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Isolation and differentiation of neural stem/progenitor cells from fetal rat dorsal root ganglia

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To find a promising alternative to neurons or schwann cells (SCs) for peripheral nerve repair applications, this study sought to isolate stem cells from fetal rat dorsal root ganglion (DRG) explants. Molecular expression analysis confirmed neural stem cell characteristics of DRG-derived neurospheres in terms of expressing neural stem cell-specific genes and a set of well-defined genes related to stem cell niches and glial fate decision. Under the influence of neurotrophic factors, bFGF and NGF, the neurospheres gave rise to neurofilament-expressing neurons and S100-expressing Schwann cell-like cells by different pathways. This study suggests that a subpopulation of stem cells that reside in DRGs is the progenitor of neurons and glia, which could directly induce the differentiation toward neurons, or SCs.

dorsal root ganglia, cell culture, stem/progenitor cells, immunocytochemistry, induced differentiation

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Due to their self-renewal capacity and multiple differentiation potential, neural stem cells (NSCs) have become an attractive and possibly unlimited donor source for cell replacement therapy to treat neurological disorders [1,2]. Accumulating evidence indicates the presence of NSCs in the developing and adult central nervous system (CNS) of mammalian organisms, including humans [3–6]. By contrast, there are few studies concerning the isolation of NSCs from the peripheral nervous system (PNS) including dorsal root ganglia (DRGs) [7–11]. During PNS development, DRGs are derived from the precursors in the neural crest. After peripheral nerve injury, the number of sensory neurons in adult DRGs initially decreases and recovers to the normal level several months later, implying the presence of multipotent stem cells in DRGs [12]. Hence, exploring whether or not DRGs contain a specialized niche for NSCs will contribute to the understanding of neurogenesis in PNS and the development of NSCs-based therapeutic approaches. Here we aimed to isolate neural stem/progenitor cells from fetal rat DRGs, and further investigated their proliferation and differentiation *in vitro* for harnessing the potential application of peripheral NSCs for the treatment of diseases in both CNS and PNS.

1 Materials and methods

1.1 Animals

Animals were obtained from the Laboratory Animal Center of Nantong University, and all animal experiments were conducted according to NIH Guidelines for the care and use of laboratory animals, and approved by the Administration Committee of Experimental Animals, Jiangsu Province,

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1.2 Cell culture

After Sprague-Dawley (SD) rats on embryonic day 18 were sacrificed by cervical dislocation under anesthetization, DRGs were removed from rat fetuses, and placed in a DMEM/F12 culture medium containing 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). The surface membrane was stripped from DRGs, which were then cut into small pieces for digestion with tryptase (Worthington Biochemical, Lakewood, NJ) at 37°C for 5 min, and gently triturated with a fire-polished pasteur pipette until most of tissues were dissociated into single cells. The mixture was passed through a 70-μm cell strainer, and centrifuged at 1000 r min^{-1} for 10 min. After the supernatant was discarded, the cells were re-suspended in the serum-free DMEM/F12 medium supplemented with 2% B27 (Gibco, Carlsbad, CA), 10 ng mL^{-1} recombinant human basic fibroblast growth factor (bFGF, Sigma, St. Louis, MO), 10 ng mL^{-1} epidermal growth factor (EGF, Sigma), and 20 ng mL^{-1} Neuregulin1-β1 (R&D Systems, Inc), and plated at a density of 1×10^6 cells mL^{-1} in 75 cm^2 cell culture flasks (Corning) for incubation at 37°C and 5% humidified CO_2. After 7–10 d culture, the cells formed small, proliferating cell spheres, which were gently triturated into a quasi-single cell suspension, and re-plated for weekly passage with the medium being refreshed every 2–3 d. The morphological change of cell aggregates was observed under a phase-contrast microscope (Nikon, Japan).

1.3 Subcloning assay

A 96-well plate-based limited-dilution method was used for the subcloning assay. The single-cell suspension derived from one primary sphere in the medium was inoculated in a 96-well plate (0–1 cells per 100 μL per well). Fresh medium was added to the wells with cells 7 d after initial seeding. The spheres resulting from the primary sphere subcloning were designated the secondary spheres. The tertiary spheres (clusters) were defined by the spheres (clusters) formed from the subcloning of the hand-picked secondary spheres.

1.4 Characterization of the cells

Flow cytometry and immuocytochemistry were used to detect the expression of nestin, p75 neurotrophin receptor (NTR) and the incorporation of 5′-Bromo-2′-deoxyuridine (BrdU) in cell aggregates as per standard protocols. The cells were fixed with 4% paraformaldehyde for 30 min. After being incubated with 0.4% Triton X-100 for 10 min at room temperature, they were blocked with 10% normal goat serum (Invitrogen) overnight at 4°C, after which primary antibodies against nestin (1∶500, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) or p75NTR (1∶200, Santa Cruz) were added for 4-h incubation, followed by reaction with secondary antibodies conjugated to FITC or TRITC fluorophores (1∶500, Sigma) for 1-h in the dark. BrdU labeling was conducted according to the kit instructions (Roche Applied Science, Indianapolis, IN). Labeling with Hoechst33342 (1 μg mL^{-1}, Sigma) was also performed. The images were captured on a DMR fluorescent microscope (Leica Microsystems, Wetzlar, Germany), and the positive ratio of nestin and BrdU was detected by flow cytometry (BD Biosciences, Rockville, USA).

1.5 Differentiation of the cells

In order to examine cell differentiation, the cell aggregates were allowed to incubate for 10 d in the DMEM/F12 culture medium added with 2% fetal bovine serum (FBS) instead of bFGF and EGF, followed by immunochemistry with antibodies against glial fibrillary acidic protein (GFAP, 1∶500, Dakocytomation, Glostrup, Denmark) and neurofilament (NF-200, 1:200, Sigma).

The secondary cell aggregates were induced under the influence of nerve growth factor (NGF) or bFGF to undergo directed differentiation. In brief, 50–100 cell aggregates were plated onto poly-L-lysine-coated plastic dishes, and cultured in the DMEM/F12 medium respectively added to 20 ng mL^{-1} NGF or 20 ng mL^{-1} bFGF. After 10-d culture, the cells were fixed and subjected to immunocytochemistry, in which primary antibodies against GFAP, NF-200 or S100β (1:200, Sigma) were respectively used.

1.6 RNA extraction and semiquantitative reverse transcription-polymerase chain reaction

The gene expression profiles of DRG tissues and DRG-derived cell aggregates in the undifferentiated and differentiated forms were detected by RT-PCR. Total RNA was isolated and the cDNA was synthesized with an Omniscript RT Kit according to the supplier's protocol (QIAGEN GmbH, D-40724 Hilden). The RT product (1 μL) was amplified in a 25 μL reaction volume consisting of 10 pmol of primer sets, 0.25 U of Taq DNA polymerase (Takara, Wuhan, China), and PCR buffer (20 mmol L^{-1} Tris-HCl, 50 mmol L^{-1} KCl, 2.5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} dithiothreitol and 1 mmol L^{-1} dNTP, pH 8.4). The PCR conditions were 94°C for 5 min (denaturation), an optimal temperature for 30 s (annealing) and 72°C for 30 s (extension) for a total of 30 cycles. Glyceraldehyde phosphate dehydrogenase (GAPDH) served as an internal control. The oligonucleotide primers used are shown in Table 1.

1.7 Western blot analysis

DRG-derived cell aggregates in the undifferentiated and

Table 1 Oligonucleotide primers used in RT-PCR

Targets	Gene locus	Primers (5′–3′)	Anneal temperature (°C)	Length (bp)
Oct4	NM_001009178	F: TGGAGGAAGCTGACAACAACG R: CATAGCCTGGAGCACCAAAGTG	62	354
Sox2	NM_001109181	F: GGTTACCTCTTCCTCCCACTCC R: TTCTAGTCGGCATCACGGTTT	62	396
Sox10	NM_019193	F: CTCCTGCCCTGATGACC R: GCCTCCCTGAAAGACTTGA	60	172
Pax6	NM_013001	F: CAGACCTCCTCGTACTCCTGC R: CCAACTGATACCGTGCCTTCT	60	426
Wnt1	NM_001105714	F: AGGTGAAAGGGCAAGGAAAG R: GCTGGCAGACAAGAGGAGTG	60	305
Sox9	XM_343981	F: GAAACTTCAGTGGGAGCGACAA R: GGCGAGCACTTAGCAGAGGC	62	192
Mash1	NM_022384	F: GCCAACAAGAAGATGAGCAAGG R: CCGCCATAGAGTTCAAGTCGT	62	169
Hes1	NM_024360	F: AATAAACCCTCAACTGCTCC R: GATAGGCTTTGATGACTTTCTGT	55	261
Olig1	NM_021770	F: AAAATTCCAACCACGTTCCAC R: GGCTACTGTCAACAACCCAAAA	60	364
NeuroD	NM_019218	F:TTTCACGATTAGAGGCACG R: TTCCAAAGGCAGTAACGAC	55	170
GAPDH	NM_017008	F: GGTGCTGAGTATGTCGTGGAG R: CAGTCTTCTGAGTGGCAGTGAT	56	292
GFAP	NM_017009	F: TGTGAAGGTCTATTCCTGGTTGC R:TTAAAGTCTAGGCGATACTCCGTAC	55	181
Nefh	NM_012607	F: AGAGGAGTGGTTCCGAGTGA R: TTCTGTAAGCAGCGATCTCAAT	61	344
S100 β	NM_013191	F: ATAGCACCTCCGTTGGACAG R: GTCCTATGGGGACAATGGTG	60	383
p75NTR	NM_012610	F: CCAGCCCACACTTCTCTCTC R: CTGACATTAAGGGCCGTGTT	60	386
TrkA	NM_021589	F: GCAGGGACATCTACAGCACA R: CAGACTCCTAGCCCAGAACG	60	384
nestin	NM_012987	F: AACCACAGGAGTGGGAACTG R: GGGTCCTCTAGCCCTACCAC	56	345
GAP-43	NM_017195	F: GGCTCTGCTACTACCGATGC R: CTGTCGGGCACTTTCCTTAG	62	384
FGFR-1	NM_024146	F: CTCTGTGGTGCCTTCTGACA R: TTCACCTCGATGTGCTTCAG	60	234
FGFR-2	NM_012712	F: ACTGGACCAACACCGAAAAG R: CTCCACCAGGCAGGTGTAAT	60	239
FGFR-3	NM_053429	F: TCTGGTCCTTTGGTGTCCTC R: TGAGGATGCGGTCTAAATCC	60	228
FGFR-4	NM_001109904	F: CTCTCTCTGGCCCTGTTTTG R: TCGCTCCTTTGAGGATGAGT	60	150

differentiated forms were lysed in the cell/tissue protein extraction reagent (Biocolors, Shanghai, China) with protease inhibitor on ice. Whole-cell lysates, whose protein concentrations had been measured by a BCA Protein Assay Reagent kit (Biocolors), were resolved by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad), which were blotted with 5% non-fat dry milk in a TBST buffer and allowed to incubate with the primary antibodies: anti-FGFR4, anti-TrkA (both from Abcam, Cambridge, MA), anti-FGFR1,2,3, anti-p75NTR (both from Santa Cruz Biotechnology, Inc, Santa Cruz, CA), or anti-β-actin (Sigma), respectively. After reaction with the second antibody goat anti-mouse-IRDye (1∶10000) or donkey anti-rabbit-IRDye (1∶10000), the membranes were washed with TBS/T and scanned with an Odyssey Infrared imager (LiCor, Lincoln, NE). The data were analyzed with the software attached to the imager. β-actin was used as an internal control for normalizing the loaded protein.

2 Results

2.1 Characterization of the cells

We examined the morphological change of the cells isolated from fetal rat DRGs during *in vitro* culture. After 3-d culture, some floating cells or small aggregates (clusters) occurred in the medium, and 7 d later, small aggregates of 4–10 cells significantly increased. After 2-weeks culture, dense cell aggregates (spheres) were observable (Figure 1A). An increase of cell number, coupled with the formation of cell spheres, indicated self-proliferation of the DRG-derived cells. These newly born aggregates were maintained for more than 3 months. A limited-dilution method with 96-well plates was used for the subcloning assay, 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. Of 672

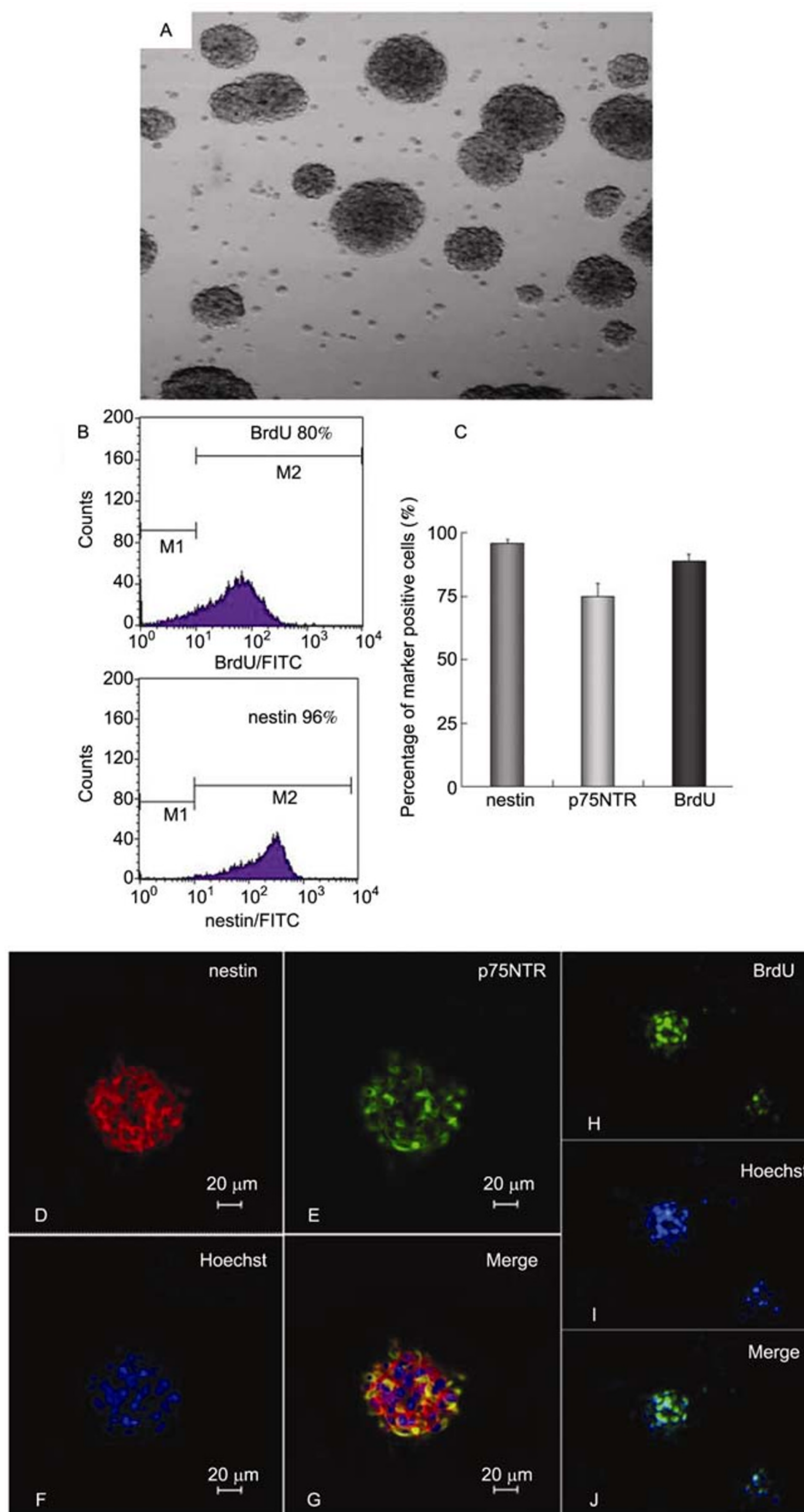


Figure 1 Characterization of primary cell spheres isolated from DRGs. A phase contrast micrograph showing the morphology of DRG-derived cells (A). Magnification×100. Representative flow cytometry images (B) showing the percentage of nestin positive or BrdU positive cells. The histogram (C), obtained for 10 randomly selected cell spheres immunostained, showing the percentage of nestin positive or BrdU positive cells. Anti-nestin (D) and anti p75NTR (E) immunocytochemistry of densely packed cell spheres, the nuclei of which were counterstained with Hoechst 33342 (F), and the merger of (D), (E) and (F) is shown in (G). The cell spheres stained with BrdU (H) or Hoechst 33342 (I). The merger of (H) and (I) is shown in (J).

cells from the primary spheres, 237 secondary neurospheres were formed, accounting for cloning efficiencies of 35.2%. We further examined the cloning efficiency of secondary spheres. Of 847 individual cells from the secondary spheres,

344 tertiary spheres were formed. The cloning efficiency for spheres from the secondary spheres was 40.6%. Immunocytochemistry (Figure 1D and E) showed that a high proportion of the floating cell aggregates expressed *nestin* and *p75NTR*, the markers of neural stem/progenitor cells [2,13], and both markers were co-localized in the same cell sphere (Figure 1G). BrdU labeling indicated that a portion of newly born cells were mitotically active, showing a feature of cell proliferation (Figure 1H–J). The positive ratio of *nestin* and BrdU was respectively 96% and 89%, according to flow cytometry (Figure 1B and C). These results collectively indicated that DRG-derived cell aggregates contained neural stem/progenitor cells, and were designated as neurospheres.

2.2 DRG-derived neurospheres in the differentiation medium

The neurospheres were cultured in differentiation conditions after removal of bFGF and EGF in the presence of FBS. After 10-d culture, immunochemistry was applied to individual clones, neuronal and glial cell lineages were observed to coexist in single clones, or the clones respectively showed the marker of neuronal and glial cell lineages (Figure 2). The number of differentiated clones was counted, with 7.4% of the clones exclusively differentiating into neurons, 23% into glial cells, and 69.6% into both glia and neurons.

In the culture medium added to NGF, DRG-derived neurospheres stopped proliferation in most clones, and grew on the surface of coverslips forming cell monolayers, a majority of which were NF-positive, displaying a bipolar or multipolar neuron appearance (Figure 3A). By contrast, in the culture medium added to bFGF, DRG-derived cells became spindle-shaped with the typical *in vitro* morphology of Schwann cells (SCs) as previously described [20]. The differentiated cells expressed *GFAP* and *S100β*, the markers for SCs (Figure 3B).

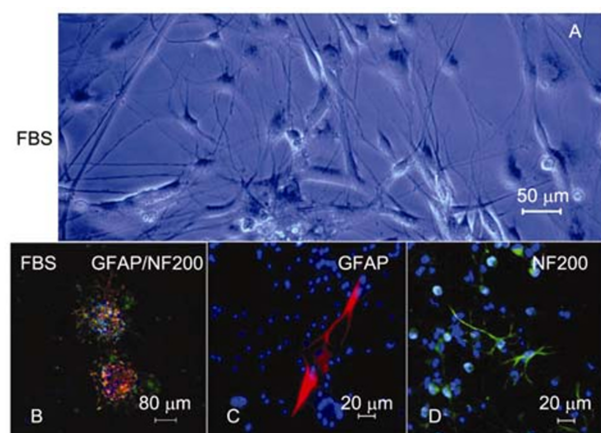


Figure 2 Differentiation of secondary cell spheres derived from DRGs after being cultured in FBS. A phase contrast micrograph showing the differentiated cell phenotypes (A). Fluorescent micrographs respectively showing differentiated cell spheres double immunostained with NF200 and GFAP (B), and GFAP positive (C) or NF 200 positive (D) cells. Scale bar, 50 (A), 80 (B), and 20 (C and D) μm.

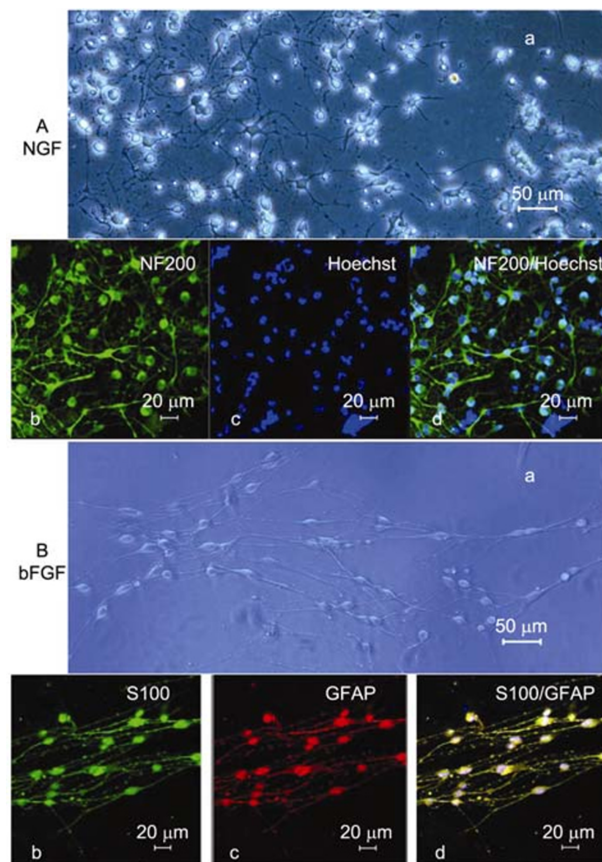


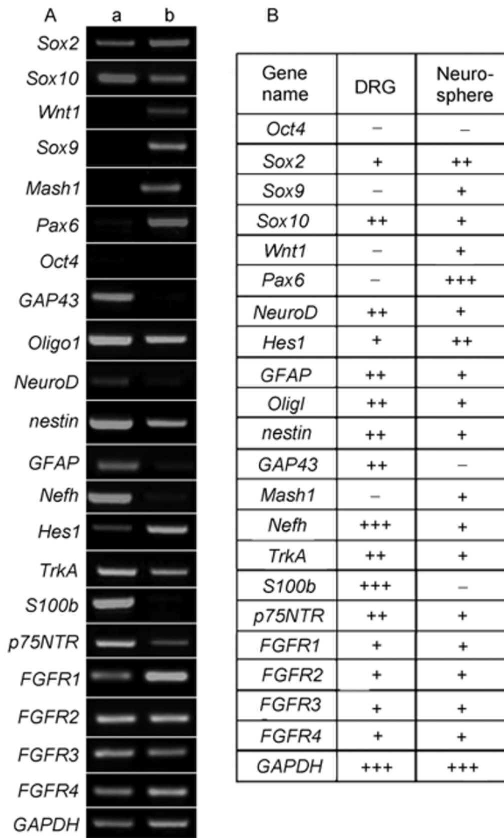
Figure 3 Directed differentiation of secondary cell spheres derived from DRGs after being cultured in the medium respectively added to NGF (A) or bFGF (B). The phase contrast micrograph showing the appearance of differentiated neuron-like cells (A(a)) or differentiated SC-like cells (B(a)). A fluorescent micrograph respectively showing differentiated cell spheres immunostained with *NF200* (A(b)) or Hoechst 33342 (A(c)). The merger of (A(b)) and (A(c)) is shown in (A(d)). A fluorescent micrograph respectively showing differentiated cell spheres immunostained with *S100β* (B(b)) or *GFAP* (B(c)). The merger of (B(b)) and (B(c)) is shown in (B (d)). Scale bar, 50 (A(a) and B(a)) and 20 (A(b–d) and B(b–d)) μm.

2.3 DRG-derived neurospheres express stem cell-related genes

We further compared the gene expression profiles of DRG tissues, and neurospheres isolated from DRGs by using RT-PCR data (Figure 4). RT-PCR data indicated that the neurospheres expressed proneural basic helix-loop-helix transcription factors (*NeuroD*, *Mash1*, and *Oligo1*), myelinating genes (*Sox10* and *Sox2*), morphogens and paired-box genes involved in the maintenance of the NSC niche (*Wnt1* and *Pax6*), as well as neural crest-specific genes (nestin and *p75NTR*).

2.4 Differentiation of DRG-derived cells under growth factors

We detected the gene expression of undifferentiated neurospheres and differentiated cells by RT-PCR, all of which



Gene name	DRG	Neuro-sphere
Oct4	−	−
Sox2	+	++
Sox9	−	+
Sox10	++	+
Wnt1	−	+
Pax6	−	+++
NeuroD	++	+
Hes1	+	++
GFAP	++	+
Oligl	++	+
nestin	++	+
GAP43	++	−
Mash1	−	+
Nefh	+++	+
TrkA	++	+
S100b	+++	−
p75NTR	++	+
FGFR1	+	+
FGFR2	+	+
FGFR3	+	+
FGFR4	+	+
GAPDH	+++	+++

Figure 4 The gene expression profiles, as respectively revealed by representative RT-PCR bands, of DRG tissues (A(a)), DRG-derived cell spheres (A(b)). *GAPDH* served as the internal control. B, Tabulated results of RT-PCR for the tested genes. The intensities of signal are scored in four arbitrary units: –, not detectable (no signal); +, detectable (weak signal); ++, easily detectable (moderate signal); +++, strongly detectable (strong signal).

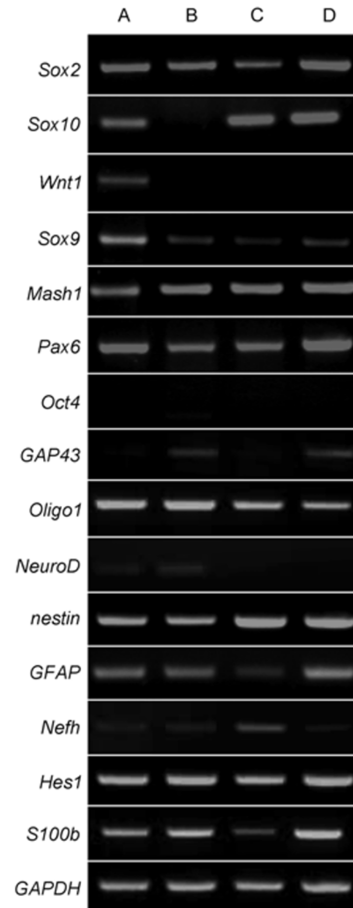


Figure 5 The gene expression profiles, as revealed by representative RT-PCR bands, of neurospheres (A), and differentiated cells after being cultured in the medium respectively added to FBS (B), NGF (C), or bFGF (D). GAPDH served as the internal control.

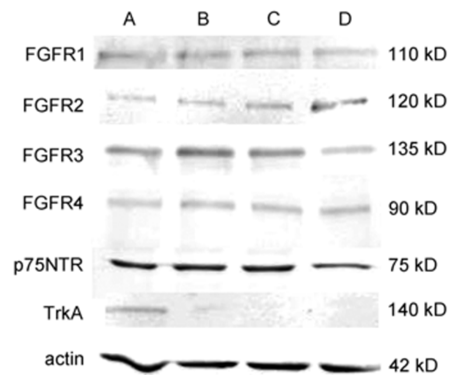


Figure 6 Expression levels of *FGFRs*, *p75NTR*, *TrkA* protein in DRG-derived cell aggregates in the undifferentiated and differentiated forms, as shown by the representative Western blot bands. A, DRG-derived cell spheres; B–D, differentiated cells after being cultured in a medium respectively added to FBS, NGF, or bFGF. *β-actin* served as the internal control.

expressed such genes as *Sox2*, *Sox9*, *Pax6*, *Mash1*, *Oligo1*, *nestin*, *Nefh*, *GFAP*, *GAP43* and *S100β*, while the differences in selected inherent characteristics were also noted (Figure 5). Likewise, Western blot analysis showed the different expression levels of the bFGF receptor (FGFRs) and the NGF receptor (*Trks* family and *p75NTR*) in the cells cultured in the medium with different additives (Figure 6).

3 Discussion

In this study, we isolated the neural stem cell or progenitor cells from the DRG. We found that the cells formed neurospheres and generated secondary and tertiary spheres by cloning assays. The cells in the cloned spheres differentiated into neuronal and glial forms. Based on previously established criteria [3,9], we conclude that the cells from DRG are most likely neural crest progenitors.

To investigate multipotency, a key characteristic of stem cells, of DRG-derived neurospheres, immunochemistry was applied to individual clones cultured in the presence of FBS, suggesting that DRG-derived neurospheres could differentiate into different cell lineages. In the culture medium added with NGF, DRG-derived neurospheres displayed a bipolar

or multipolar neuron appearance (Figure 3A). By contrast, in the culture medium added to bFGF, DRG-derived cells turned spindle-shaped with the typical *in vitro* morphology of Schwann cells (SCs). These results demonstrated that DRG-derived cells, identified as neuronal stem/progenitor cells, have multiple differentiation potential, and their differentiation is influenced by environmental cues.

We detected several neural stem cell-specific genes, consistent with the view that the cells from DRG explants are likely neural progenitor cells. Those genes expressed in the neurospheres, including *Sox2*, *Sox10*, *Olig1*, *NeuroD*, *nestin*, *GFAP*, *S100β*, *Nefh*, *GAP43*, *FGFRs* (FGF receptors), *p75NTR*, and *TrkA*, were found to also be expressed in parental DRG tissues, suggesting that the gene expressions observed in the neurospheres were not an artifact of cell culture. Expressions of such genes as NeuroD, TrkA, Hes1 and Nefh were noticeably reduced or lost in the neurospheres as compared to DRG tissues. This result might be because untreated DRG tissues originally contained a higher abundance of these genes than their derived neurospheres or because these genes were down-regulated as a consequence of cell culture. Conversely, the genes that were up-regulated in the neurospheres included *Sox2*, *Sox9*, *Wnt1*, *Mash1*, *GFAP*, *Pax6*, *FGFRs* and *GAP43*. This gene expression profile is consistent with results obtained in cloning analysis and immunocytochemistry. Especially, the presence of such NSC-specific genes, as *Sox2*, *Pax6*, and *Mash1*, provided further evidence that neural stem/progenitor cells could be isolated from DRGs. As Wnt signaling is known to promote sensory neurogenesis in early NSCs [14] and participate in the maintenance of NSCs [15], the *Wnt1* expression in the neurospheres might suggest an involvement of Wnt signaling in the formation of neural progenitor cells. The expression of *Hes1*, one of the *Notch1* downstream effectors, in the neurospheres may underpin the migratory and proliferating features of neural progenitor cells. The expression of *Sox2*, *Oligo1* and *GFAP* in the neurospheres might imply their glial potential and possible neural crest origin, and the upregulation of *Sox10*, a transcription factor for NSCs, might be further evidence supporting their possible neural crest origin [16–19]. RT-PCR data represented the average characteristics of cell population in neurospheres, rather than the properties of individual cells. Gene expression analysis offered substantial evidence that a subpopulation of DRG-derived cells was likely composed of neural crest progenitor cells with multipotency and self-renewal capacity. DRG-derived neurospheres expressed nearly all of the stem cell-related genes found in NSCs, indicating that the neurospheres might have a close molecular relationship with NSCs.

After culturing the cells in a medium with different additives, the increased expression of *NeuroD*, *Hes1* and *Mash1*, proneural genes of basic HLH transcription factors, in differentiated cells suggested the neuronal differentiation of DRG-derived neurospheres. In accordance with the migratory capacity of NSCs, several genes related to stem cell migration and trafficking were up-regulated in the neurospheres and differentiated cells. The differentiated cells induced by NGF and bFGF, rather than those induced by FBS, were observed to express *Sox10*, the high mobility group transcription factor. Considering the role of *Sox10* for glial specification, we assumed that FBS-induced differentiation of the neurospheres toward glial cells might be mediated by a different pathway without the involvement of *Sox10* as compared with bFGF-induced differentiation, while NGF-induced differentiation failed to generate glial cells possibly due to the lack of instruction concerning the commitment to a glial fate [21–24]. Otherwise, bFGF treatment of the progeny of NSCs increased the proliferative rate of precursors and the number of SC-like cells generated, whereas the number of differentiating neurons was significantly reduced, which was explainable by their different receptors. *FGFRs* were identified in many early precursors and in the oligodendrocyte lineage and astrocytes, and multiple *FGFRs* expression partly determined cell lineage-specific [25]. The expression of *TrkA*, *TrkB*, *TrkC* and *p75NTR* is a characteristic of neural crest progenitors during development [26,27], whereas neurotrophins are essential for cell survival, proliferation, and differentiation [28,29]. The observation that DRG-derived neurospheres co-expressed the neurotrophin receptors such as *FGFRs*, *Trks* family and *p75NTR* suggests that the neurospheres responded to neurotrophins, and thus explains why the neurotrophins, including NGF and bFGF, were responsible for the survival, proliferation and directed differentiation of the neurospheres. However, *TrkA* was not expressed in the differentiated cells induced by NGF. This suggests that the expression of *TrkA* in the neurospheres was unlikely to be regulated by NGF. The absence of TrkA in the neurospheres also suggests that the neurospheres may sit at an earlier developmental stage than the sensory neuronal progenitors. The details of how neurotrophins affect the survival, proliferation and differentiation of the stem/progenitor cells isolated from DRGs need further investigation.

To summarize, we isolated the neural stem/progenitor cells from DRGs, and found that different neurotrophins induced the differentiation of DRG-derived cells toward neurons, glia cells or SCs, as shown by morphological observation and immunochemistry with cell markers. Our findings suggest that DRG-derived neural stem/progenitor cells represent a promising alternative to neurons or SCs for cell-based therapeutic strategies including peripheral nerve repair applications.

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