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Original Article

Identification of neurospheres generated from human dental pulp stem cells in xeno-/serum-free conditions

Yoko Kawase-Koga [a, *], Yasuyuki Fujii [a, b], Daiki Yamakawa [a], Marika Sato [a], Daichi Chikazu [a]

[a] Department of Oral and Maxillofacial Surgery, Tokyo Medical University Hospital, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
[b] Department of Oral Health and Diagnostic Sciences, School of Dental Medicine, University of Connecticut Health, Farmington, CT, 06030, United States

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ABSTRACT

Introduction: Cell-based therapies require an emerging alternative treatment using easily harvested cell sources. Neural stem cells derived from various tissues, including brain, bone marrow, skin and retina can give rise to both neurons and glial cells. Recently, human dental pulp stem cells (DPSCs) and stem cells from human exfoliated deciduous teeth (SHED) were demonstrated to have mesenchymal stem cell-like abilities such as self-renewal and multi-lineage differentiation, including neuron and glial cells. Moreover, DPSCs and SHED show a higher proliferation rate and a higher number of population doublings compared with adult bone marrow stromal stem cells. Therefore, DPSCs are a useful source that can be applied in cell replacement therapy for various neurological disorders. Generally, the conventional culture methods for DPSCs have used serum, therefore the undefined components in culture medium may complicate investigations of the molecular mechanisms that control the self-renewal and differentiation of DPSCs. However, neural stem cells proliferate to form 'neurospheres' in suspension *in vitro* in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). No study to date has obtained neurospheres from DPSCs in serum-free conditions in primary culture. Thus, the aim of this study was to establish a method for the proliferation and neural differentiation of DPSCs in xeno- and serum-free conditions in primary culture.
Methods: DPSCs were obtained from the dental pulp of wisdom teeth from healthy individuals (18–41 years old) and cultured in conventional medium containing 15% fetal bovine serum and xeno-/serum-free medium. We evaluated the proliferation of DPSCs, neurosphere generation, and neural differentiation under xeno-/serum-free conditions by flow cytometry, immunohistochemistry, and real-time polymerase chain reaction.
Results: In proliferation medium without xeno/serum, DPSCs can proliferate and generate neurospheres, however, the neurospheres had limited self-renewal ability. Under differentiation conditions, class III β-tubulin (TUBB3) and microtubule-associated protein (MAP2) were more significantly expressed in neurospheres derived from DPSCs in xeno-/serum-free culture conditions than in DPSCs in conventional culture conditions.
Conclusions: Our result demonstrated that neurosphere generation from DPSCs in xeno-/serum-free culture may be an accessible source for clinical cell replacement therapies for neuronal degenerative diseases.

1. Introduction

Neural stem cells (NSCs) for cell-based therapy are predicted to be one of the most important treatments for central and peripheral nervous system lesions such as spinal cord injury, stroke, and Alzheimer's disease [1,2]. NSCs derived from various tissues, including brain, bone marrow, skin, and retina can give rise to both

* Corresponding author. Fax: +81 3 3342 1723.
E-mail address: kogay@tokyo-med.ac.jp (Y. Kawase-Koga).
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Abbreviations

DPSCs	human dental pulp stem cells
SHED	human exfoliated deciduous teeth
EGF	epidermal growth factor
bFGF basic	fibroblast growth factor
TUBB3	III β-tubulin
MAP2	microtubule-associated protein
NSCsNeural	stem cells
MSCs	mesenchymal stem cells
GFAP	glial fibrillary acidic protein
FBS	fetal bovine serum
α-MEM	alpha modification of Eagle's medium
KSRKnockOut™	Serum Replacement
NEAA	non-essential amino acids
PBS	phosphate-buffered saline
FITC	fluorescein isothiocyanate
PE	phycoerythrin
PFA	paraformaldehyde
SEM	standard error of the mean
TGF-β	transforming growth factor-β

neurons and glial cells [3–6]. *In vitro*, NSCs proliferate to form 'neurospheres' in suspension in defined serum-free culture medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [6,7]. Once plated under adherent conditions for long-term culture, neurospheres have the ability to differentiate into neurons, astrocytes, and oligodendrocytes with migration of the cells out of the neurospheres [8,9].

Dental pulp stem cells (DPSCs) are mesenchymal stem cells (MSCs) that have the potential to differentiate into multiple cell lineages, such as adipogenic, neurogenic, and osteogenic cells [10–12]. Furthermore, stem cells from human exfoliated deciduous teeth (SHED) have high proliferation ability, self-renewal, and multi-lineage differentiation to neuron and glial cells [13]. Compared with other MSCs, DPSCs and SHED are easily obtained from extracted teeth by low-invasive surgery without any ethical issues and exhibit several different characteristics in their osteogenic, adipogenic, and oncogenic potential. In addition, DPSCs of neural crest origin express several neural markers such as nestin and astrocytic markers such as glial fibrillary acidic protein (GFAP) [10]. DPSCs are considered an appropriate cell source for nerve regeneration, and several studies reported the differentiation of DPSCs into neural-like cells *in vitro* and *in vivo* [14–16]. However, little is known about the cellular and molecular mechanisms of neural differentiation in DPSCs.

To date, there have been no studies demonstrating the primary culture of cells obtained from human DPSCs in xeno-/serum-free medium to form neurospheres that differentiate into neurons. Generally, the conventional culture method for DPSCs is the use of high concentrations (10%–20%) of serum to maintain cell viability [10–16]. Therefore, undefined components in the medium can complicate experiments examining the molecular mechanisms that control DPSC self-renewal and differentiation.

Moreover, for the clinical application of stem cells, the use of animal components such as fetal bovine serum (FBS) that are at risk of transmitting pathogens and immune responses to recipients must be considered [17,18]. FBS contains not only growth factors, nutrients, and hormones but also numerous uncharacterized constituents that vary in composition, which can transfer pathogens that interfere with standardized cell preparations [17]. Additionally, FBS may be added to stem cells during culture, generating an immune response in the host cells and leading to rejection of transplanted cells if repeat injections are required [18].

To overcome these issues, we established a method for the isolation and proliferation of DPSCs and their differentiation into neurons under xeno- and serum-free conditions in primary culture.

2. Methods

2.1. Cell isolation and primary culture

DPSCs were obtained from the dental pulp of wisdom teeth from healthy patients (n = 3–5, 18–41 years old) at Tokyo Medical University Hospital. This procedure was approved by the institutional ethics committee of the Faculty of Medicine, Tokyo Medical University (approval no.3486) and was performed after receiving written consent from all patients. DPSCs were collected as described previously [10]. Briefly, the dental pulp tissue was minced into pieces and digested in a solution containing 3 mg/ml collagenase type I (Sigma–Aldrich) for 45 min at 37 °C, and single-cell suspensions were obtained by passing the cells through a 70-μm cell strainer. The isolated cells were cultured on 100-mm dishes (BD, Franklin Lakes) at the density of 1×10^6 cells in normal conditions (alpha modification of Eagle's medium (α-MEM, GIBCO/BRL) supplemented with 15% FBS (Biowest) and 1% penicillin/streptomycin (P/S; Wako Pure Chemical Industries)) and serum-free conditions (knockout DMEM (GIBCO/BRL) containing 20% KnockOut™ Serum Replacement (KSR, GIBCO/BRL), 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol (Life Technologies), 4 ng/ml basic fibroblast growth factor (bFGF, Pepro Tech), and 0.1% P/S). To passage DPSCs in conventional condition, the cells were digested with Trypsin, for DPSCs in serum free condition, the cells were dissociated by AccutaseTM (Nacarai Tesque).

2.2. Neurosphere culture

The formation of neurosphere cultures was performed as described previously with minor modifications [3]. DPSCs were mechanically triturated into single cells with Accutase™. These cells were plated at a cell density of 10 cells/μl on uncoated 24-well dishes and cultured in DMEM/F12 (Gibco) with N2 supplement (Gibco), B27 supplement (Gibco), 10 ng/ml bFGF, and 20 ng/ml EGF (Invitrogen). The number of primary neurospheres per well was counted after 7 days of culture. For the self-renewal analysis of NSCs, primary neurospheres were dissociated with Accutase™ and passaged at a cell density of 10 cell/μl using the same culture conditions as in the primary culture.

2.3. Flow cytometry analysis

Cells isolated from neurospheres were suspended with 0.25% trypsin, washed with phosphate-buffered saline (PBS; GIBCO BRL), triturated into single cells, and filtered through a 70-μm cell strainer. After suspension, cells were blocked with 10% FBS for 10 min at 37 °C, and were immunolabeled for 90 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated anti-human CD14, FITC-human CD34, FITC-human CD44, FITC-human CD81, phycoerythrin (PE)-conjugated human CD90, and FITC-human CD105 antibodies (BioLegend). Cells were then washed and fixed in 4% paraformaldehyde (PFA) for 10 min at 4 °C. Cells that were not treated with fluorescent antibodies were used as controls. The cell surface expression of markers was assessed using a FACS Verse flow cytometer (BD Biosciences). Data were analyzed using the FACS software (FlowJo, FLOWJO, LLC).

2.4. Differentiation of sphere colonies and dental pulp stem cells

For the differentiation analysis of neurospheres from DPSCs, neurospheres and DPSCs from conventional culture conditions were cultured in DMEM/F12 supplemented with B27 supplement, N2 supplement, and 1% FBS (Gibco) on poly-L-lysine (Sigma)- and laminin (Invitrogen)-coated 24-well dishes for 21 days.

2.5. Immunohistochemistry

Cells and neurospheres were washed with PBS and fixed in 4% paraformaldehyde for 10 min at 37 °C, followed by treatment with absolute methanol for 10 min at 4 °C and blocking in PBS containing 1% FBS for 10 min. The samples were then incubated with primary antibodies at 37 °C overnight, rinsed twice with PBS, and incubated with the secondary antibodies for 1 h. The samples were observed under a confocal microscope LSM700 (Carl Zwiss, Thornwood, NY). Primary antibodies were as follows: anti-nestin (Covance), anti-N-FM (Millipore), and TUBB3 (Abcam). Secondary antibodies were as follows: Alexa 594-conjugated donkey anti-mouse IgG (Molecular Probes, Eugene, OR), Alexa 594-conjugated donkey anti-rabbit IgG (Molecular Probes), and R-PE-conjugated rabbit anti-rat IgG (Southern Biotechnology Associates, Inc., Birmingham, AL).

2.6. Real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA from cultured cells was isolated with TRIzol (Invitrogen) and cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. qPCR was performed in a Light Cycler 96 (Roche Diagnostics) using THUNDERBIRD SYBR qPCR Mix (Toyobo) under the following conditions: 95 °C for 60 s and then 45 PCR cycles at 95 °C for 10 s, 65 °C for 30 s, and 72 °C for 45 s mRNA expression was normalized against *GAPDH* levels. Primer sequences are shown in Table 1.

2.7. Statistical analysis

All experiments were repeated independently in triplicate, and data were reported as means ± standard error of the mean (SEM). Statistical significance was evaluated using Mann–Whitney U test or one-way ANOVA for comparisons using IBM SPSS statistics software (version 25.0; IBM Japan, Tokyo, Japan). A P-value of <0.05 was considered statistically significant.

Table 1
Sequence information of primers used for quantitative real-time polymerase chain reaction.

Gene	Primer sequences (forward and reverse, 5'–3')
GAPDH	GAAGGTGAAGGTCGGAGTCA
	GAAGATGGTGATGGGATTTC
NES	AACAGCGACGGAGGTCTCTA
	TTCTCTTGTCCCGCAGACTT
SOX2	CCCAGCAGACTTCACATGT
	CCTCCCATTTCCCTCGTTTT
VIM	GAGAACTTTGCCGTTGAAGC
	CTAACGGTGGATGTCCTTCG
TUBB3	CTCAGGGGCCTTTGGACATC
	CAGGCAGTCGCAGTTTTCAC
NEFM	TCGTCATTTGCGCGAATACC
	GCCAATTCCTCTGTAATGGC
MAP2	AACCCTTTGAGAACACGACA
	TCTTTCCGTTCATCTGCCA

3. Results

3.1. Characterization of DPSCs in xeno-/serum-free culture conditions

To test whether our culture conditions of xeno-/serum-free medium affected the development of DPSCs, we undertook primary culture of DPSCs collected from the dental pulp of wisdom teeth from healthy individuals to form DPSCs in medium containing KSR, L-glutamine, NEAA, bFGF, and 2-mercaptoethanol compared with conventional culture medium containing α-MEM and 15% FBS for 14 days (Fig. 1). We found that DPSCs in xeno-/serum-free conditions were well developed from the dental pulp of wisdom teeth. The morphology of DPSCs was different between conventional culture conditions and in xeno-/serum-free conditions. DPSCs in conventional culture indicated fibroblasts-like cells, however DPSCs in serum-free conditions revealed neuronal-like cells (Fig. 2A, B).

To further characterize DPSCs in xeno-/serum-free conditions, we analyzed the expression of cell surface molecules by flow cytometry. Cells freshly isolated from DPSCs in conventional culture conditions and in xeno-/serum-free conditions were dissociated using trypsin, followed by staining with specific antibodies. The cells were positive for CD90, CD44, CD105 and CD81 mesenchymal stem cell markers, but were negative for the hematopoietic stem cell markers CD14 and CD34 (Fig. 2C). These data are in accordance with previous studies of DPSC surface expression patterns [2]. However, the expression levels of CD90 only slightly differed between xeno-/serum-free conditions and conventional culture conditions, at 76.8% and 97.6%, respectively. Interestingly, these expression patterns were similar between conventional conditions and our study conditions even though these morphological patterns were different.

3.2. Identifying self-renewable DPSCs in complete xeno-/serum-free culture conditions

To address whether xeno-/serum-free culture conditions affect the development of DPSCs, we cultured DPSCs collected from primary culture in knockout DMEM and 20% KSR to form neurospheres in medium containing bFGF and EGF (Fig. 1). As expected, some of the cells either adhered to the plastic or died, and small spheres of floating cells had formed by 3–5 days, which proliferated to generate larger spheres. Thus, we found that numerous neurospheres were developed from DPSCs in xeno-/serum-free culture conditions (Fig. 3A, C), consistent with previous studies of rat DPSCs [19]. The average diameter of primary neurospheres was 97 μm. Furthermore, to investigate whether surviving primary neurospheres from DPSCs in xeno-/serum-free culture conditions have the capacity for self-renewal, we dissociated the primary neurospheres into single cells and tested whether they could form new neurospheres. The surviving cells from primary neurospheres could form secondary neurospheres, however, the formation numbers were low and the neurospheres were small (Fig. 3B, C). Moreover, the single cells from secondary neurospheres could form tiny neurospheres, however their number and size were more reduced compared with P1 neurospheres (primary neurospheres were termed as passage 0; P0) (Fig. 3C). These results suggest that the self-renewal capacity of DPSC-derived neurospheres was limited. These data are in accordance with previous studies of rat DPSC-derived neurospheres (see Fig. 4) [19].

We next assessed whether the surviving neurospheres expressed stem-cell markers. Expression of the neural stem cell marker nestin was detected in neurospheres at P0 (Fig. 3D) and P1 (data not shown), suggesting that surviving neurospheres from DPSCs in xeno-/serum-free conditions have the potential for 'stemness'.

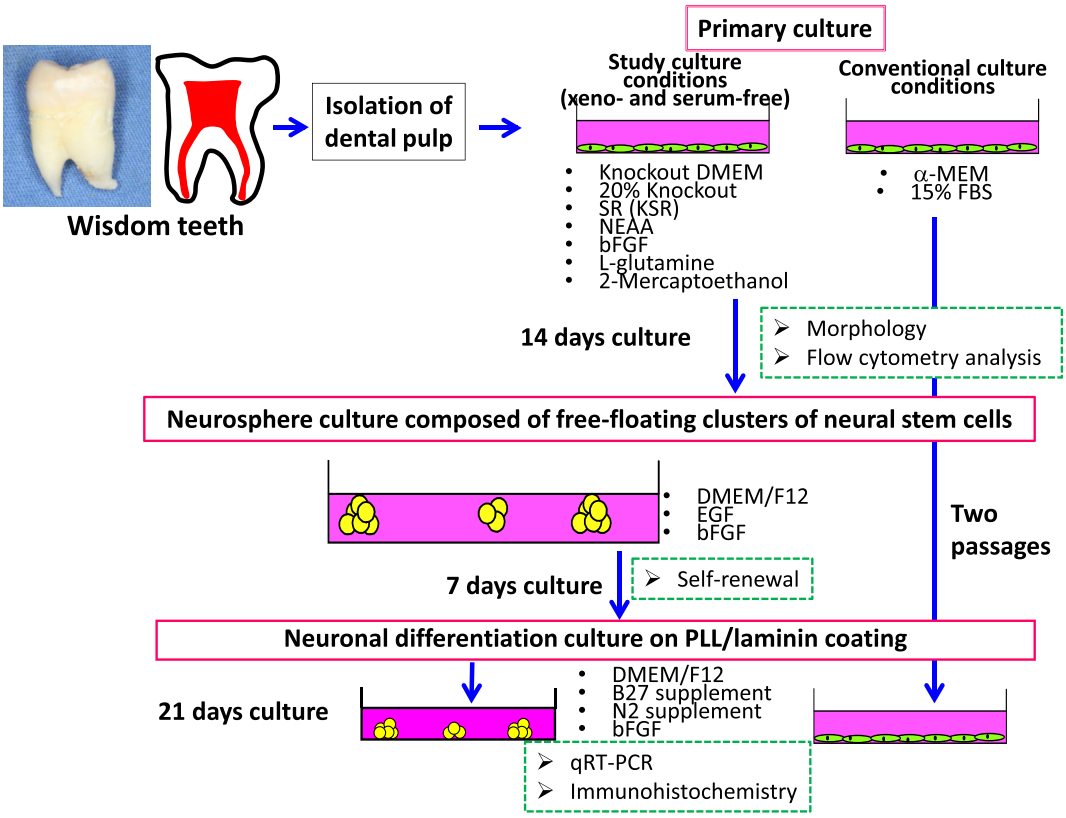

Fig. 1. Schema of our experimental strategy. Human dental pulp stem cells were isolated from wisdom teeth and cultured for 14 days in two different conditions: xeno-/serum-free medium and conventional culture medium containing 15% FBS. DPSCs from both conditions were morphologically evaluated and analyzed by flow cytometry. After primary culture for 7 days, DPSCs in xeno-/serum-free conditions formed neurospheres that were composed of free-floating clusters of neural stem cells. Then, cells were investigated for self-renewal. After formation of the neurospheres, they were cultured in differentiation medium for 21 days. After 21 days, the cells from neurospheres were evaluated for the expression of neural cell markers by qRT-PCR and immunohistochemistry.

3.3. Neural differentiation of neurospheres derived from DPSCs in xeno-/serum-free culture conditions

To investigate whether neurospheres derived from DPSCs can differentiate into neurons, we cultured neurospheres derived from DPSCs in neural differentiation conditions compared with DPSCs in conventional culture conditions (Fig. 1). Three weeks after the neural differentiation process, we found that nestin, TUBB3, a neuron-specific marker, and NF-M, which is found in the intermediate filaments of neurons, were more visible in neurospheres derived from DPSCs compared with DPSCs derived in conventional culture conditions. Furthermore, qRT-PCR revealed that neurospheres derived from DPSCs significantly upregulated the expression of microtubule associated protein (*MAP2*) for activation of neurons at 21 days ($P < 0.05$). Moreover, *NES*, *SOX2* marker of neural stem cells, *VIM* marker of radial glial cells, and *TUBB3* were expressed at higher levels in neurospheres derived from DPSCs than in DPSCs derived in conventional culture conditions; however, these expression levels were not significantly different. These results suggested that neurospheres derived from xeno-/serum-free culture conditions showed a higher neural differentiation capacity compared with the cells cultured in conventional FBS-containing medium.

4. Discussion

In this study, we isolated cells from DPSCs in xeno-/serum-free culture conditions from primary culture, generated neurospheres in suspension in xeno-/serum-free conditions, and differentiated them into neural cells with xeno-/serum-free conditions. Previous studies indicated that DPSCs proliferate and differentiate into neurons in serum-free conditions, however, these studies used serum for a minimum of 12 h of DPSC cell separation as primary culture [17]. In other words, it was reported that neural culture is not performed in serum-free medium for isolation procedures. To our knowledge, only one study has reported primary culture that achieved proliferation of human DPSCs in KSR medium without serum, however, to assess the potential of neurons, maintenance medium with 20% FBS was used [20]. Therefore, we developed a serum-free culture method covering all steps from isolation to neural stem cell culture and neural differentiation induction culture.

Stem cells are generally maintained in culture medium supplemented with serum (FBS), which includes animal-derived components important for cell proliferation because they contain numerous extracellular matrix molecules and growth factors that aid cell adhesion, proliferation, and differentiation [21]. However, to harness the clinical applications of stem cells, the use of FBS in cell culture must be considered because of its risks in transmitting pathogens and initiating immune responses in recipients [17,18]. In MSCs, even maintenance in serum-free conditions may not apply to humans due to MSC-specific differentiation [21]. Moreover, MSCs in serum-free conditions without growth factors or cytokines are not able to proliferate. This is probably because serum induces intracellular calcium oscillations that are essential for stem cell proliferation and differentiation [22]. In *in vitro* culture, serum is used as a neutralizing agent for trypsin, not only for cell maintenance but also for sub-culturing steps. A previous report showed that aggregation of cells was sometimes due to the absence of serum proteins

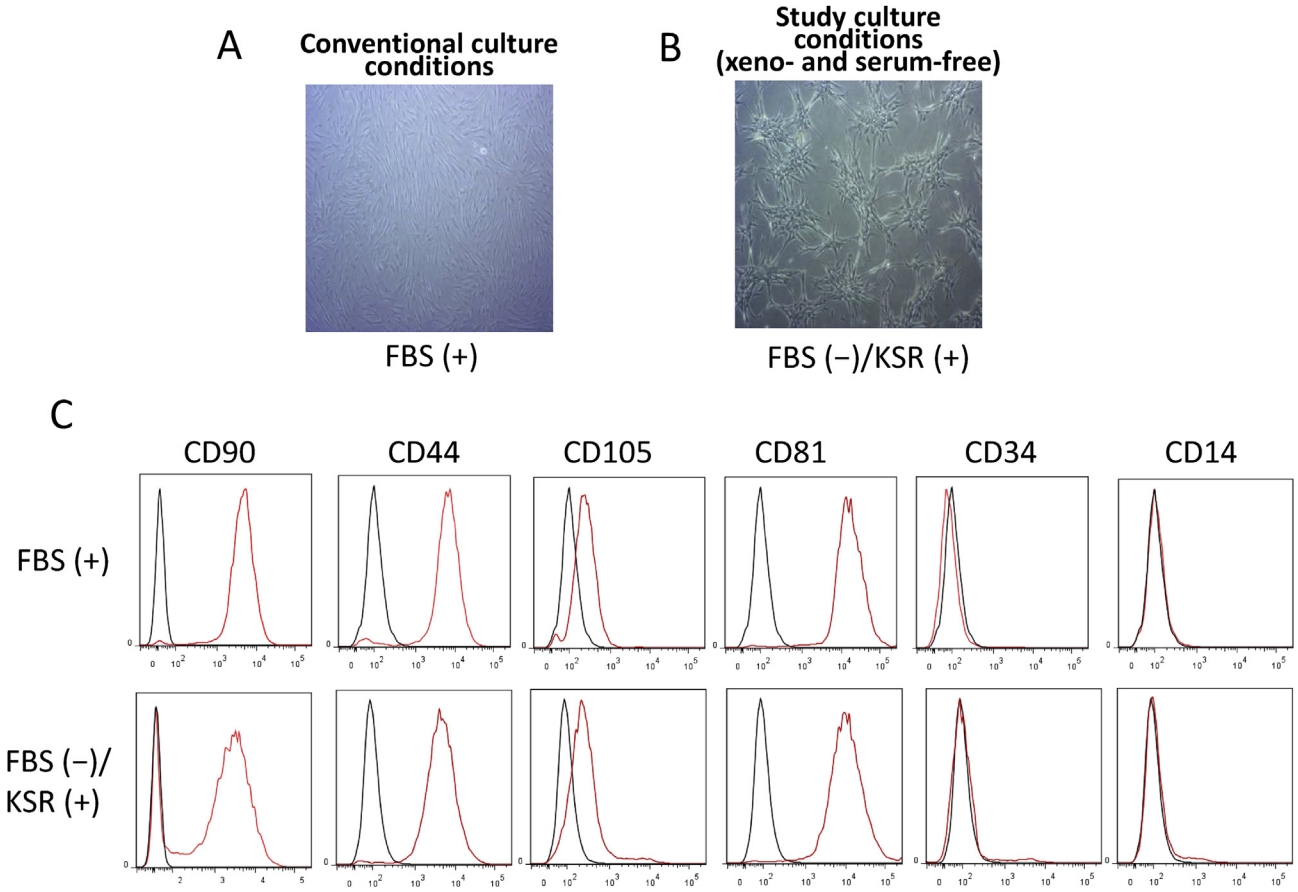

Fig. 2. Characterization of DPSCs in complete xeno-/serum-free culture conditions. (A) Morphology of DPSCs in conventional culture conditions and (B) xeno-/serum-free culture conditions in primary culture on day 14. (C) Flow cytometric analysis of cell surface markers on DPSCs in xeno-/serum-free culture conditions. DPSCs in the primary culture were surface-stained with FITC-conjugated CD14, CD34, CD44, CD81, and CD105 and PE-conjugated CD90. The black curve represents control cells and the red curve represents cells positive for these markers. The experiments were repeated three times and one representative experiment is presented.

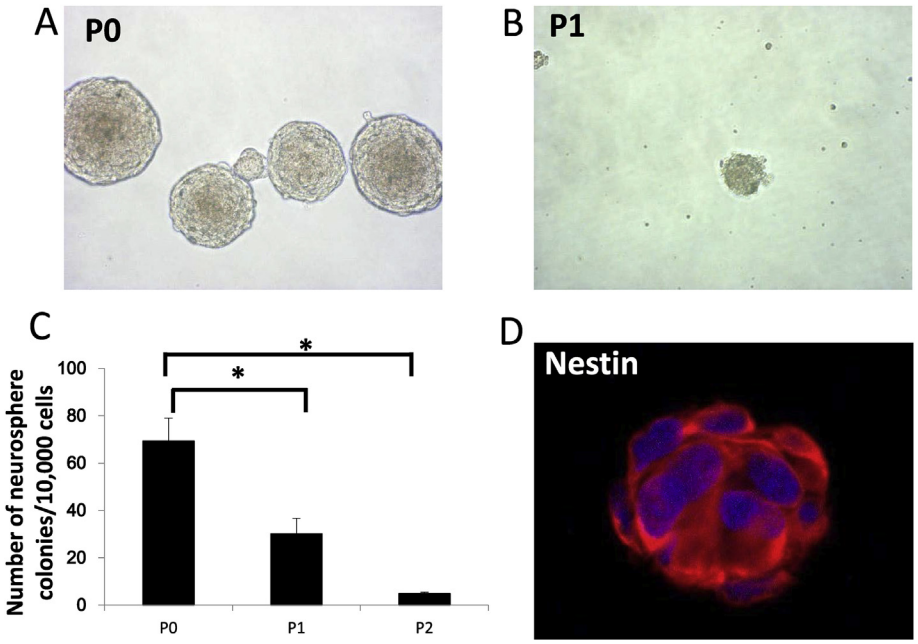

Fig. 3. Limitations of the self-renewal potential of neurospheres derived from DPSCs in xeno-/serum-free culture conditions. (A, B) Neurospheres from DPSCs in complete xeno-/serum-free culture conditions at P0 (A) and P1 (B). (C) The number of colonies of neurospheres derived from DPSCs/10,000 cells in xeno-/serum-free culture conditions at P0, P1, and P2. N = 3; $^{*}P < 0.05$. (D) Immunocytochemistry staining for nestin in neurospheres at P0 from DPSCs in xeno-/serum-free conditions. DAPI was used for nuclear staining.

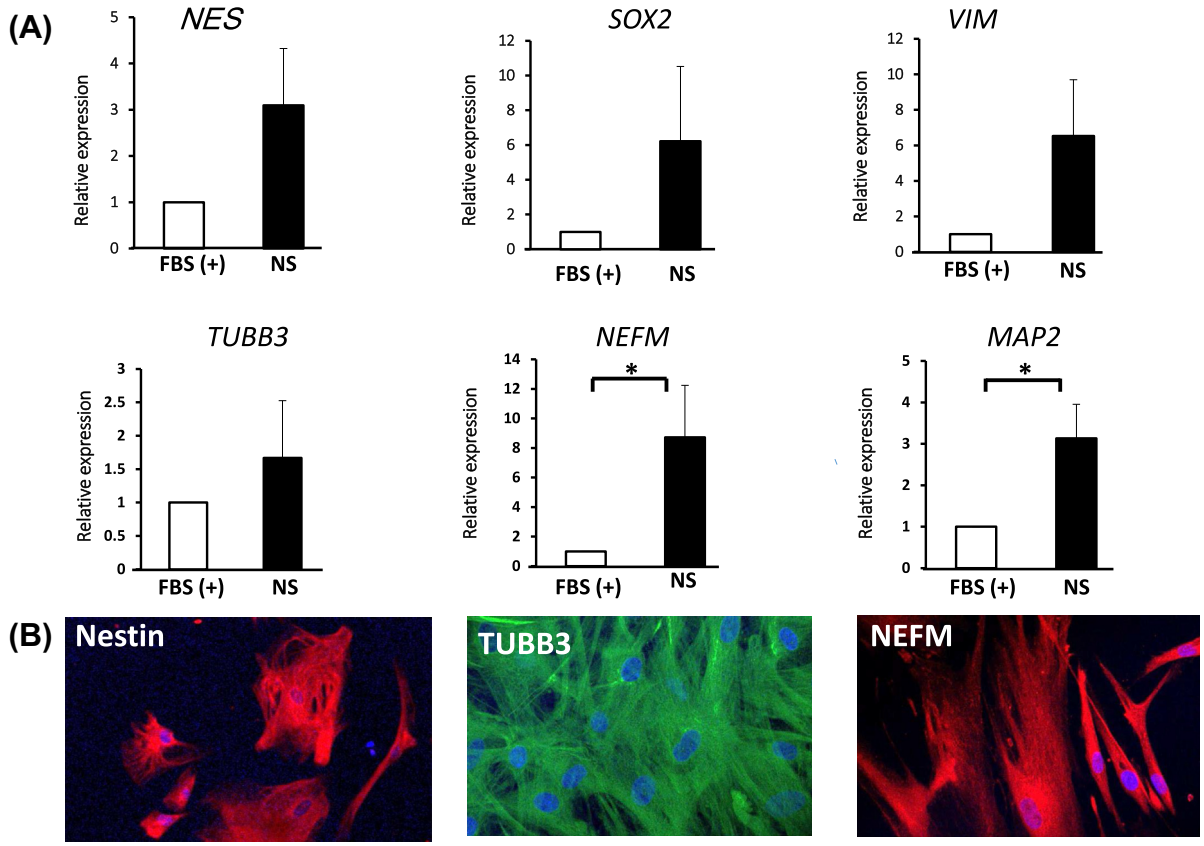

Fig. 4. Neural differentiation of the neurospheres derived from DPSCs in complete xeno-/serum-free culture conditions. (A) Relative mRNA expression of neuron marker genes, *NES*, *SOX2*, *VIM*, *TUBB3*, *NEFM*, and *MAP2* in neurospheres derived from xeno-/serum-free conditions. DPSCs in neural differentiation conditions at day 21. Expression levels in DPSCs from conventional culture conditions were set as 1. N = 3; $^{*}P < 0.05$. (B) Immunocytochemical staining of nestin, TUBB3, and NFM in neurospheres derived from xeno-/serum-free DPSCs in neural differentiation conditions at day 21. DAPI was used for nuclear staining.

during the dissociation steps when passaging and sub-culturing [23]. An alternative method for avoiding the use of FBS in cell sub-culture is to replace trypsin with a less harmful protease. In this study, to settle these issues for neutralization and cell aggregation, we performed gentle trituration to obtain separate single cells using Accutase™, which contains proteolytic and collagenolytic enzymes to gently break down the cell adhesion structure on the outside of cells, and requires no neutralization steps such as serum or inhibitory agents. The viability and cell proliferation ability of DPSCs sub-cultured with Accutase™ were no different from those when trypsin was used for neutralization. Therefore, it can be safely used for the sub-culturing steps of embryonic stem cell and NSC culture. It was very important that there was no use of serum during DPSC culture and deactivation during the sub-culturing steps in the present study.

In 2000, Gronthos et al. first demonstrated isolation of a clonogenic and highly proliferative population of cells from adult human dental pulp [11]. DPSCs have the potential to differentiate into multiple cell lineages, such as adipogenic, neurogenic, and osteogenic cells [10–12]. Furthermore, Miura et al. reported in 2003 that stem cells from SHED also demonstrated high proliferation ability, self-renewal, and multi-lineage differentiation to cells including neuron and glial cells [13]. Compared with other MSCs, DPSCs and SHED are easily obtained from tooth extraction surgery without any ethical issues. Therefore, DPSCs are considered to be an essential cell source for regenerative medicine and tissue engineering. To apply DPSCs to regenerative medicine, it is critical to obtain a sufficient number of cells. DPSCs have a greater proliferative capacity than MSCs but it is unclear whether this is sufficient for clinical application. In osteogenic differentiation, we previously reported the development of a novel approach for bone regenerative medicine and demonstrated the osteogenic differentiation of DPSCs with TH, a small osteogenic small molecule [24]. Therefore, it is considered that DPSCs have enough cells necessary for neural regenerative medicine using such osteogenic small molecules.

DPSCs are stem cells derived from ectoderm originating from migrating neural crest cells. They exhibit fibroblast-like morphology in plastic adhesion culture and have the characteristics of MSCs [25]. A previous study showed DPSCs have high mRNA expression of various neural crest development genes and neural crest-related genes, which share NSC properties such as *NES* and *GFAP* [10]. Furthermore, DPSCs express MAP2, neurofilament protein N-FM, TUBB3, oligodendrocyte-associated CNPase, and GFAP, suggesting that DPSCs have similar capacities to neural cells. In our study, neurospheres from DPSCs in xeno-/serum-free conditions highly expressed neural cell markers including nestin, SOX2, Vimentin, TUBB3, N-FM, and MAP2.

Neurospheres are thought to contain a mixture of NSCs and progenitor cells that can develop in appropriate environmental conditions. For neurosphere formation, cells respond to EGF, bFGF, or both growth factors to proliferate and form spheroid structures in suspension culture [3]. In our conditions, our data suggested that EGF- and bFGF-responsive cells proliferated and formed

sphere colonies by continuing to grow and generate large spheres with the initial colony-forming cells. After sub-culture, the cells from the initial neurospheres could form secondary neurospheres, however, the number and size formed were revealed to be low and small, respectively. Tertiary neurospheres from DPSCs could not form colonies of spheres, however, they could be sub-cultured. Previous studies showed that the generation of secondary or tertiary spheres did not match the criteria for self-renewal and the assumption of stem cell adaptation based on limited self-renewal was insufficient in rat [19]. Thus, as previously reported in rat DPSCs, our DPSCs were difficult to characterize as 'stem cells' and may instead be 'progenitor cells' with the ability to produce tertiary spheres following passage. In a previous study of rat neurospheres from DPSCs, DPSC spheres were negatively formed by transforming growth factor-β (TGF-β) [19]. TGF-β has the capacity as a potential inhibitor of various cell types, such as hematopoietic, epithelial, and endothelial cells [25]. However, TGF-β accelerate the proliferation of fibroblasts [26], neurosphere from skin-derived stem cells [27] and mammalian DPSCs *in vitro* [28]. As Sasaki et al. reported in rat DPSCs, neurospheres derived from human DPSCs may produce TGF-β, and self-renewal of DPSC spheres may be prevented by TGF-β signaling; thus, it if it is possible to suppress TGF-β activity, sphere proliferation and passage limitations may be improved [19].

To address these questions, further studies are required to elucidate and more accurately characterize DPSC neuronal differentiation.

5. Conclusions

We demonstrated that cells derived from DPSCs in xeno-/serum-free conditions proliferated as in conventional culture conditions, had the ability to generate neurospheres, and differentiated into neurons. DPSCs under our xeno-/serum-free conditions may be an accessible source for clinical cell-replacement therapies for neurodegenerative diseases.

Author contributions

Y.K–K conceived and designed the study, collected samples, performed experiments, analyzed data, and wrote and edited the manuscript. Y.F. collected samples, analyzed data, and wrote the manuscript. D.Y. collected samples, performed experiments, and analyzed data. M.S. analyzed the data. D.C. conceived and designed the study and collected samples. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

There are no conflicts of interest associated with this study.

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