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Article

Formation of Three-Dimensional Spheres Enhances the Neurogenic Potential of Stem Cells from Apical Papilla

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Abstract: Cell-based neural regeneration is challenging due to the difficulty in obtaining sufficient neural stem cells with clinical applicability. SCAPs originating from embryonic neural crest with high neurogenic potential could be a promising cell source for neural regeneration. This study aimed to investigate whether the formation of 3D spheres can promote SCAPs neurogenic potential. **Material and methods:** 3D SCAPs spheres were first generated in 256-well agarose microtissue mold. The spheres and single cells were individually cultured on collagen I coated μ-Slide for 4 and 7 days. Cell morphological changes, neural marker expression, and neurite outgrowth were evaluated under a confocal microscope. Secretion of BDNF and NGF-β was measured by ELISA kits. **Results:** Pronounced morphological changes were noticed in a time-dependent manner. The migrating cells' morphology changed from fibroblast-like cells to neuron-like cells. Compared to the 2D culture, neurite length, number, and the expression of neural markers, including Nestin, β-tubulin III, NeuN, and MAP-2 were significantly increased in the 3D spheres, while the secretion of BDNF and NGF-β was markedly downregulated at day 7. **Conclusion:** The formation of 3D spheres enhanced the neurogenic potential of SCAPs, suggesting the advantage of using the 3D spheres of SCAPs for the treatment of neural diseases.

Keywords: neurogenic potential; neurites; SCAPs; sphere; stem cells

1. Introduction

The Neural injuries and diseases can lead to severe physical and mental impairment, such as hypoesthesia, paralysis, and dementia, negatively affecting the patient's life [1]. Injuries to neural tissue cause cell damage and necrosis, inflammation, ischemia, and cell signaling alteration, leading to demyelination, axonal degeneration, and an inhibitory environment for regeneration [2]. Various strategies have been investigated, including drug delivery, cell-based approach, and tissue engineering, however, effective and predictable therapies are still lacking [3]. Stem cell therapy, including mesenchymal stem cells (MSCs), neural stem cells, neural progenitor cells (NPCs), embryonic stem cells, and induced pluripotent stem cells, is a promising treatment, via supporting autologous neural proliferation or self-differentiation to replace the injured neural cells, and immunomodulation [4]. However, pre-clinical and clinical studies on stem cell therapy only demonstrated limited benefits, suggesting that a combined treatment is needed [4,5].

Dental stem cells, which express multiple embryonic stem cell markers, including OCT-4, NANOG, SSEA-3, SSEA-4, etc., can differentiate into multiple tissue-specific cells [6]. In particular, dental stem cells may serve as promising cell sources for neural tissue

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regeneration since they share a common origin with neural crest cells and possess high neurogenic potential [7]. Compared to dental pulp stem cells (DPSCs), stem cells from apical papilla (SCAPs) proliferate 2- to 3-fold greater, and have higher proliferative and regenerative capacity and telomerase activity [8,9]. SCAPs express neural markers, including Nestin, β-tubulin III, neuronal nuclear protein (NeuN), neurofilament protein (NFM), and neuron-specific enolase [8]. In addition, SCAPs secrete a sufficient amount of brain-derived neurotrophic factor (BDNF) and can stimulate neurite elongation in-vitro and in-vivo [10]. In this context, SCAPs are an attractive source for neural regeneration.

To achieve successful cell-based therapies, a substantial number of cells are required for transplantation, which necessitates extensive ex-vivo cell expansion. However, long-term in-vitro culture and serial passages by using the conventional two-dimensional (2D) dish, are quite likely to evoke continuous changes in MSCs. Stem cells may gradually lose self-renewal ability, differentiate into bone cells or adipocytes spontaneously, and undergo senescence [11]. To overcome these deleterious effects and their possible consequent functional alterations, 3D cell culture has been employed in various studies, which can better simulate the growth state of cells in-vivo, promote cell self-renewal, inhibit their differentiation, and maintain the original characteristics of cells [11,12]. For example, short-term spheroid formation of adipose-derived stem cells (ASCs) can enhance their stemness, angiogenic capacity, and chemotaxis and thereby increase their therapeutic potential for tissue regeneration [13]. Similarly, the expression of stemness markers, NANOG, TP63, and CD44 in the spheroids of human dental pulp cells (DPCs) was much higher than within the monolayer cultures. The expression of neural markers, NFM, and β-tubulin III in the spheroids, was increased in a time-dependent manner. Intriguingly, cells in the spheroids spontaneously differentiated into neuron-like cells after 1-week cultivation without any neural induction in a serum-free medium [14]. Kim BC et al also found that three-dimensional (3D) SCAP clusters strongly expressed neuronal maturation markers, such as NFM, than non-aggregated cells under neural inductive culture conditions [15]. Although many studies have been performed to investigate the neural differentiation of dental stem cells, the induction methods are highly variable. The most noticeable variables are the duration of neurosphere formation, the duration of induction, the medium used, etc. Nevertheless, the optimal conditions for high-yielding neural cells from dental stem cells are unmet. Therefore, this study aimed to investigate the effects of 2D vs 3D culture without neural induction on early cell fate, neurite outgrowth, and differentiation potential of SCAPs.

2. Materials and Methods

Unless otherwise stated, chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Culture medium and supplements were purchased from Gibco-Invitrogen (Carlsbad, CA, USA). All plastic labware consumables were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA).

2.1. SCAPs isolation and characterization

The study protocol for procuring SCAPs from human dental tissue was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB. IRB Reference Number: UW 21-378). After obtaining informed consent, SCAPs were isolated from the freshly extracted third molars of seven adults (aged 19 - 21 years) at the Oral and Maxillofacial Clinic in Prince Philip Dental Hospital. Briefly, the apical papilla was gently removed from the apex, and SCAPs were isolated as previously described [9]. SCAPs were cultivated in α-minimum essential medium (α-MEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin antibiotic solution (ThermoFisher Scientific, Inc. Waltham, MA, USA) at 37 °C in a 5% CO2 incubator. The medium was changed every two days. SCAPs were characterized by flow cytometric analysis based on their expression of CD45, CD73, CD90,

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and CD105, as well as multi-lineage differentiation assays using osteogenic and neurogenic induction protocols [16-18], and chondrogenic and adipogenic protocols (GUXMX-90041, GUXMX-90031, Cyagen, Santa Clara, CA, USA) according to the manufacturer instructions. Passages 3 to 6 were used for these experiments.

2.2. Formation and culture of SCAPs 3D spheres

Upon reaching 70 to 80% confluence, SCAPs were digested with 0.5% (w/v) trypsin-EDTA. 2 × 105 cells in 190 μL medium were transferred into each well of the 256-well agarose microtissue mold (MicroTissues, Inc, Sharon, MA, USA) and cultured for 3 days to generate spheres [19]. Then, 15 - 20 spheres were collected from the mold and seeded on the top of μ-Slide (Cat. No. 80427, ibidi GmbH, Lochhamer Schlag, Gräfelfing, Germany) coated with rat tail collagen I (Cat. No. 354236, Corning, NY, USA). Alternatively, SCAPs were seeded in the 6-well plates for 3 days and then digested using 0.5% (w/v) trypsin-EDTA. 2 × 105 cells were cultured on the top of μ-Slide coated with rat tail collagen I. The culture medium was α-MEM containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin antibiotics. The medium was changed every 3 days. The samples were evaluated at days 4 and 7.

2.3. Immunofluorescence assessment of the neuronal markers

The expression of neural markers (Nestin, β-tublin III, Microtubule-associated protein-2 (MAP-2), and NeuN) was analyzed by immunocytochemistry at days 4 and 7. Briefly, the cells were fixed with 4% (v/v) paraformaldehyde for 20 minutes, permeabilized with 0.1% (w/v) Triton X-100 for 10 min, and blocked in PBS with 1% (w/v) Bovine Serum Albumin (BSA) for two hours. Then, the samples were incubated with primary antibodies at 4 ℃ overnight. The following antibodies were utilized; rabbit anti-MAP-2 (1:200 dilution) (Cat. No. AB5622-I Merck KGaA, Darmstadt, Germany), rabbit anti-NeuN (1:100 dilution) (Cat. No. 702022, Invitrogen, Carlsbad, CA, USA), mouse anti-β-tubulin III (1:500 dilutions) (Cat. No. AB78078, Abcam, Cambridge, UK), mouse anti-Nestin (1:200 dilution) (Cat. No. 33475S, Cell Signaling Technology, Danvers, MA, USA). The excessive primary antibodies were washed with 10% PBS. The samples were then incubated with the corresponding secondary antibodies (1:200), i.e., goat anti-mouse secondary antibody conjugated to Alexa fluor 488 (Cat No. ab150117, Abcam) or goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (Cat No. ab6718, Abcam) for 2 h in the dark at room temperature. After washing the excess secondary antibodies with 10% PBS, the samples were imaged under a laser scanning confocal microscope (LSM900, ZEISS, Germany) using a 10 × objective lens under the specific excitation/emission wavelengths for Alexa Fluor 488 (490/580 nm) and Alexa Fluor 594 (565/700 nm). All the images were processed and analyzed using ZEN Blue software (ZEISS, Germany).

2.4. Neural markers expression, and neurites measurement

The expression of Nestin, β-tubulin III, MAP-2, and NeuN was measured by the fluorescent density with ImageJ software using the following formula. Integrated density – (Area of selected cell × Mean fluorescence of background readings). All the measurements were taken using NeuronJ plugins in ImageJ software (NIH Image, Bethesda, MD, USA). The scale was set according to the reference scale within the image. Neurites were defined as a process extending from the cell body ≥ 20 μm (20). Six cells were randomly selected, which were out of the sphere and completely within the frame of the image. The data collected from four images were used for statistical analysis.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Both 3D sphere and 2D culture of SCAPs were cultivated in 6-well plates for another four days. 5 × 105 cells were used in both groups. The supernatants were collected and centrifuged at 1500 rpm (Centrifuge 5420; Eppendorf, Hamburg, Germany). BDNF and

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nerve growth factor-beta (NGF-β) were measured by the ELISA kits according to the manufacturer's instructions (ab212166 and ab193760, Abcam).

2.6. Statistical Analysis

All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 27.0 (SPSS Inc., Chicago, Ill., USA). Kolmogorov-Smirnov normality test and Levene's homogeneity test were conducted. Two-way ANOVA was undertaken to analyze the expression of Nestin, β-tubulin III, MAP-2, NeuN, and neurites. While an independent T-test was used to analyze the ELISA results. The threshold for statistical significance was set at *P < 0.05; **P < 0.01.

3. Results

3.1. SCAPs characterization

Under phase-contrast light microscopy, SCAPs displayed a fibroblastic-like appearance of spindle-shaped (Figure 1A). Flow cytometry results showed that SCAPs expressed typical mesenchymal markers CD73 (100%), CD90 (100%), CD105 (66.1%), and lack the expression of CD45 (1.76%) (Figure 1B). Multiple differentiation assay was conducted to verify the multilineage differentiation potential of SCAPs. SCAPs differentiated into osteoid tissue after 21 days of induction, which was Alizarin Red positive (Figure 1C). Cultured SCAPs in small molecules neural induction medium, expresses a morphological changes along with neural markers including β-tubulin III and MAP-2 (Figure 1D). After 27 days of adipogenic induction, adipocytes were identified by Oil Red (Figure 1E). SCAPs formed chondroid tissue after 28 days and were positive for Alcian Blue (Figure 1F).

3.2. Neural markers expression

The expression of Nestin in the 3D spheres was 7.51 ± 1.25 and 9.93 ± 1.5 at days 4 and 7, and the difference was statistically significant (P < 0.01). The expression of Nestin in the 2D culture was 1.1 ± 0.09 and 1.14 ± 0.18 at days 4 and 7, and the difference was not significant (P > 0.05). The expression of Nestin at both time points was significantly higher in the 3D spheres than that in the 2D culture (P < 0.01) (Figure 2).

The expression of NeuN in the 3D spheres was 1.18 ± 0.18 and 2.8 ± 1.9, respectively, and the difference between both time points was statistically significant (P < 0.01). The expression of NeuN in the 2D culture was 0.35 ± 0.024 and 0.21 ± 0.05 at days 4 and 7, respectively, and no significant difference was found between them (P > 0.05). At both time points, NeuN expression was significantly increased in the 3D spheres compared to the 2D culture (P < 0.01) (Figure 2).

The expression of β-tubulin III in the 3D spheres was 2.41 ± 0.82 and 10.3 ± 1.54, respectively, and the difference between both time points was statistically significant (P < 0.01). The expression of β-tubulin III in the 2D culture was 3.12 ± 0 .45 and 3.04 ± 0.37 at days 4 and 7, respectively, and no significant difference was found between them (P > 0.05). The expression of β-tubulin III was not significantly different between the 2D and 3D cultures at day 4 (P > 0.05), while the expression was significantly higher in the 3D spheres compared to the 2D culture at day 7 (P < 0.01) (Figure 3).

The expression of MAP-2 in the 3D spheres was 2.83 ± 0.42 and 2.75 ± 0.41, respectively, and the difference between both time points was not significant (P > 0.05). The expression of MAP-2 in the 2D culture was 1.7 ± 0 .24 and 1.77 ± 0.35 at days 4 and 7, respectively, and no significant difference was found (P > 0.05). At both time points, MAP-2 expression was significantly higher in the 3D spheres than that in the 2D culture (P < 0.01) (Figure 3).

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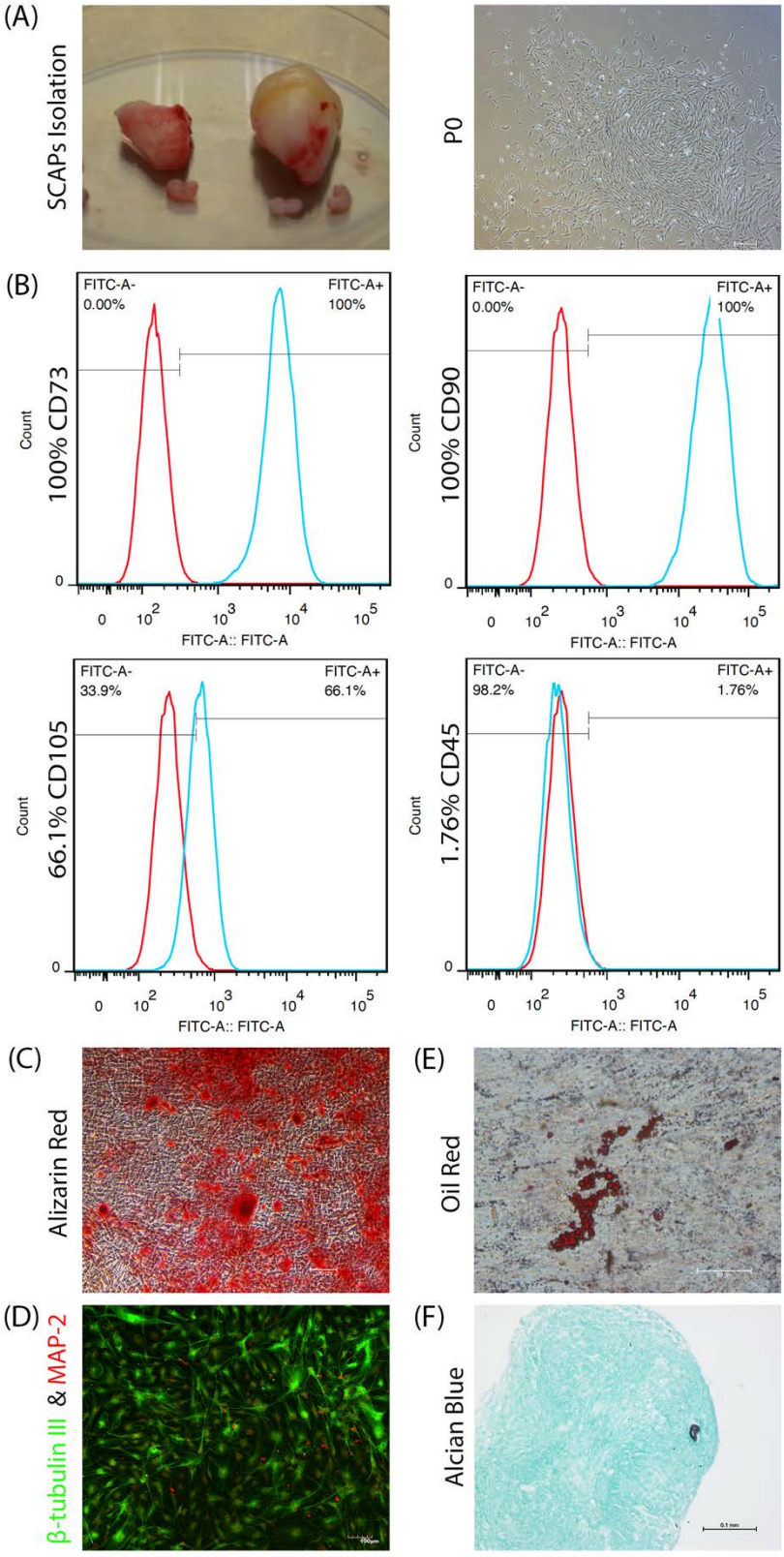

Figure 1. (A) Microscopic Images for SCAPs isolation; (B) Flow cytometric results for SCAPs characterization; Microscopic images for (C) osteogenic, (D) neuro-genic, (E) adipogenic and (F) chondrogenic multi-differentiation assay.

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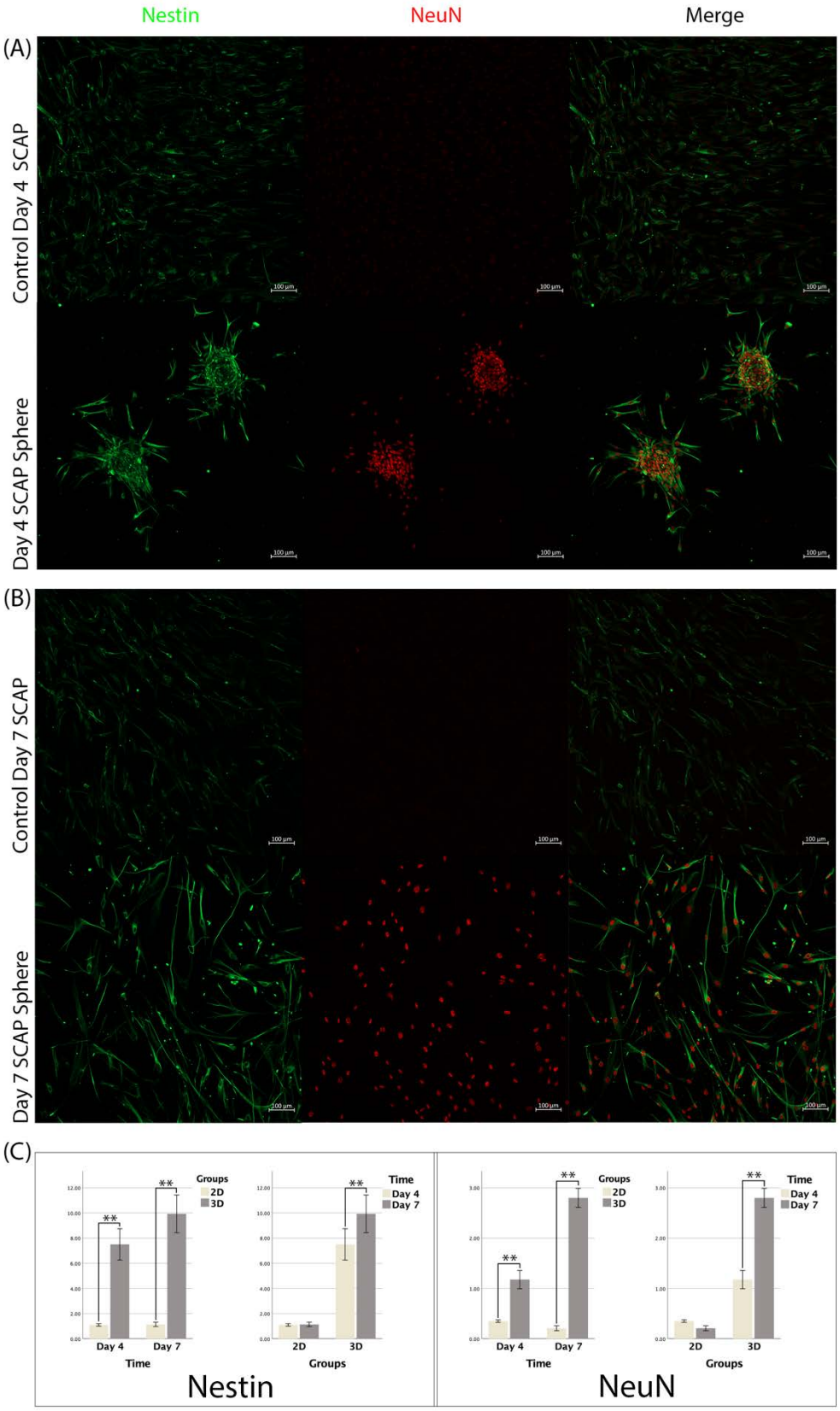

Figure 2. (A, B) Immunocytochemical staining for 3D SCAPs sphere and 2D SCAPs for detecting Nestin and NeuN expression at different time points; (C) Neural markers expression analysis of 2D and 3D SCAPs in two time points (**: P < .001).

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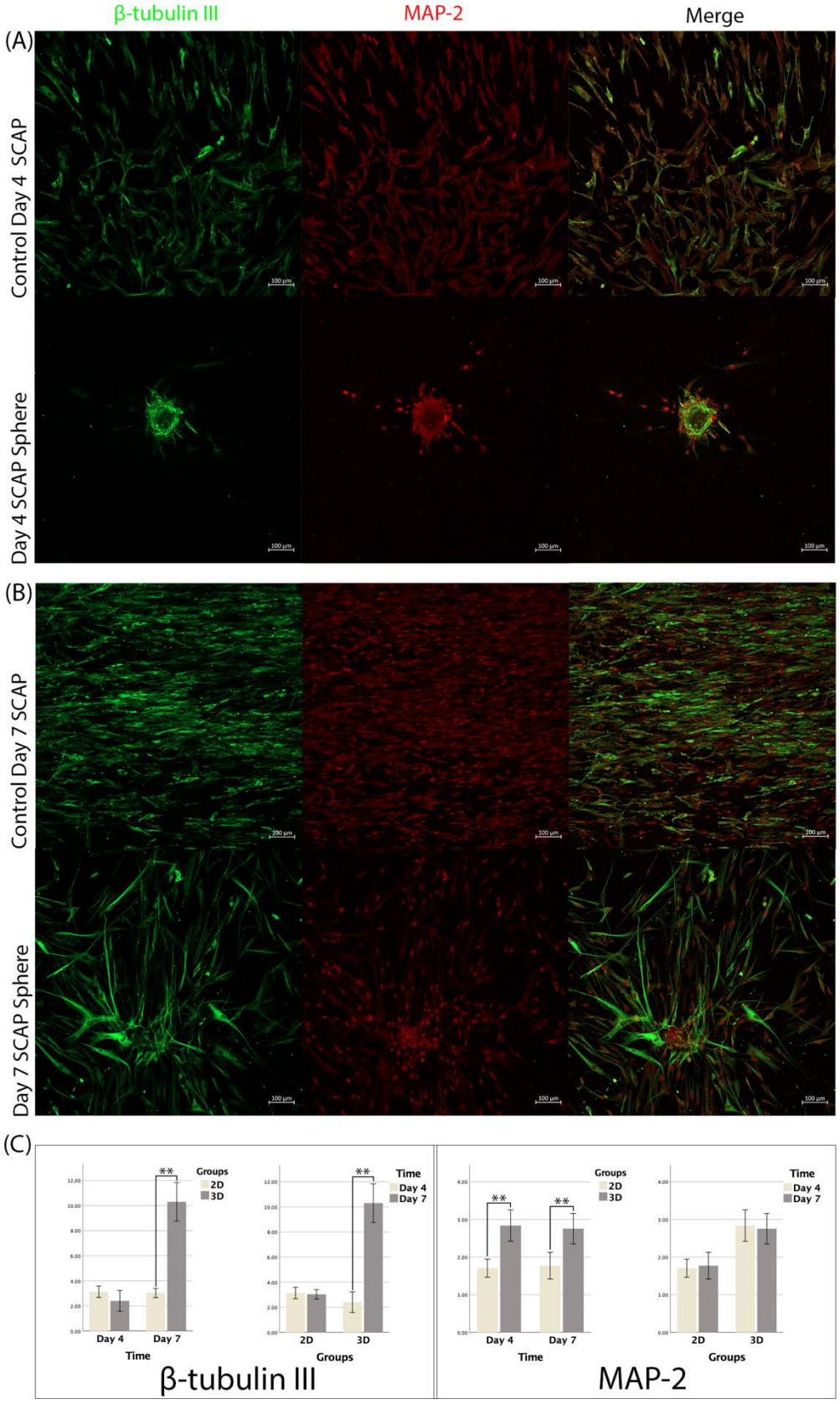

Figure 3. (A, B) Immunohistochemical staining for 3D SCAPs sphere and 2D SCAPs for detecting βIII and MAP-2 expression in different time points; (C) Neural markers expression analysis of 2D and 3D SCAPs in two time points (**: P < .001).

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3.3. SCAPs morphological changes and neurites measurement

SCAPs spheres were standardized using the same number of cells in agarose molds. The 3D spheres of similar size were formed after three days of culturing. All spheres were ranging from 150 to 200 μm. 3D spheres attached well to the collagen I coated glass, followed by cell migration out of the spheres. The migrating cells' morphology changed from fibroblast-like cells to neuron-like cells, which were round with long neurites. The typical neuron-like morphology was apparent on day 7.

The neurite number in the 3D spheres was 2.38 ± 0.37 and 2.58 ± 0.44 at days 4 and 7, respectively, and the difference was not significant ($P > 0.05$). The neurite number in the 2D culture was 1.5 ± 0.14 and 1.5 ± 0.14 at days 4 and 7, respectively, and also no significant difference was found ($P > 0.05$). There was significantly more neurite outgrowth in the 3D spheres than that in the 2D culture at both time points ($P < 0.01$) (Figure 4).

The mean length of neurites in the 3D spheres was 64.12 ± 8.02 and 143 ± 8.65, respectively, and the difference was statistically significant ($P < 0.01$). The mean length of neurites in the 2D culture was 51.7± 5.7 and 58.8 ± 6.28 at days 4 and 7, respectively, and no difference was found ($P > 0.05$). The mean neurite length was significantly different between the 2D and 3D cultures ($P < 0.05$) at both time points (Figure 4).

The total neurite length in the 3D spheres was 1002.38 ± 266.55 and 2880.95 ± 771.7 at days 4 and 7, respectively, and the difference was statistically significant ($P < 0.01$). The total neurite length in the 2D culture was 462.03 ± 22.93 and 530.52 ± 87.75 at days 4 and 7, respectively, and no significant difference was found ($P > 0.05$). No difference in the total neurite length at day 4 was found between the 2D and 3D cultures ($P > 0.05$), while the total neurite length was significantly increased in the 3D spheres at day 7 ($P < 0.01$) (Figure 4).

3.4. Secretion of BDNF and NGF by SCAPs spheres

After being cultured in α-MEM for 7 days, ELISA assay was carried out to determine the neurotrophic secretion by the 2D and 3D culture. The level of BDNF expressed by 2D culture was 118.91 ± 7.13 pg/mL, which was significantly higher than that was secreted in the 3D SCAPs spheres (63.68 ± 10.13 pg/mL) ($P < 0.01$) (Figure 4). The mean level of NGF-β was 15 ± 0.91 pg/mL in SCAPs 2D culture, which was also significantly higher than that in the 3D SCAPs spheres (3.16 ± 1.78 pg/mL) ($P < 0.01$).

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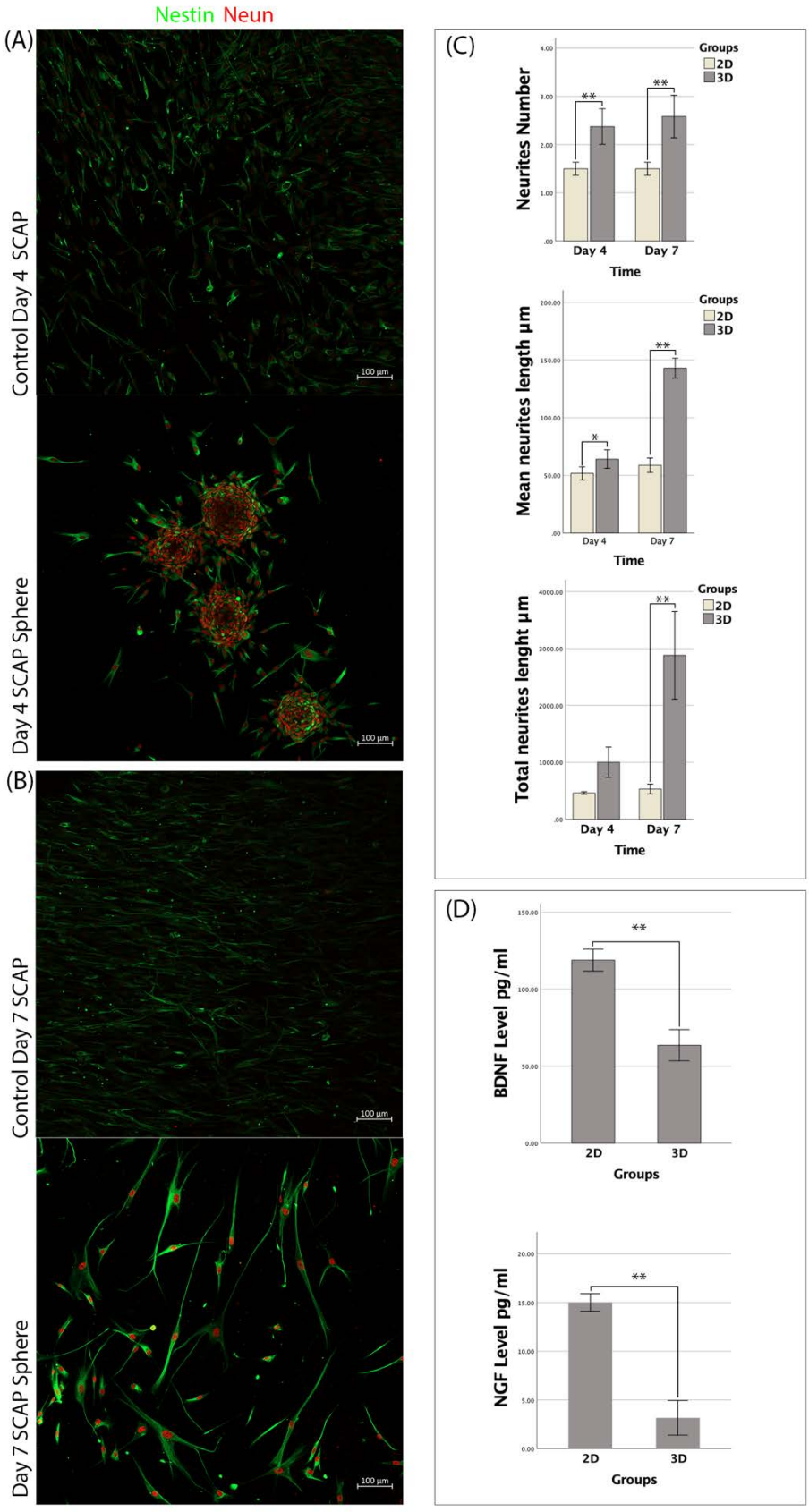

Figure 4. (A, B) Nestin and NeuN immunohistochemical staining showed changes in cell morphology on days 4 and 7; (C) Neurites number, mean length and total length analysis; (D) ELISA results for the expression of BDNF & NGF in pg/mL. Note (*: $P < 0.05$ / **: $P < 0.001$).

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4. Discussion

Nestin is a known marker of neural stem cells or progenitor cells. It is also constitutively expressed in dental stem cells, such as DPSCs, SCAPs, and periodontal ligament stem cells [8,21]. Since dental stem cells express neural crest-associated markers, it is not difficult to differentiate them into neurons under neural inductive settings both in-vitro and in-vivo [21,22]. Following neural induction, the early neural markers of neural stem cells/progenitor cells, Nestin and Musashi1, will be downregulated while the markers of immature and mature neurons, β-tubulin III, NeuN, and MAP-2, could be gradually increased in a time-dependent manner [23,24]. Our previous study also demonstrated that neuronal differentiation of dental stem cells exhibits a similar pattern of neural marker expression [17]. However, after long-term culture and multiple passaging in 2D conditions without any specific induction, MSCs may naturally undergo osteogenic or chondrogenic differentiation with the upregulation of the corresponding genes and proteins while losing their stemness markers and neurogenic potential [25]. While the 3D sphere conditions may be able to suppress the inherent traits of SCAPs toward osteogenic or chondrogenic differentiation by increasing neurotrophins receptors [26]. In this respect, the formation of 3D spheres could be a viable way for SCAP expansion before neural induction. In addition, transplantation of 3D spheres of SCAPs may lessen the detrimental effects of the harsh local microenvironment on cell survival, and more importantly prevent the transplanted cells from differentiating into other lineage fates, causing the formation of non-neural tissue. Interestingly, without any neural induction, 3D sphere-derived DPCs can spontaneously convert into neuron-like cells positive for neural markers, HuC/D and P75, in a serum-free medium [14]. These studies highlighted the importance of 3D culture, neural induction medium, and serum-free medium in neural differentiation of dental stem cells. In this study, we investigated the combined effects of 3D spheres cultured in α-MEM with 10% FBS on SCAP neurogenic potential, since α-MEM with 10% FBS is commonly used for MSC isolation, characterization, and expansion, compared to serum-free medium. The subsequent impact of α-MEM with 10% FBS on the SCAP neurogenic potential may affect SCAP neuroprotection and neuroregeneration capacity [25,26]. It was found that Nestin expression level was not changed in 2D culture in α-MEM with 10% FBS from day 4 to 7, while it was significantly increased in the 3D spheres. The expression of Nestin was 7-fold higher in the 3D spheres than that in 2D culture. The results strongly suggest that the formation of 3D spheres can enhance SCAP properties like neural stem cells/progenitor cells in a serum-containing medium.

It was shown that DPSCs and SCAPs cultured in neurosphere culturing condition (DMEM/F12 (1:1) supplemented with bFGF, 20 ng/mL EGF, and N2 supplement) formulate spheres that possess the characteristics of NSCs [27]. Similarly, several studies showed that the sphere formation of dental stem cells enhanced their stemness and differentiation ability [28-30]. The 3D spheres may provide favourable conditions for neural regeneration via increasing intra-sphere and intra-cellular communication at the ultrastructural level. Furthermore, cells within the sphere exhibit prominent nucleoli, Golgi apparatus, and enlarged endoplasmic reticulum, demonstrating an elevated metabolic activity [31]. Nevertheless, sphere size is crucial to maintaining a healthy condition. In this study, the generated SCAP spheres were 150 to 200 μm since oxygen diffusion would be compromised beyond 200 μm [19]. 3D sphere formation not only maintains the stemness of the embedded MSCs but also leads to the upregulation of several immature and mature neural markers, such as β-tubulin III, MAP-2, and NeuN [29,31,32]. In this study, we found that the immature and mature neural markers were upregulated in the 3D spheres of SCAPs, and the mature neural markers, such as MAP-2, and NeuN, are substantially expressed in the cells which have already migrated from the spheres. The induced cells may comprise a mixture of NPCs and neuronal cells, as demonstrated by the expression of early, immature, and mature neuronal markers. However, when SCAPs were cultured in 2D conditions, both immature and mature neural markers were not upregulated in the absence of

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a neural induction medium, suggesting the critical role of a neural induction medium in monolayer culture.

The migrating cells' morphology in the 3D spheres was round with long neurites, resembling neuron-like cells. The typical neuron-like morphology was more apparent on day 7. In line with the morphological changes, the neurite number, the mean length of neurites, and the total length of neurites in the 3D spheres were increased from day 4 to 7 and were much higher than those in the 2D culture. These results suggested that 3D sphere-derived SCAPs have superior neurogenic differentiation capacity than the 2D cultured cells. The results are corroborated by the previous study which reported that short-term spheroid formation of ASCs can increase their therapeutic potential for tissue regeneration [13].

Neurotrophins play critical roles in regulating neural cell survival and differentiation, and neurite outgrowth. It was reported that BDNF and NGF-β, the low-molecular-weight proteins of neurotrophins, can significantly increase the neurite length of neural stem cells [33] (pp. 547-550). Neurotrophins initiate signaling cascades by binding with high-affinity tyrosine kinases (Trk) or low-affinity neurotrophin P75 receptors (Figure 5) [33]. Multiple studies showed that dental stem cell-derived spheres express more Trk and P75 neurotrophin receptors than single-cell culture [26,27,34,35]. Therefore, BDNF and NGF-β secreted by SCAPs under 3D conditions could be more efficiently utilized and subsequently promote neural differentiation of SCAPs, which was corroborated by the much lower levels of BDNF and NGF-β found in the 3D spheres in this study than that in the 2D culture. This suggestion is further supported by the fact that several immature and mature neural markers were highly upregulated, and more typical neuron-like cells were found on day 7 in the 3D conditions.

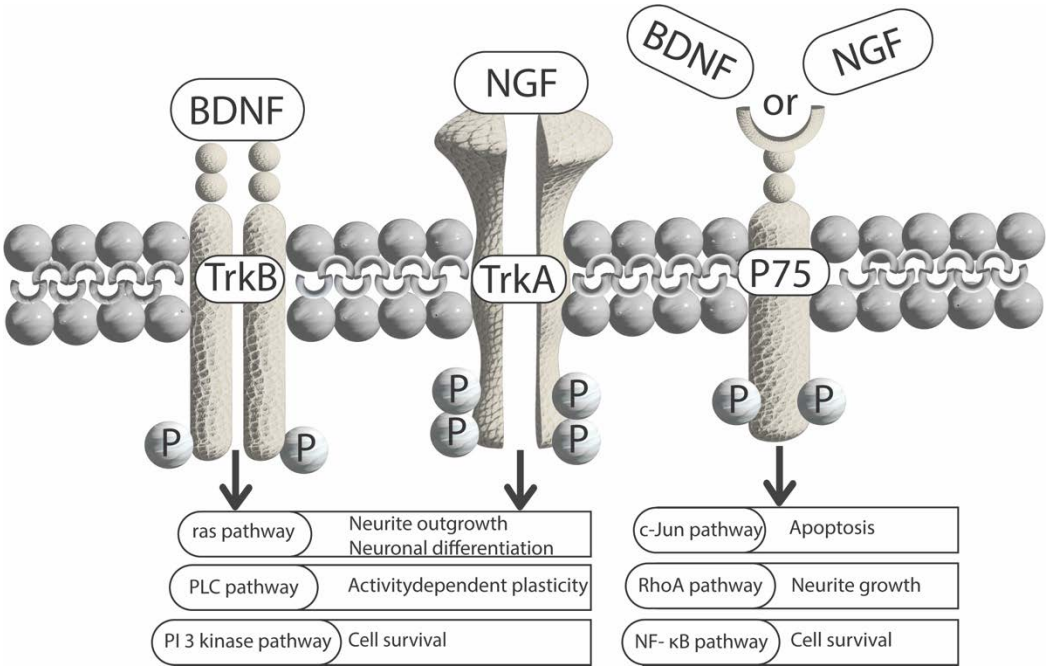

Figure 5. Diagram of neurotrophin signaling and their receptors which can lead to a diversity of cellular responses. BDNF mainly binds to TrkB , NGF mainly binds to TrkA, and both could bind to P75. [33] (pp. 547-550). [phospholipase C (PLC) / phosphoinositol 3 (PI 3) kinase / nuclear factor κB (NF-κB)]

5. Conclusions

This study illustrated that the formation of 3D spheres enhanced the neurogenic potential of SCAPs in terms of neural marker expression and neurite length and number. Signaling cascades initiated by BDNF and NGF-β could be a key contributing factor. The

formation of 3D spheres could be a viable way for SCAP expansion and neural differentiation.6. Patents

This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Author Contributions: Conceptualization, M.S.B. and C.Z.; methodology, M.S.B., H.L. and J.Z.; software, M.S.B.; validation, H.L. and J.L.; formal analysis, M.S.B.; investigation, M.S.B. and J.Z.; resources, C.Z. and A.Z.; data curation, M.S.B. and J.Z.; writing—original draft preparation, M.S.B.; writing—review and editing, C.Z. and J.K.; visualization, T.Z.; supervision, C.Z.; project administration, B.Y.; funding acquisition, C.Z. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB. IRB Reference Number: UW 21-378).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All the datasets are available online and have been duly referenced within the text.

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Conflicts of Interest: The authors declare no conflict of interest.

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