals were euthanized with an overdose of halothane before perfusion through the heart with cold saline to flush the blood cells from the system before tissue dissection at ambient temperature (20–25°C). Isolated DRGs were pooled in a Petri dish containing D-Hanks’ solutions (Gibco, Grand Island, NY, http://www.invitrogen.com) on ice. DRGs were cleaned of nerve fibers, connective tissues, and capsule membranes; rinsed; and transferred to a

Correspondence: Xin-Fu Zhou, Ph.D., Department of Human Physiology, Flinders University, GPO Box 2100, Adelaide 5001, South Australia, Australia. Telephone: 61-8-8204-5814; Fax: 61-8-8204-5768; e-mail: xin-fu.zhou@flinders.edu.au  Received February 9, 2007; accepted for publication May 11, 2007; first published online in STEM CELLS EXPRESS May 24, 2007. ©AlphaMed Press 1066-5099/2007/
$30.00/0 doi: 10.1634/stemcells.2007-0080

A limited-dilution method with 96-well plates was used for the subcloning assay. The single-cell suspension derived from one primary/secondary sphere in the cloning medium was inoculated in 96-well plate (0–1 cell per 100 μl well). Fresh cloning medium was added to the wells with cells (100 μl per well) 7 days after initial seeding. Two weeks later, the number of wells with clusters or spheres was counted. The primary sphere was obtained from explant culture. The spheres resulting from the primary sphere subcloning were called the secondary spheres; similarly, the tertiary spheres (clusters) were defined by the spheres (clusters) formed from subcloning of the hand-picked secondary spheres. The number of clusters and spheres was counted, and the cloning efficiency, as a percentage (% of total cells), was calculated against the total number of live cells initially seeded.

*significant difference between the number of the secondary clusters and the number of the tertiary clusters (p < .05, X² test).

**Table 1.** Subcloning efficiency of adult dorsal root ganglia-derived spheres

<table>
<thead>
<tr>
<th>Clusters (%)</th>
<th>Primary → secondary</th>
<th>Secondary → tertiary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spheres (%)</td>
<td>138 (26.9)</td>
<td>188 (39.6)*</td>
</tr>
</tbody>
</table>

A 2-hour incubation with species-specific and/or isotype-specific secondary antibodies was performed first to visualize intracellular antigens in cell samples and in all of the tissue sections, respectively. The immunocytochemical procedures was validated by omitting the primary antibodies or by using nonimmune serum instead of the primary antibodies, and negative staining controls comprised sections from naïve, uninjected animals and tissue treated with the primary antibody.

**Table 1.** Subcloning efficiency of adult dorsal root ganglia-derived spheres

<table>
<thead>
<tr>
<th>No. of clones (%)</th>
<th>Clusters</th>
<th>Primary → secondary</th>
<th>Secondary → tertiary</th>
</tr>
</thead>
<tbody>
<tr>
<td>(of total cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spheres (%)</td>
<td>8 (1.6)</td>
<td>5 (1.1)</td>
<td></td>
</tr>
</tbody>
</table>

For cloning identification, single secondary sphere/clusters were plated on laminin-, poly-L-lysine-, and fibronectin-coated coverslips in 24-well plates or 8-well chamber Lab-Tech slides (Nunc, Rochester, NY, http://www.nuncbrand.com) and were covered with 50 μl of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1) containing 15% fetal calf serum (FCS; Gibco), supplemented with penicillin/streptomycin and L-glutamine for 30 minutes in an incubator to allow the spheres/clusters to attach on coverslips. The attached spheres/clusters were then washed briefly once with differentiation medium (DM; similar to PCM but with the addition of 2 μM forskolin and the omission of bFGF and EGF) and cultured for 2 weeks in 500 μl of the same medium with or without 2% FCS, which is referred to as either serum-containing medium (SCM) or serum-free medium (SFM). For phenotype differentiation induced by combination of neurotrophins, the secondary clusters/spheres plated on poly-L-lysine and laminin-coated coverslips were fed by DM supplemented with a set of growth factors at 50 ng/ml, including recombinant human β-nerve growth factor (NGF) (Peprotech), brain-derived neurotrophic factor (BDNF) (Regeneron, Tarrytown, NY, http://www.regeneron.com), neurotrophin (NT)-3 (Regeneron), and glial growth factor 2 (GGF-2; gift of Dr. Mark Marchionni, Cambridge Neuroscience, Inc., Cambridge, MA) and cultured for 2 weeks, with the medium changed every 4 days.

**Sample Preparation for Immunostaining**

For cell culture, cells were fixed by 4% paraformaldehyde with 2% sucrose for 30 minutes at room temperature or acid ethanol (5:95, vol/vol) for 15 minutes at −20°C, according to the antigens revealed, and processed for immunofluorescent staining. For cytosin and cell smears, the free-floating cells or clusters were collected by centrifugation, washed with Hanks’ solution several times, and cytospin on slides. The dried samples were fixed with ice-cold acetone, rehydrated, and stored at −20°C for further processing. Cell suspensions were also spread on the gelatin-coated slides as cell smear, fixed with acid ethanol, and processed for indirect immunofluorescence. For tissue section, explant pieces taken from in vitro culture were rinsed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 4 hours, and cryoprotected in 30% sucrose overnight. DRGs samples from perfused rats or tissue pieces from explant culture were cryostat sectioned at 15 μm and processed for immunofluorescence staining or 5-bromo-2′-deoxyuridine (BrdU) labeling.

**Immunocytochemistry**

The generic protocol for immunohistochemistry was used as described previously [3, 25], with slight variations for each antigen. Permeabilization with 0.5% Triton X-100 (in PBS) for 15 minutes and for 2 hours was performed first to visualize intracellular antigens in cell samples and in all of the tissue sections, respectively. Samples were reincubated for a minimum of 30 minutes (for cells) or 2 hours (for tissue section) at room temperature in blocking buffer (Tris-buffered saline containing 5% donkey serum, 0.1% gelatin, 0.3% Triton X-100, and 1% BSA) followed by overnight incubation at 4°C with the primary antibodies in blocking buffer and a 2-hour incubation with species-specific and/or isotype-specific secondary antibodies. Double-labeling or triple-labeling experiments were performed by simultaneously incubating samples in appropriate combinations of primary antibodies followed by non-cy-reactive secondary antibodies (Alexa Fluor 488-conjugated secondary antibodies, 1:500; Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) or Cy-conjugated secondary antibodies (1:500; Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com). Sources of primary antibodies used and their concentrations are summarized in supplemental online Table 1. In some samples, nuclei were counterstained with 4′,6-diamidino-2-phenylindole. The specificity of the light microscopically immunocytochemical procedures was validated by omitting the primary antibodies or by using nonimmune serum instead of the primary antibodies. To reveal BrdU labeling of DRG or explants, cryostat sections were treated and stained as described by Valero et al. [26]. Anterior subventricular zone, gut, and injured sciatic nerve dissected from BrdU-injected rats were used as positive controls, and negative staining controls comprised sections from naïve, un.injected animals and tissue treated without the primary antibody.
RNA Extraction and Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from the cultured secondary spheres/clusters, acute rotated adult DRG, and positive control samples using TRI Reagent (Sigma-Aldrich) according to the supplier’s protocol. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed in triplicate on each gene (supplemental online Table 2) as described previously [27] with slight modifications. Briefly, the purity and concentration of RNA were assessed by spectrophotometry. To generate cDNA, total RNA was reverse-transcribed into cDNA by using 200 IU of SuperScript III reverse transcriptase (Super-Script III first-strand cDNA synthesis kit; Invitrogen) in a total reaction volume of 50 μl, following the supplier’s protocol. Two microliters of reverse-transcription product was amplified by PCR in a 50-μl reaction volume containing 10 pmol of primer sets, 0.25 U of EXTaq DNA polymerase (Takara, Shiga, Japan, http://www.takara.co.jp), PCR buffer (pH 8.4; final concentrations of 20 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl2,1 0m M dNTP), the protocol for the thermal cycler was as follows: denaturation at 94°C for 5 minutes, followed by 30–38 cycles at 94°C (30 seconds), optimal annealing temperature (supplemental online Table 2) (1 minute), and 72°C (45 seconds), with the reaction terminated by a final 10-minute incubation at 72°C. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as the internal control. Control experiments without reverse transcriptase or without template cDNA revealed no nonspecific amplification. When PCR results were negative, cDNAs from different rat tissues or rat cell lines (details given in the Fig. 4 legend) were run as positive controls in parallel with the negative samples to eliminate the possibility of false negative results.

In Vivo BrdU Labeling and In Vitro Chasing

The procedures for left spinal nerve transection have been detailed previously [25]. All axotomized neurons were retrogradely labeled with Fluororuby (FR) by soaking the proximal stumps in FR solution (1% dissolved in saline; Molecular Probes). Immediately after surgery, rats were injected intraperitoneally with BrdU (50 mg/kg) and then every 12 hours for 3 consecutive days. Twelve hours after the last injection, the injured DRG and the contralateral uninjured DRG were dissected and subjected to explant culture as described above. Injured DRGs dissected from rats perfused with 4% paraformaldehyde were sampled for in situ BrdU detection. Cells migrating out from the explant culture were smeared onto gelatin-coated slides and stained for BrdU and the appropriate markers. The explants were collected at different time points and samples prepared as detailed above. Cultured and uncultured DRG were sectioned and stained for BrdU and appropriate markers.

Image Acquisition

Stained samples were viewed using epifluorescent microscopy. If necessary, the stained samples were viewed with a laser scanning confocal microscope (Bio-Rad 2000 confocal image system, with Lasersharp 2000 acquisition software, model 1024; Bio-Rad, Hercules, CA, http://www.bio-rad.com). The digitized images taken from the epifluorescent microscope were adjusted for brightness and contrast, color-coded, and merged, when appropriate, using the Adobe Photoshop 7.0 program (Adobe Systems Inc., San Jose, CA, http://www.adobe.com); no other alterations were made.

Statistical Analysis and Cell Counting

The number of positive-stained cells was counted using the NIH ImageJ program, and the percentage of total cells expressing positive marker was determined in three to six coverslips (>200 cells) in triplicate experiments. Data were analyzed statistically using the SPSS 12.0 program (SPSS, Inc., Chicago, http://www.spss.com). The χ² test was used for comparing the subcloning efficiency of primary and secondary spheres and for comparing frequency of lineage differentiation under SCM and SFM. The paired t test was used for analyzing the data presented as the mean ± SEM.
DRG-Derived Cells Have Limited Self-Renewal Capacity

Self-renewal and multipotency are hallmarks of stem cells. We next tested whether individual cells derived from the neurospheres could form new neurospheres that differentiated subsequently into multiple cell types. Primary neurospheres and secondary neurospheres were dissociated into single-cell suspensions and reseeded at clone density in cloning medium. One day after seeding, only individual cells and no clusters were observed. In all cases, clonally derived spheres were visible within 2 weeks after reseeding. Of 506 cells from the primary spheres, 136 clusters and 8 secondary neurospheres were formed, accounting for the cloning efficiencies of 26.9% and 1.6%, respectively. We further examined cloning efficiency of secondary spheres. Of 475 individual cells from the secondary spheres, 188 tertiary clusters and 5 tertiary spheres were formed. The cloning efficiencies for clusters and spheres from the secondary spheres were 39.6% and 1.1%, respectively (Table 1). These data showed that a subpopulation of migrating cells from adult DRG had a capacity to self-renew to a certain extent.

Phenotypes of Clusters and Spheres in the Differentiation Medium Are Different in the Presence and Absence of Serum

The clusters and spheres were cultured in differentiation conditions after removal of bFGF and EGF in the presence (SCM) and absence (SFM) of 2% fatal calf serum. Two weeks after differentiation, proliferation stopped in most clones, and they grew on the surface of the coated coverslips as a monolayer and differentiated into different types of cells. The triple-labeling technique was used to define the phenotypes of these clones, and the results revealed the following types of clones: neuron (N)-only clones (Fig. 3A); glia (G)-only clones (Fig. 3B); smooth muscle (S)-only clones (Fig. 3C); N and G clones (Fig. 3D); N and S clones (Fig. 3E); G and S clones (Fig. 3F); and N, G, and S clones (Fig. 3G). Statistical analysis on the 37 clones grown in SFM and the 42 clones grown in SCM revealed distinctly different differentiation patterns. As shown in Table 2, in SFM, 10.8% of the DRG-derived clones/clusters differentiated into neurons only, 35% into glial cells only, and 48% into both glia and neurons. In contrast, in the presence of fatal calf serum, DRG-derived progenitor cells were more likely to differentiate into smooth muscle cells (15% S only, 40% G/S, and 30% N/S) (Fig. 3H). No clone expressed all three markers in serum-free culture, and only 5% of clones expressed the three markers in the SCM (Fig. 3H). These data showed that DRG-derived stem cells differentiate predominantly into neuronal and glial cell types, in particular in the absence of serum. This suggests that they are useful in the repair of nervous system. On the other hand, serum promoted their differentiation toward mesoderm-lineage cells. The emigrating cells from the frozen stocks could also form clusters without loss of multilineage potentials (supplemental online Fig. 1), further supporting the self-renewing ability.

Figure 1. Cells migrating from adult dorsal root ganglia (DRG) explants form clusters and spheres. The phase contrast micrographs show that the cells emigrated from DRG explants 1 d ([A], arrow) after in vitro culture. Cell budding could be seen around the blocks ([B], arrow). One w after culture, loose-packed suspended cellular aggregates (clusters) could be found ([C], arrow), and there were floating single cells and small clusters in the medium. After 2 w in culture, a few spherical structures could be found in flasks, and the sizes of these multicellular aggregates varied from several cells to large, solid spheres (the tight-packed floating cellular aggregates indicated by the arrow in [D]). The larger spheres could be formed, as shown by arrows, after 3 w ([E] and 4 w ([F]) in culture, respectively. Scale bar = 100 μm. Abbreviations: d, days; w, weeks.
Glial fibrillary acidic protein (GFAP), Ngn1, Msi-1, olig1, Mash1, BDNF, REST, coREST, and MAP-LC3 were expressed in both DRG and spheres. This suggests that the gene expression observed in the spheres was not an artifact of cell culture. Expression of the REST, coREST, Bmi-1, NeuroD, BDNF, MAP-LC3, AC133, and Brn3a genes, most noticeably, was reduced or lost in the spheres, as compared with the whole tissues. This finding suggests that uncultured tissues expressed these genes at higher levels than their sphere-initiating cells and their progeny or that these genes were downregulated as a consequence of cell culture. The genes that were upregulated in the spheres include Sox2, Egr2, EdnrB, Id4, Wnt1, Notch1, Delta1, GFAP, Ngn1, Msi-1, Pax6, Beclin1, Twist1, Snail1, and FoxD3. These results demonstrate that spheres from adult DRG expressed migration-related genes (CXCR4, EdnrB), self-renewal genes (Sox10, Bmi-1), proneural basic helix-loop-helix transcription factors (NeuroD, Ngn1, Hes1, Mash1, olig1), inhibitors of differentiation (Id2, Id4), myelinating genes (Sox10, Egr2, Sox2), morphogens and paired-box genes involved in the maintenance of adult NSC niche (Notch1, Wnt1, Pax6), neuronal-specific RNA binding protein gene (Msi-1), and self-clearing/autophagy genes (Beclin1, MAP-LC3), as well as neural crest-specific genes (Twist1, Snail1, FoxD3). Jagged1, one of Notch1 ligands, was undetected in the spheres and their parental tissues; however, another Notch1 ligand, Delta1, similar to Notch1, was expressed in the spheres rather than in acutely isolated adult DRG tissue. The gene-expression profile is quite consistent with the features identified through cloning analysis and immunocytochemistry, which are migratory, limited self-renewal, neural-lineage differentiation, and adult neural crest origin. No AC133 mRNA was detected in the spheres, although the cytoplasmic labeling was evident. A typical densely packed sphere coexpressing nestin and p75NTR, nuclei were counterstained by DAPI. Scale bar = 50 μm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; p75NTR, p75 neurotrophin receptor.

Figure 2. Characterization of primary clusters/spheres derived from adult dorsal root ganglia explants by immunofluorescence. (A–L): Cytospun clusters collected from the suspension culture for 2 weeks were labeled for nestin (green in [A, D]), p75NTR (green in [G, J]), ErbB2 (B), ErbB4 (E), TrkA (H), and TrkB (K); (C, F, I, L) show the same fields in merged images of (A, B), (D, E), (G, H), and (J, K), respectively. Arrows in (A–L) show the colabeled cells (yellow in [C, F, I, L]). Unlike other receptors examined in the present study, ErbB2 and ErbB4 were predominantly present in nuclei (B, E), although the cytoplasmic labeling was evident. (M–P): A typical densely packed sphere coexpressing nestin and p75NTR, nuclei were counterstained by DAPI. Scale bar = 50 μm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; p75NTR, p75 neurotrophin receptor.

www.StemCells.com
analysis also showed that they had different expression levels of the same set of genes examined here (supplemental online Fig. 1C), suggesting region specificity of adult stem cells in the nervous system.

Differentiation of DRG-Derived Cells Under Growth Factors Challenge

We did not detect any sensory neuron phenotypes by specific neuropeptide probes of substance P (SP), calcitonin gene-related peptide (CGRP), and vanilloid receptor-like 1; under the differentiation of SFM or SCM; or in the presence of NGF or GGF-2 alone (data not shown). It was considered that these challenges are not sufficient for transmitter phenotype acquisition. Because the emigrated cells expressed Trks and p75NTR (receptors for neurotrophins) and ErbB2 and ErbB4 (receptors for glial growth factor 2), we attempted to apply the combination of their ligands (NGF, BDNF, NT-3, and GGF-2) to induce possible functional differentiation. The application of combined growth factors induced NF200+/H11001 cells with long processes, and some neurons bore monopolar or pseudobipolar neurites that extended for several millimeters and aligned with the GFAP+ differentiated cells (Fig. 5A–5E). Nestin+/PGP9.5+ containing cells were also found (Fig. 5F–5H). These data indicate that under these conditions, DRG-derived clusters could generate mature neurons synthesizing neuropeptide and mature myelin; and Schwann cell myelination [32], was also detected in the present culture. The amased P0 staining was dominant, and some cells were double-labeled with GFAP (Fig. 5O–5Q), suggesting that Schwann cell precursors were undergoing maturation by synthesizing the myelin protein. These results indicate that the secondary spheres derived from adult DRG could give rise to mature neurons synthesizing neuropeptide and mature myelin.
The critical question that arose from these data was that of the identity of the neural progenitor cells. To address this issue, we conducted in vivo BrdU pulsing and in vitro chasing tests. Three days after axotomy, the cells in the injured DRG were clearly labeled by FR and BrdU (Fig. 6A–6D), and no neuronal BrdU labeling was observed. Most injured DRG were labeled by FR and BrdU (Fig. 6H, 6J), and fewer BrdU cells were found around neurons (Fig. 6I). This phenomenon indicates that the emigrating cells are likely SGCs in the explant culture. We then identified the characteristics of migrating cells with immunocytochemistry. Of 86 floating cells analyzed, none contained Fluororuby fluorescence, indicating that no emigration of mature neurons occurred in the explant culture. We did not observe any floating cells stained with βIII-tubulin, an earlier marker for immature neurons (data not shown), suggesting that no “late-differentiation” involving in adult neurogenesis, proliferation, self-renewal, and self-clearing/autophagy. (A): Tabulated results of RT-PCR for the tested genes. The intensities of signal are scored in five arbitrary units: −, not detectable (no signal); ±, barely detectable (minor signal); +, detectable (weak signal); ++, easily detectable (moderate signal); ++++, strongly detectable (strong signal). Full gene names are given in supplemental online Table 2. (B): The representative bands of the PCR products run in 2% agarose gel electrophoresis. The left lane is cDNA from embryonic rat motor neuronal cell line (NSC34) used as a positive control; the middle lane is mRNA in the adult DRG, and the right lane is mRNA in the NSP derived from adult DRG. RT-PCR for G3PDH was used as a loading control. RNA from E14 rat embryos was used as +ve for AC133, CXCR4, and Bmi-1; RNA from rat immature oligodendrocytes cell line (OLN-93) after 1 week of culture in proliferation culture medium was used as +ve for SOX10, BDNF, Beclin1, and LC3; RNA from embryonic rat motor neuronal cell line (NSC34) was used as +ve for NeuroD; RNA from adult rat bone marrow stem cell was used as +ve for AC133, CXCR4, and Bmi-1; RNA from rat immature oligodendrocytes cell line (OLN-93) after 1 week of culture in proliferation culture medium was used as +ve for SOX10, BDNF, Beclin1, and LC3; DNA from adult rat cartilage was used as +ve for SOX9; RNA from adult sciatic nerve was used as +ve for Jagged1; and RNA from adult rat forebrain-derived NSP was used as +ve for the other genes examined. Abbreviations: +ve, positive control; bp, base pairs; DRG, dorsal root ganglia; NSP, neurospheres.

### Schwann cells synthesizing P0 in vitro under long-term induction by neurotrophic factors.

The Cells Emigrating From DRG Explants Probably Come from Proliferating Glial Cells Rather Than Sensory Neurons

Table 2. Number of different kinds of clone in SCM/SFM identified by double/triple immunofluorescence

<table>
<thead>
<tr>
<th>Clone (%)</th>
<th>NGS</th>
<th>NG</th>
<th>NS</th>
<th>GS</th>
<th>N</th>
<th>G</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCM</td>
<td>4 (9.5)</td>
<td>ND</td>
<td>12 (28.6)</td>
<td>16 (38.1)</td>
<td>ND</td>
<td>4 (9.5)</td>
<td>6 (14.3)</td>
</tr>
<tr>
<td>SFM</td>
<td>ND</td>
<td>18 (48.6)</td>
<td>ND</td>
<td>2 (5.4)</td>
<td>4 (10.8)</td>
<td>13 (35.1)</td>
<td>ND</td>
</tr>
</tbody>
</table>

The single clone/cluster from subcloning assay was collected and allowed to differentiate on poly-L-lysine-, laminin-, and fibronectin-coated coverslips in the SCM and SFM for 1 week. The samples were characterized with phenotypic markers (e.g., NF200 and β III-tubulin as neuronal marker; glial fibrillary acidic protein as glial marker; and smooth muscle α-actin as myofibroblast or smooth muscle cell marker) by indirect immunofluorescent labeling (four samples in SCM, three samples in SFM, used as omitted antibody control). Forty-two clones in SCM and 37 in SFM were analyzed. The frequencies and the percentage of total clones (in parentheses) in each category are shown. Abbreviations: G, clones with markers for glia; GS, clones with markers for gli and muscle cells; N, clones with markers for neurons only; ND, not detected; NG, clones with markers for neurons and glia; NGS, clones with markers for neurons, glia, and muscle cells; NS, clones with markers for neurons and muscle cells; S, clones with markers for muscle cells only; SCM, serum-containing medium; SFM, serum-free medium.

Figure 4. Molecular profile of the adult DRG-derived spheres compared with the adult DRG. Reverse transcription-polymerase chain reaction (RT-PCR) on selected genes was performed using total RNA isolated from the adult DRG tissue and from the adult DRG-derived spheres. NSP express a set of markers of neural crest stem cell, and they also express distinct genes involving in adult neurogenesis, proliferation, self-renewal, and self-clearing/autophagy. (A): Tabulated results of RT-PCR for the tested genes. The intensities of signal are scored in five arbitrary units: −, not detectable (no signal); ±, barely detectable (minor signal); +, detectable (weak signal); ++, easily detectable (moderate signal); ++++, strongly detectable (strong signal). Full gene names are given in supplemental online Table 2. (B): The representative bands of the PCR products run in 2% agarose gel electrophoresis. The left lane is +ve for AC133, CXCR4, and Bmi-1; RNA from rat immature oligodendrocytes cell line (OLN-93) after 1 week of culture in proliferation culture medium was used as +ve for SOX10, BDNF, Beclin1, and LC3; DNA from adult rat cartilage was used as +ve for SOX9; RNA from adult sciatic nerve was used as +ve for Jagged1; and RNA from adult rat forebrain-derived NSP was used as +ve for the other genes examined. Abbreviations: +ve, positive control; bp, base pairs; DRG, dorsal root ganglia; NSP, neurospheres.
p75NTR, and GFAP] used in our in vitro analysis). To see whether the cells that emigrated from injured DRG had different features from uninjured DRG, we characterized the cells from injured and uninjured DRG explant cultures. Both kinds of cells coexpressed nestin and GFAP (supplemental online Fig. 3A, 3B), and there was no significant difference between numbers of colabeled cells (supplemental online Fig. 3C). This shows, first, that axotomy or injury did not result in remarkable changes in the characteristics of emigrating cells, and second, that the emigrated cells from injured DRG in the explant culture were not an artifact caused by axotomy. Thus, this experiment demonstrated that neural progenitor cells from DRG were most likely SGCs of incorporating BrdU and expressed nestin and GFAP, which were located around somata of sensory neurons.

**DISCUSSION**

In the present study, we examined whether there are neural progenitor cells in the adult DRG that may contribute to the neuronal addition after nerve injury. We found that the cells emigrating from adult DRG explant culture formed neurotrophic factors promote neurogenesis and maturation of neurons and glia derived from progenitors of adult dorsal root ganglia (DRG). (A–Q): Images taken from cultured DRG clusters differentiated in the presence of neurotrophic factors (NGF, BDNF, NT-3, and glial growth factor 2; each concentration, 50 ng/ml) in differentiation medium for 2 weeks. (A–E): Micrographs from sample stained for neurofilament 200 (red) and glial fibrillary acidic protein (GFAP) (green). (A): Montage of 16 photos taken under epifluorescence microscope at ×10 with dry objectives (numerical aperture [NA], 0.40) The region in the white box in (A) consists of a three-dimensional (3D) construction of 36 images (B–E) taken by confocal laser scan microscope at ×60 with oil objectives (NA, 1.40) and with a Z-step of 0.4 μm (Z-stack bottom = 0; Z-stack top = 14). (B, C): Projection of cy3 (NF200, red) and cy2 (GFAP, green) channels withchroma 31,002 and chroma 31,001 filter blocks, respectively. (D): Merged projection of (B, C). Arrows in (B, D) mark a cell with a long process extending several millimeters, as seen in (A). (E): y- and z-axes of (D). (F–H): A typical cell, shown in confocal images, was positive for mature pan-neuronal markers PGP9.5 (F) and nestin (G) and their colocalization (H). (I–N): Images taken under an epifluorescence microscope at ×40 with dry objectives (NA, 0.85). The differentiated cells expressed neuropeptide SP (green in [I]), CGRP (green in [L]), S100β (red in [J]), and p75NTR (red in [M]). (K, N): Same-field merged images of (I, J) and (I, M), respectively. Blue (K, N) marks the DAPI staining. Arrows in (I–K) show the cells stained by SP and S100β (yellow). (Q): Same-field 3D construction confocal images (16 images) taken at ×20 with oil objectives (NA, 0.8) with a Z-step of 0.5 μm. (O): Cells stained by peripheral myelin marker P0. (P): Glial marker GFAP labeling. (Q): Merged projection of (O, P). Arrows in (O–Q) mark containing cells; arrowheads in (O–Q) mark P0+/GFAP− cells. Abbreviations: μ, μm; CGRP, calcitonin gene-related peptide; GFAP, glial fibrillary acidic protein; P0, protein zero; p75NTR, p75 neurotrophin receptor; SP, substance P.

**Figure 5.** Neurotrophic factors promote neuritogenesis and maturation of neurons and glia derived from progenitors of adult dorsal root ganglia (DRG). (A–Q): Images taken from cultured DRG clusters differentiated in the presence of neurotrophic factors (NGF, BDNF, NT-3, and glial growth factor 2; each concentration, 50 ng/ml) in differentiation medium for 2 weeks. (A–E): Micrographs from sample stained for neurofilament 200 (red) and glial fibrillary acidic protein (GFAP) (green). (A): Montage of 16 photos taken under epifluorescence microscope at ×10 with dry objectives (numerical aperture [NA], 0.40) The region in the white box in (A) consists of a three-dimensional (3D) construction of 36 images (B–E) taken by confocal laser scan microscope at ×60 with oil objectives (NA, 1.40) and with a Z-step of 0.4 μm (Z-stack bottom = 0; Z-stack top = 14). (B, C): Projection of cy3 (NF200, red) and cy2 (GFAP, green) channels with chroma 31,002 and chroma 31,001 filter blocks, respectively. (D): Merged projection of (B, C). Arrows in (B, D) mark a cell with a long process extending several millimeters, as seen in (A). (E): y- and z-axes of (D). (F–H): A typical cell, shown in confocal images, was positive for mature pan-neuronal markers PGP9.5 (F) and nestin (G) and their colocalization (H). (I–N): Images taken under an epifluorescence microscope at ×40 with dry objectives (NA, 0.85). The differentiated cells expressed neuropeptide SP (green in [I]), CGRP (green in [L]), S100β (red in [J]), and p75NTR (red in [M]). (K, N): Same-field merged images of (I, J) and (I, M), respectively. Blue (K, N) marks the DAPI staining. Arrows in (I–K) show the cells stained by SP and S100β (yellow). (Q): Same-field 3D construction confocal images (16 images) taken at ×20 with oil objectives (NA, 0.8) with a Z-step of 0.5 μm. (O): Cells stained by peripheral myelin marker P0. (P): Glial marker GFAP labeling. (Q): Merged projection of (O, P). Arrows in (O–Q) mark containing cells; arrowheads in (O–Q) mark P0+/GFAP− cells. Abbreviations: μ, μm; CGRP, calcitonin gene-related peptide; GFAP, glial fibrillary acidic protein; P0, protein zero; p75NTR, p75 neurotrophin receptor; SP, substance P.
spheres and generated secondary and tertiary spheres by cloning assays. More importantly, the cells in the cloned spheres differentiated into neuronal, glial, and smooth muscle antigenic-positive cells. Based on criteria established previously [34] and recently [35], we conclude that the emigrating cells from adult DRG are most likely neural crest progenitors. Several lines of evidence suggest that these cells are most likely SGCs around neuronal cell bodies.

We failed to generate any neurospheres by dissociated cultures despite repeated efforts. However, by taking advantage of the neural crest features of migration and chemotropism, we succeeded in isolating the neural progenitors by DRG explant culture. The fact that the dissociated culture failed but the explant culture succeeded in generating stem/progenitor cells could be due to several fundamental differences between explant cultures and dissociated cultures. The cell-cell interactions and cellular environment are maintained in explant culture but are lost in dissociated cultures. The cell-cell interaction and immediate environment may be important for the initiation and maintenance of mitosis by neural crest progenitors. Cell proliferation may be driven by mitogens or growth factors from neurons or nearby glial cells by autocrine and paracrine mech-

Figure 6. Migrating cells from dorsal root ganglia (DRG) explants are likely satellite glial cells, as determined by in vivo BrdU pulsing and in vitro chasing. (A–D): Images of DRG sections. Neurons were retrogradely labeled in vivo by FR (red), and proliferating cells were labeled in vivo by BrdU immunofluorescence (green). (A, B): Pictures of the same field. (C): Merged image of (A, B). (D): Enlarged image of the boxed region in (C), demonstrating that most BrdU+ cells encircled neuronal profiles in vivo. Scale bar = 100 μm (C), 25 μm (D). (E–J): Images stained for BrdU (green) in DRG tissues (FR-labeled and BrdU-pulsing in vivo) taken from explant culture for 3 d (E), 1 w (F), 2 w (G), and 3 w (H–J). (I): High-magnification image showing the region in the white box in (H). (J): A typical image showing BrdU+ cells segregated at the border of DRG explants after 3 w in vitro culture. (K–T): Images taken from slide smears of floating cells collected from suspension of DRG (FR-labeled and BrdU-pulsing in vivo) explant cultured for 1 w. The smears were immunostained by BrdU and GFAP (K), Nestin and p75NTR (L), TrkA (M), TrkC (N), ErbB4 (Q), and TrkB (R), respectively. (K): Two cells colabeled with GFAP (green) and BrdU (red). (L): Cells coexpressing nestin and p75NTR (yellow). (P): Merged image of (M–O) showing cells expressing TrkA and TrkC. (T): Merged image of (Q–S) demonstrating the staining of ErbB4 and TrkB. The yellow areas in (P) and (T) show the double-labeled cells. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; d, days; DAPI, 4′,6-diamidino-2-phenylindole; FR, Fluororuby; GFAP, glial fibrillary acidic protein; p75NTR, p75 neurotrophin receptor; w, weeks.
anisms, as these cells upregulate transforming growth factor-α [36], bFGF [37], and neurotrophins [38] after axotomy. Dissociated DRG cells may lose these signals from their immediate environment. They may also lose the extracellular matrix, which is important for cell survival and migration [39]. In addition, enzymatic digestions may have detrimental effects on the progenitors by removing cell surface receptors and mechanical dissociation may cause cell stress and phenotypic instability in vitro. Thus, our study has provided a simple, economical, and effective means of isolating neural progenitors from adult DRG and may have practical application for isolation and purification of adult stem/progenitor cells from other tissues.

**DRG-Derived Cells in Explant Culture Demonstrate Characteristics of Progenitors**

We have characterized the cells that emigrated from adult DRG in vitro cultures by several methods. First, we examined the behaviors and number of cells generated in the culture of each DRG at different time points. We found that the cells emigrating from the explants were very active and proliferative and formed clusters in the first several days, and some of these clusters increased their size and formed spheres. The number of cells increased with time within the first week of cultures. The behavioral changes of the cells suggest that these cells are mitotic and may have self-renewal capacity. In fact, the cloning assay from hand-picked primary spheres showed that approximately 20%–30% of these cells formed multicellular clusters, and approximately 1%–1.5% of the cells formed solid spheres, as measured by limited-dilution analysis. These results suggest that the emigrating cells contain stem/progenitor cells with a limited self-renewal capacity.

Immunohistochemical data showed that a high proportion of the floating cells expressed neural progenitor markers such as nestin [40] and p75NTR [34]. Both markers are often colocalized in the same cells, suggesting that some of these cells are neural crest progenitor cells. In addition, these cells also coexpress the neurotrophin receptors Trks, together with p75NTR, suggesting that these cells may respond to neurotrophins and be dependent on neurotrophins for their proliferation, survival, and differentiation. The expression of TrkA, TrkB, TrkC, and p75NTR is a characteristic of neural crest progenitors during development [35, 41], whereas neurotrophins are essential neurotrophic factors for their proliferation, survival, and differentiation [42]. Whether neurotrophins affect proliferation and survival of the progenitor cells isolated from adult DRG needs further investigation. However, we investigated the function of neurotrophins on the morphology and differentiation of the progenitors. The addition of combination of neurotrophins resulted in promotion of neuritogenesis and maturation of neurons and Schwann cells. In the presence of neurotrophins, some cells generated typical morphology of sensory neurons with monopolar or pseudopolar long processes. Some cells synthesize sensory neuron peptides substance P and CGRP. Determination of whether these “sensory neuron-like” cells have functional properties requires an electrophysiological examination.

One of the key characteristics of stem cells is their multipotency. We examined the multipotency of the clone cells from the adult DRG by double and triple labeling of individual clones grown in the presence or absence of FCS under differentiation conditions. We demonstrated that neuronal, glial, and smooth muscle cell lineages coexisted in single clones. The data suggest that the cells isolated from adult DRG are multipotent and differentiate into different cell lineages. At least some of these cells are derived from neural crest, as they differentiated into sensory neuron-like phenotype, P0-expressing Schwann cells, and smooth muscle cells. We found that FCS significantly affects their fate. In the absence of serum, these cells tend to differentiate into glial and neuronal cell lineages, whereas in the presence of FCS, these cells preferentially differentiated into smooth muscle cells. Although most clones were multipotential, some were unipotential and only differentiated into either glia, or neuron, or smooth muscle cells, alternatively. These studies not only support the note that these cells are multipotential progenitor cells but also suggest that the differentiation can be influenced by environmental cues.

**Gene Expression Profile Further Defines the Neural Crest Progenitors**

We detected several neural stem cell-specific genes, such as Sox2, Notch1, Pax6, and MASH1, consistent with the view that the cells from DRG explants are likely neural progenitor cells. The expression of Wnt1 in sphere preparations suggests a role of Wnt signaling in DRG-derived progenitors. It has been demonstrated that Wnt signaling promotes sensory neurogenesis in early NCSCs [43] and participates in the maintenance of NCSCs [44]. The expression of Notch1 and one of its downstream effectors, Hes1, in our sphere preparations probably underpins the migratory and proliferating features of the progenitors. The existence of Delta1 (Notch1 ligand) in the spheres perhaps underlies the glial fate decision during differentiation [45] and probably involves in the maintenance of stem cell/progenitor status/niches [46, 47]. A number of genes relating to self-renewal and proliferation are expressed by DRG-derived progenitors, including Msi-1 [48], Bmi1 [49], and helix-loop-helix (HLH) transcriptional regulators Id2 and Id4 [50, 51]. The upregulation of Id4 may underlie the proliferation capacity of the progenitors.

The increased expression of proneural genes of basic HLH transcription factors NeuroD, neurogenin 1, Hes1, and Mash1, along with the decreased expression of REST and coREST (the neuronal gene repressor/silencer [52]), is in agreement with the neuronal differentiation of the progenitors observed in this study. On the other hand, the expression of Sox2, Egfr, olig1, and GFAP indicates their glial potential and possible neural crest origin. Further evidence that supports neural crest origin is the upregulation in the spheres of Sox10, Twist1, Snail1, and FoxD3, a set of transcription factors for NCSCs [53–56]. In accordance with the migratory capacity of NCSCs, several genes related to stem cell migrations and trafficking were upregulated in our preparations. These include endothelin receptor-B [57, 58] and the chemokine receptor CXCR4 [59, 60]. It is surprising that sensory neuron progenitor-specific Pou transcription factor Brn3a was not expressed in the spheres. This suggests that the expression of TrkA in the DRG-derived progenitors was unlikely to be regulated by Brn3a, whose role is reported to be the acquisition of sensory neuron phenotypes [61–64]. The absence of Brn3a in the spheres also suggests that the progenitors isolated from adult DRGs may sit at an earlier developmental stage than the sensory neuron progenitors.

In addition, both adult DRG spheres and adult heart-derived cardiospheres [7] have multipotent potentials with neural crest features. The gene expression profile of DRG spheres presented here is quite consistent with that of cardiac neural crest cells isolated from adult heart [7], and both expressed Msi-1, Mash-1 and GFAP, all of which were detectable in the sphere-initiating cells. Adult DRG-derived spheres, however, may have some different inherent characteristics (e.g., expression of Brn3a or neurotrophic responsiveness). The RT-PCR data represent the average characteristics of cell population in spheres, rather than the properties of individual cells. Our gene expression analysis
provides substantial evidence in support of our conclusion that at least a subpopulation of emigrating cells, from the adult DRG explants, is likely to consist of neural crest progenitors with characteristics of multipotency, migration, and limited self-renewing capacity.

**Progenitors Isolated from the Adult DRG Are Likely to Have Originated from SGCs In Situ**

Several lines of evidence suggest that the DRG progenitors are likely to have originated from SGCs. First, the results from in vivo BrdU labeling and in vitro chasing experiments showed that the cells emigrating from adult DRG are BrdU+. The majority of BrdU+ cells in situ were tightly apposed to neurons and BrdU+ cells but lost their normal perineuronal configuration when cultured in vitro. With the time in culture, they detached from neuronal somata and emigrated out from the center of the explants toward the edge. Second, the emigrating cells expressed GFAP, a glial cell marker, demonstrating their glial origin. Most importantly, BrdU+ cells surrounding neurons in situ were also GFAP-positive. After nerve injury, GFAP+ cells surrounding neurons also expressed the proliferation marker. This suggests that these cells undergo proliferation in vivo after nerve injury. Third, these cells also expressed the progenitor markers p75NTR and nestin, both in the culture medium and in situ in the DRG after nerve injury (supplemental online Fig. 3). Finally, progenitors of adult DRG have molecular features similar to those of radial glia or neurogenic glial precursors in adult CNS, which have been identified as neural stem cells per se [65] and may be a counterpart of NSCs of glial origin in the CNS. Some peripheral myelinated glia (e.g., Schwann cell precursors isolated from embryonic sciatic nerve [66] and P0-positive cells from adult heart [71]) have been demonstrated to have neurogenic potential in vitro and in vivo. Thus, SGCs are probably also kinds of neurogenic cells as well, at least in vitro, as shown in the present study.

Recent studies by others also support the possibility that SGCs are likely candidates for ganglionic progenitor cells. A subpopulation of SGCs in the adult DRG [16], vestibular and spiral ganglia [67], was found to be nestin-positive. In the normal adult DRG, we found, similar to other reports, that SGCs incorporated BrdU [68, 69]. SGCs in neonatal DRG are multipotential precursors that can differentiate into oligodendrocytes, astrocytes, and Schwann cells [70]. In addition, SGCs express EGF receptors [36] and fibroblast growth factor receptors [71], which provide a molecular basis for the in vitro expansion mediated by mitogens. Furthermore, SGCs are the progeny of postmigratory neural crest cells [72] and neural crest boundary cap cells [73]. Therefore, SGCs, as the neural crest derivatives, may have a broader developmental potential [66] and greater adult plasticity [28]. The characteristics of SGCs may underlie its phenotype instability in vitro [74, 75] and the capacity of multipotential differentiation and self-renewing, as demonstrated in this study.

It is also very important to carefully interpret the present results, which were mainly based on in vitro analysis, not on in vivo long-term reconstitution assay. At present, our in vitro assay alone does not allow us to fully characterize the adult DRG-derived stem cells. Even though it is quite difficult to purify the SGCs in an in vitro culture, both technically and methodologically [70], an additional in vitro long-term assay is needed to clarify this point. We truly recognize that sustained lineage tracing in an appropriate in vivo model by genetic or transgenic approaches is likely to be the best functional definition of SGCs as stem cells.

In conclusion, a subpopulation of cells emigrating from the adult DRG explants in vitro expresses neural crest progenitor markers nestin and p75NTR and possesses stem cell characteristics of multipotency and self-renewal ability. These stem-like cells most likely originate from SGCs. Our data strongly suggest that adult DRG might contain neural crest progenitors, which may participate in the ongoing and reactive neurogenesis in the adult.

Acknowledgments

We are grateful to Prof. J.M. Polark (Imperial College London, London, U.K.) for antibodies to SP and CGRP; Prof. R.A. Rush (Flinders University, Adelaide, Australia) for PGP9.5; Prof. L. Reichardt (University of California San Francisco, San Francisco, CA) for TrkA; TrkB, and TrkC; Prof. M.V. Chao (New York University, New York, NY) for p75NTR; Dr. J.J. Archelos (Graz University, Graz, Austria) for P0; and Dr. Mark Markchonion (Cambridge Neuroscience, Inc., Cambridge, MA) for rhGFP2 protein. We also thank Prof. Ian Gibbins (Flinders University) for training on confocal techniques; Dr. John Y.J. Wang (Flinders University) for statistics analysis; and J.X. Mi, J. Zhong, Dr. Li Li, and Dr. Yongjun Fan for technical assistance and discussions. We thank Dr. Steve Johnson (Flinders University), Dr. Ernest Aguilar (Flinders University), Antony Zaknic (Flinders University), and Dr. Heather Young (University of Melbourne, Melbourne, VIC, Australia) for critical reading of the manuscript. Some monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, http://www.uiowa.edu/~dshbwww) under the auspices of National Institute of Child Health and Human Development, NIH, and maintained by the University of Iowa. This work was supported by Grants 375109 and 375110 from the National Health and Medical Research Council of Australia.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

**REFERENCES**


Farel PB. Late differentiation contributes to the apparent increase in sensory neuron number in juvenile rat. Brain Res Dev Brain Res 2003;141:91–98.


See www.StemCells.com for supplemental material available online.