

In vitro neural/glial differentiation potential of periodontal ligament stem cells

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Abstract

Introduction: It is known that periodontal ligament stem cells (PDLSCs) can differentiate into cementoblast-like cells, adipocytes and collagen-forming cells. However, whether PDLSCs are able to differentiate into Schwann cells and which method is best for their neural induction remain unknown. We attempted to determine whether PDLSCs possessed the potential for neural differentiation *in vitro*.

Materials and methods: We isolated and multiplied PDLSCs from periodontal ligaments obtained from the teeth ($n = 24$) of 8-month-old beagle dogs. Four protocols with different chemicals and growth factors were adopted to induce the PDLSCs to differentiate into Schwann cells. Immunohistochemistry, RT-PCR and qRT-PCR were performed to investigate the *in vitro* neural differentiation potential of PDLSCs.

Results: We compared the 4 different protocols and showed that all 4 protocols could successfully induce PDLSCs to express nestin, GFAP and S100, markers for Schwann cells. Further, qRT-PCR revealed relative differences in the expression levels of these 3 genes in differentiated PDLSCs obtained by different protocols.

Conclusions: We conclude that PDLSCs have neural/glial differentiation potential *in vitro* and that neural/glial differentiation can be induced in PDLSCs if suitable protocols are followed. We also found that supplementing the growth medium with suitable growth factors is more effective than applying chemicals alone. While nerve growth factor is more effective than platelet-derived growth factor for inducing neural/glial differentiation in PDLSCs, pre-induction of PDLSCs with dimethyl sulphoxide yields better results than those obtained with all-*trans*-retinoic acid.

Key words: periodontal ligament, stem cells, neural/glial differentiation, Schwann cells.

Introduction

Seo *et al.* [1] isolated periodontal ligament stem cells (PDLSCs) for the first time in 2004, and the stem-cell characteristics of PDLSCs have since been verified repeatedly by different groups [2-5]. Kawanabe *et al.* reported that about 3.9% of periodontal ligament cells (PDLs) express early mesenchymal stem-cell markers such as STRO-1 and CD146/MUC18 [4]; another authors reported that approximately 30% of PDLs could form single-cell colonies, and then 30% of these colonies express STRO-1 [5].

In vitro, PDLSCs can differentiate into cementoblast-like cells, adipocytes and collagen-forming cells, and when transplanted into immunocompromised rodents, PDLSCs can generate a cementum/PDL-like structure [1, 3-5]. Considering the periodontal tissue regeneration ability of PDLSCs, we reasoned that it would be ideal to seed these cells around a tooth implant to induce periodontal ligament reconstruction. Schwann cells (SCs) are known to play a pivotal role in the regeneration, development and function maintenance of peripheral nerves [6-8], but until now, whether PDLSCs could differentiate into SCs and whether such differentiated periodontal ligament stem cells (dPDLSCs) could improve the sensation and perception of tooth implants was unknown.

Bone marrow stromal cells (BMSCs) can differentiate into neural-like cells under certain conditions [9-11]. Recently, adipose-derived stem cells (ADSCs) have also been shown to undergo neural differentiation *in vitro* [12-15]. Therefore, we proposed that PDLSCs could also differentiate into neural-like cells or SCs *in vitro*. To test this hypothesis, we attempted to induce PDLSCs to undergo neural differentiation by using 4 different protocols: protocol A adopted chemicals only; protocol B added some growth factors; protocol C replaced platelet-derived growth factor with nerve growth factor; and the only difference between protocols C and D was that all-*trans*-retinoic acid was used in protocol D instead of DMSO in C. We then compared their neural/glial induction efficiency. Our positive results show that differentiated PDLSCs have a great potential to be applied in nerve tissue engineering around the tooth implant and hence may improve its sensation and perception in the future.

Materials and methods

All animal experiments in this report were approved by the Ethical Guideline Committee for Animal Care, West China College of Medical Sciences, Sichuan University.

Isolation, culture and identification of PDLSCs

Twenty-four teeth from 4 normal 8-month-old beagle dogs were collected under aseptic conditions and placed in phosphate-buffered saline (PBS) solution supplemented with 1% (v/v) penicillin/streptomycin for 5 min. According to the report of Seo *et al.* [1], the periodontal ligament was gently separated from the surface of the tooth root and then dissociated with 0.25% trypsin (Sigma, UK) and 0.1% collagenase (Sigma, UK) for 90 min at 37°C. The solution was passed through a 75- μ m filter to remove undissociated tissue, neutralized with alpha-Modified Eagle Medium (α -MEM;

Hyclone, USA) containing 20% (v/v) fetal bovine serum (FBS) (Hyclone, USA) and centrifuged at 1000 \times g for 8 min. Then, the cell pellet was resuspended in α -MEM containing 20% (v/v) FBS with 1% (v/v) penicillin/streptomycin solution and cultured in an incubator at 37°C with 5% carbon dioxide. The culture medium was first changed 7 days later and then changed regularly at 3-day intervals. The cells were passaged with trypsin/EDTA (Sigma, UK) when required.

To isolate PDLSCs, we used single-colony selection [1, 2]. Briefly, single-cell suspensions of PDLSCs (1-2 cells/200 μ l) were seeded into 96-well culture plates at a density of 1-2 cells/well. The plates were then incubated at 37°C with 5% carbon dioxide. The medium was changed every 3 days. After 2 weeks, aggregates of more than 50 cells were scored as colonies; these were digested, pooled together and transferred to 6-well culture plates for propagation. The morphology of these PDLSCs was observed under a phase-contrast microscope. PDLSCs were identified by seeding cells onto coverslips and immunostaining with antibodies against STRO-1, cytokeratin, vimentin (1 : 100 dilution) (Santa Cruz, CA, USA) and CD146/MUC18 (1 : 100 dilution) (Zymed Laboratories, USA). The coverslips were examined using an Olympus IX 70 inverted phase-contrast microscope.

The potential of PDLSCs to differentiate into multiple phenotypes has been investigated, as previously described [1, 2, 14]. Briefly, to induce differentiation into the osteogenic phenotype, the cultures were treated with 100 μ g/ml ascorbate, 0.1 μ M dexamethasone and 10 mM β -glycerophosphate for 3 weeks. The cells were then fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature and washed 3 times with PBS. To stain any calcium deposits, the cells were then incubated with 1% (w/v) alizarin red solution. To induce differentiation into the adipogenic phenotype, the cells were treated with 0.5 mM isobutyl-methylxanthine (IBMX), 1 μ M dexamethasone, 10 μ M insulin and 200 μ M indomethacin for 3 weeks. The cells were then fixed with 10% (v/v) formalin for 60 min and washed in H₂O. Lipid clusters in the cells were stained with oil red O.

Protocols used to induce PDLSCs to differentiate into SCs

Undifferentiated PDLSCs (uPDLSCs), which were at 3 or 4 passages, about 3 weeks after primary culture and 2 weeks after single colony isolation, were used as the control group. To induce differentiation of the PDLSCs to the SC phenotype, the growth medium was removed from sub-confluent cultures and replaced with medium supplemented with 1 mM β -mercaptoethanol

(Sigma-Aldrich, UK) for 1 day. Then, 4 different protocols were followed to induce differentiation of the PDLSCs [15].

1. Protocol A: The cells were washed and cultured in α -MEM without FBS supplemented with 2% dimethyl sulphoxide (DMSO) for 5 h.
2. Protocol B: As in protocol A, the cells were first washed. The medium was replaced with a differentiation medium, i.e. cell-growth medium supplemented with 10 ng/ml platelet-derived growth factor (PDGF) (PeproTech Ltd., UK), 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech Ltd., UK), 14 μ M forskolin (Sigma, USA) and 10 ng/ml brain-derived neurotrophic factor (BDNF) (PeproTech Ltd., UK). The cells were incubated for 2 weeks under these conditions, and fresh medium was added approximately every 3 days.
3. Protocol C: This protocol was the same as Protocol B, except that 10 ng/ml nerve growth factor (NGF) (PeproTech Ltd., UK) was used instead of 10 ng/ml PDGF.
4. Protocol D: The cells were washed and cultured in fresh medium supplemented with 35 ng/ml all-*trans*-retinoic acid (Sigma, USA). After 3 days, the cells were washed, and the medium was replaced with a differentiation medium, as described in protocol C. The only difference between the last two protocols was that DMSO in protocol C was replaced by all-*trans*-retinoic acid in protocol D.

Immunocytochemistry

According to former reports [1, 2, 14], uPDLSCs and dPDLSCs were seeded on glass coverslips for 1 day and fixed in 4% (w/v) paraformaldehyde in PBS (pH = 7.5) for 30 min. They were then washed with PBS, before treatment with 3% (v/v) H₂O₂ for 5 min. The coverslips were incubated for 1 h with blocking buffer, i.e. 5% normal goat serum (Sigma, UK) in 0.1% Triton X-100 in PBS and then with primary antibodies, including STRO-1 (1 : 100), cytokeratin (1 : 100), vimentin (1 : 100), CD146/MUC18 (1 : 100),

S100 (1 : 100) (Santa Cruz, USA), glial fibrillary acidic protein (GFAP) (1 : 200) (Santa Cruz, USA) and nestin (1 : 100) (Santa Cruz) at 4°C overnight. After thorough washing, the coverslips were incubated with biotinylated secondary antibodies and then with horseradish peroxidase-conjugated streptavidin. The peroxidase reaction was developed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen. The coverslips were counterstained with haematoxylin and then examined under an Olympus IX 70 inverted phase-contrast microscope (Olympus, Japan). STRO-1 was incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1 : 100) (Santa Cruz), and the fluorescence signals were detected with an Olympus IX 70 fluorescence microscope (Olympus, Japan).

RNA reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR analysis

Total RNA was extracted using the Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. RT-PCR and quantitative real-time PCR were performed as described before [2, 15]. Briefly, the yield and quality of the RNA were assessed by measuring the absorbance at 260 and 280 nm, followed by electrophoresis on a 3% (w/v) agarose gel. Total RNA (500 ng) was reverse-transcribed into complementary DNA (cDNA) with a PrimeScript™ RT Reagent Kit (Perfect Real Time, Takara, Japan) according to the manufacturer's guidelines. Primers of S100, GFAP, nestin and the internal calibrator glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (synthesized by Takara Biological Technology Co. Ltd., Dalian, China) and their related GenBank accession numbers are listed in Table I. PCR reaction solution (25 μ l: 12.5 μ l, 2 \times SYBR Premix Ex Taq™; 0.5 μ l, 10 μ mol/l forward primer; 0.5 μ l, 10 μ mol/l reverse primer; 0.5 μ l, 50 \times ROX Reference Dye1, 2 μ l, cDNA; and 9 μ l, dH₂O) was amplified using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA). The thermocycle of the PCR reaction was as follows: After an initial

Table I. Primer sets for RT-PCR and real-time PCR and GenBank accession numbers

Primer	Sequences (5'-3')	Product size [bp]	GenBank accession no.
S-100	For: ctgcacgtgttccagcagta Rev: ctgtccagtgtctccatgacc	155	XM548737
NESTIN	For: gggaagaatgtggtcaggact Rev: catcagactccctatccctgtc	165	XM547531
GFAP	For: ctggatctggagaggaagattg Rev: ttctcttaggctgctgtgag	162	XM843285
GAPDH	For: gatgctggtgctgagtatgttg Rev: agaaggagcagagatgatgacc	111	NM001003142

For. and Rev. indicate forward, reverse primer, respectively. All primers were purchased from Takara Biological Technology Co. Ltd, (Dalian, China)

denaturation at 95°C for 10 s, 45 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 31 s were performed. The PCR products were analysed using agarose gel electrophoresis (BIO-RAD, USA). During the real-time PCR, fluorescence signals were collected during annealing. The cycle of threshold value (Ct) was calculated for further statistical analysis. In order to examine the efficiency of the real-time PCR, standard curves were established using a serial dilution of the sample RNA (500 ng; S100, GFAP, nestin and GAPDH; 10 × serial dilution). The total RNA of the uPDLSCs was used as the non-template control, which was included in all experiments and in the RT-PCR in order to evaluate DNA contamination of the reagents used for the amplification. None of the experiments resulted in a positive signal from the non-template control.

Statistical analysis

All data were expressed as mean ± SEM. Statistical calculations were performed using SPSS software (SPSS, Chicago, IL, USA). Inter-group comparisons were made by a one-way analysis of

variance (ANOVA), and then Tukey's honestly significant difference (HSD) test was performed for multiple inter-group comparisons. *P* values less than 0.05 were considered significant.

Results

Under the phase-contrast microscope, the PDLSCs appeared mainly polygonal with a nucleus in the centre. Immunocytochemical assessment of the cell-surface markers on PDLSCs revealed that the cells were positive for STRO-1, CD146/MUC18 and vimentin but negative for cytokeratin (Figure 1). The results indicated that the cultured PDLSCs showed some characteristics of adult stem cells – they expressed 2 early mesenchymal stem-cell markers, STRO-1 and CD146/MUC18. Vimentin expression further indicated that the cultured PDLSCs were mesenchymal stem cells derived from the embryonic mesoderm. Lack of cytokeratin expression excluded the possibility that the cells were from the ectoderm. These results are consistent with other reports [1, 5, 16].

To investigate the potential to differentiate into multiple phenotypes of the cells isolated from the periodontal ligament, the cells were treated with

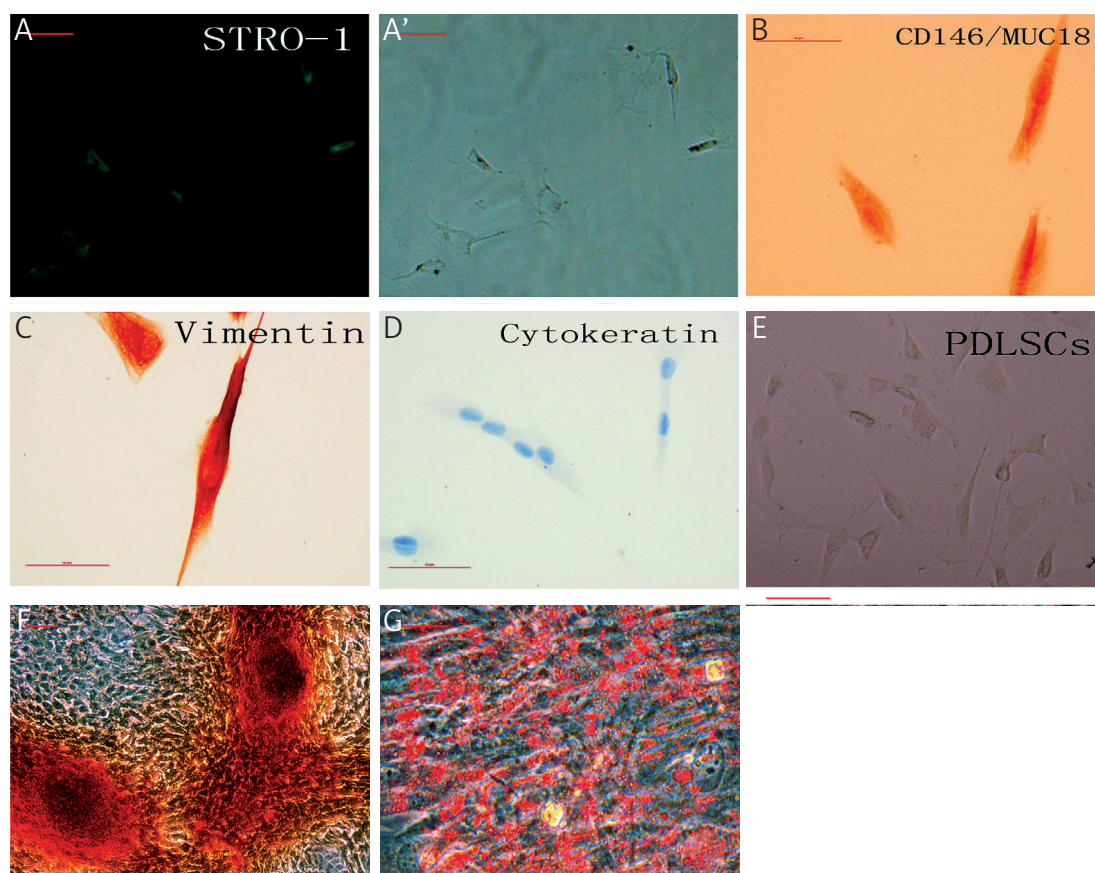


Figure 1 Immunocytochemical assessment of PDLSCs cell markers. **A** – stro-1; **A'** – live cell picture corresponding to **A**; **B** – CD146(+); **C** – vimentin (+); **D** – cytokeratin (-); **E** – PDLSCs showed little polygon morphology; **F** – alizarin red staining showed some mineralized nodule formation in PDLSCs cultures; **G** – cultured PDLSCs formed Oil Red O-positive lipid clusters. Scale bar = 50 µm

agents known to induce differentiation into the osteoblastic and adipogenic phenotypes. Differentiation into the osteoblastic phenotype was confirmed by the presence of calcium deposits, as detected with alizarin red (Figure 1F), and differentiation into the adipogenic phenotype by the presence of oil red O-positive lipid clusters (Figure 1G).

While the uPDLSCs were negative for the neural progenitor-cell marker nestin and glial cell markers S100 and GFAP, all the dPDLSCs were positive in different degrees for nestin, S100 and GFAP (Figure 2).

To confirm the immunocytochemistry results, RT-PCR was performed, and the result is shown in Figure 3. All dPDLSCs from all 4 protocols were

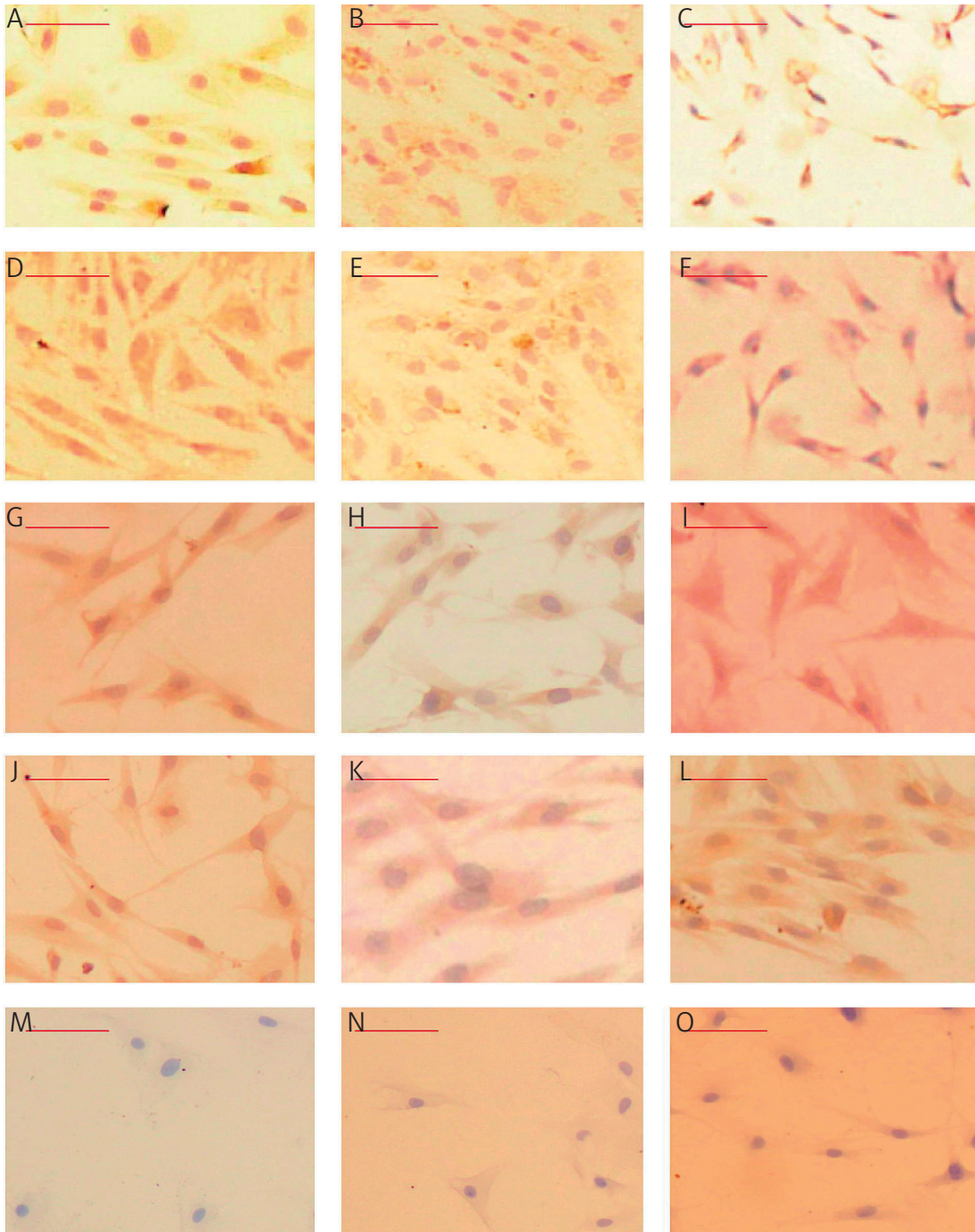


Figure 2. All differentiated PDLSCs after induction with protocol A, B, B and D showed positive in different degrees for S-100, Nestin and GFAP; but undifferentiated PDLSCs showed negative for S-100, Nestin and GFAP. Protocol A: **A.** S100 (+), **B.** Nestin (+), **C.** GFAP (+); Protocol B: **D.** S100 (+), **E.** Nestin (+), **F.** GFAP (+); Protocol C: **G.** S100 (+), **H.** Nestin (+), **I.** GFAP (+); Protocol D: **J.** S100 (+), **K.** Nestin (+), **L.** GFAP (+); PDLSCs: **M.** S100 (-), **N.** Nestin (-), **O.** GFAP (-). Scale bar = 50 μ m

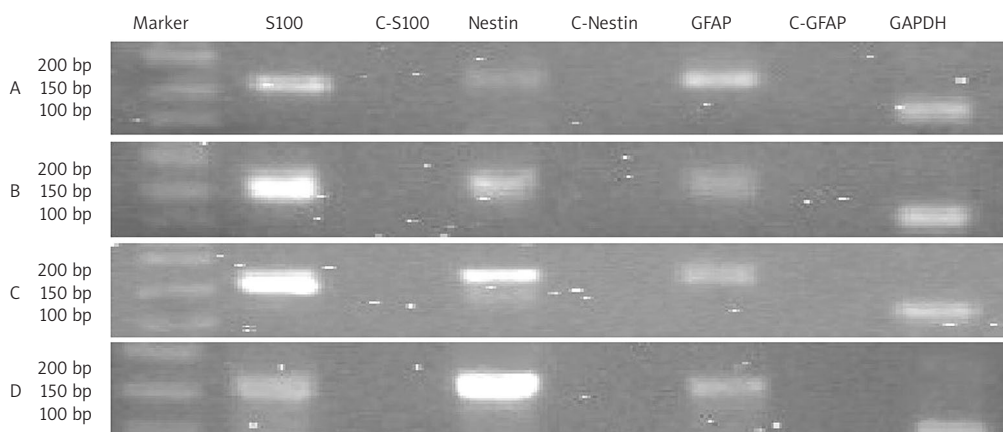


Figure 3. In above figures, all the differentiated PDLSCs from protocols A, B, C and D showed positive strips for S100, Nestin and GFAP, while the control groups (undifferentiated PDLSCs: C-S100, C-Nestin and C-GFAP) showed no positive strips for S100, Nestin and GFAP. We used GAPDH as a reference for assessing PCR efficiency

positive for S100, nestin and GFAP, but the uPDLSCs were negative for S100, nestin and GFAP. Subsequently, we performed real-time PCR to consolidate the findings. For S100 and GFAP, statistical analyses showed no statistically significant differences between protocols A and B, or C and D, respectively ($P > 0.05$). However, the

results of protocols C and D showed statistically significant differences as compared with those of protocols A and B ($P < 0.05$). Gene expression of S100 in protocols C and D was higher than that in protocols A and B (Tables II and III). For nestin gene expression, there were no statistically significant differences between protocols A and B ($P > 0.05$), but there were differences between protocols C and D ($P < 0.05$). All the results of protocols C and D were significantly higher than those of protocols A and B ($P < 0.05$), and nestin gene expression was the highest in protocol C, closely followed by protocol D (Table IV, Figure 4).

Table II. S100 gene expression relative quantity difference analyses

	Group	N	Subset for alpha = 0.05	
			1*	2*
Tukey HSD**	A	3	0.8817	
	B	3	0.9638	
	C	3		4.6976
	D	3		5.8230
	Sig.		1.000	0.664

*Means for groups in homogeneous subsets are displayed
 **Uses harmonic mean sample size = 3.000
 For groups A, B, C and D, there are no statistical differences within subset 1 or 2, but there are statistical differences between subsets 1 and 2

Discussion

In 2000, Woodbury and colleagues first demonstrated that BMSCs could be induced to differentiate into the neural phenotype *in vitro* with β -mercaptoethanol, DMSO and butylated hydroxyanisole as the neuronal induction medium. However, these expressions were not sustained for more than 6 days [17]. Thereafter, several studies

Table III. GFAP gene expression relative quantity difference analyses

	Group	N	Subset for alpha = 0.05	
			1*	2*
Tukey HSD**	B	3	0.0000	
	A	3	3.4654	
	D	3		9.1448
	C	3		10.2916
	Sig.		0.272	0.911

*Means for groups in homogeneous subsets are displayed
 **Uses harmonic mean sample size = 3.000
 For groups A, B, C and D, there are no statistical differences within subset 1 or 2, but there are statistical differences between subsets 1 and 2

Table IV. Nestin gene expression relative quantity difference analyses

	Group	N	Subset for alpha = 0.05		
			1*	2*	3*
Tukey HSD**	A	3	0.0000		
	B	3	0.6357		
	D	3		3.7274	
	C	3		5.9861	
	Sig.		0.286	1.000	1.000

*Means for groups in homogeneous subsets are displayed
 **Uses harmonic mean sample size = 3.000
 For groups A, B, C and D, there are no statistical differences within subset 1 or 2, but there are statistical differences between subsets 1 and 2

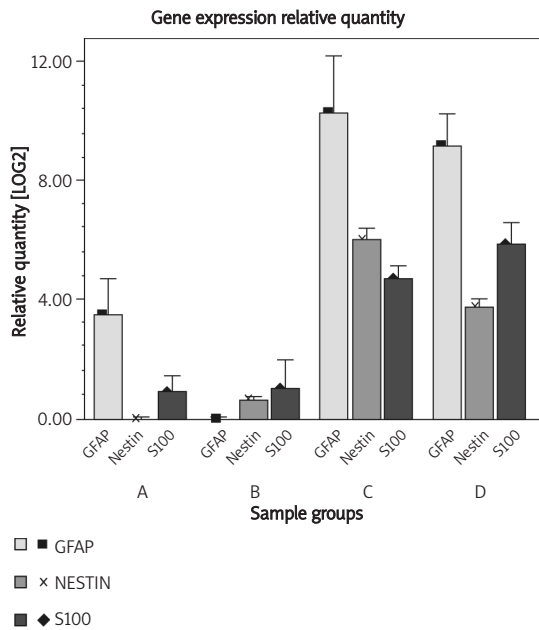


Figure 4. In the graph, bar height represents the gene expression relative quantity (mean \pm SEM). For GFAP and S100 gene expression relative quantity, statistic analyses show no statistical differences between group A and B, C and D, respectively ($P > 0.05$), but group C and D have significantly statistical differences as compared with group A and B ($P < 0.05$); for Nestin gene expression relative quantity, there are no statistical differences between group A and B ($P > 0.05$), but there are statistical differences between group C and D ($P < 0.05$), and the results of group C or D are significantly higher than those of group A or B ($P < 0.05$)

reported that stable neural differentiation of BMSCs and ADSCs could be induced by treating the cells with chemicals combined with some growth factors such as PDGF, NGF, bFGF and BDNF [14, 15, 18-22]. Based on these studies, we adopted 4 protocols for inducing the neural differentiation of PDLSCs: one with chemicals alone and the 3 others involving chemicals in conjunction with different growth factors. All 4 protocols successfully induced the PDLSCs to differentiate into SCs, suggesting that PDLSCs, like BMSCs and ADSCs, have neural differentiation potential.

Comparing protocol A with B and C showed that adding suitable growth factors is more effective than chemicals alone for inducing the neural/glial differentiation of PDLSCs. Further comparing protocol B with C, we observed that the neural/glial induction efficiency of protocol C was significantly better. NGF is a potent nerve growth factor and plays a crucial role in the development and preservation of the sensory and sympathetic nervous systems; PDGF is a potent mitogen for connective tissue cells and is involved in biological processes such as hyperplasia and embryonic neuron development. Both factors can promote

neural differentiation of PDLSCs. However, with regard to inducing PDLSCs to differentiate into SCs, NGF appears to be more effective than PDGF.

The only difference between protocol C and D was the pre-induction agent: 2% DMSO for 5 h in protocol C and 35 ng/ml all-*trans*-retinoic acids for 72 h in protocol D. All-*trans*-retinoic acid was used as a cell-differentiation factor by Sanchez and colleagues [18], and recently it was considered a pre-induction agent, which was to be used prior to the addition of cell growth factors [14]. Although the mechanism of DMSO to induce neural differentiation is still unknown, DMSO has been shown to play an important role in the neural differentiation of stem cells [16, 20]. In addition to enhanced expression of the nestin gene, we also observed that PDLSCs treated with protocols C and D expressed relatively higher levels of the S100 and GFAP genes, indicating that the neural/glial-induction efficiencies of protocols C and D for PDLSCs are comparable. Thus, considering the lower price and faster action of DMSO, we strongly recommend that DMSO be used as a pre-induction agent for the neural/glial differentiation of PDLSCs.

We conclude that PDLSCs have neural/glial differentiation potential *in vitro* and that neural/glial differentiation can be induced in PDLSCs if suitable protocols are followed. We also found that supplementing the growth medium with suitable growth factors is more effective than applying chemicals alone. While NGF is more effective than PDGF for inducing neural/glial differentiation in PDLSCs, pre-induction of PDLSCs with DMSO yields better results than those obtained with all-*trans*-retinoic acid.

Acknowledgments

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