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Original Article

Induction of retinal progenitors and neurons from dental follicle stem cell under defined conditions

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Neural differentiation

Abstract *Background/purpose:* Retinopathy affects millions worldwide with increasing prevalence. Dental-derived mesenchymal stem cells (MSCs), primarily originating from the neural crest, possess unique neural-like properties, including high expression of neural markers, making them promising candidates for neural and retinal regenerative medicine. The present study compared two isolation methods for dental-derived MSCs and investigated the potential of dental follicle stem cells (DFSCs) to differentiate into neurons and retinal progenitor cells under defined conditions.

Materials and methods: The cells of extracted dental tissues were isolated by enzymatic digestion and explant culture methods. The multi-lineage differentiation capabilities of DFSCs were confirmed by Alizarin Red staining and Oil Red-O staining after defined culture stimulation. The differentiation of neurons and retinal progenitors was induced in DFSCs and confirmed through cell morphology, immunofluorescence staining, and western blotting.

Results: The enzymatic digestion method was faster and promoted quicker proliferation of DFSCs compared to other dental sources. The expression of Alizarin Red staining and Oil Red-O staining confirmed the multi-lineage differentiation capabilities of DFSCs. Under specific conditions, DFSCs demonstrated the potential to differentiate into neural, retinal progenitor,

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ganglion, bipolar, amacrine, and Müller-like cells with morphologic changes and expression of related proteins.
Conclusion: The present study demonstrates the superior proliferation ability of DFSCs among dental cells, and possesses the potential to differentiate into retinal progenitor cells and neurons under defined conditions. Provide valuable insights into DFSCs as an effective stem cell source and their potential for future therapeutic applications in retinal-related diseases.

Introduction

The retina is part of the central nervous system, comprised of three interconnected neuronal layers: photoreceptor cells, bipolar cells, and retinal ganglion cells, positioned atop the retinal pigment epithelium. In addition to these primary neurons, the retina also contains interneuron horizontal and amacrine cells, as well as a special type of glial cell known as Müller cells.[1] During the development of the retina, retinal progenitor cells display an extraordinary differentiation potential, capable of generating different types of retinal cells.[2] The coordinated action of these cells ensures the accurate reception and transmission of visual information.

Due to the rise in diabetes and aging populations, the prevalence of retinal pathologies has increased significantly.[3] From a pathological perspective, retinal degenerative diseases can be categorized based on neuronal layer involvement. Outer retinopathy primarily compromises photoreceptor cells responsible for light perception, while inner retinopathy disrupts the function of bipolar and retinal ganglion cells.[4] However, the mechanisms of retinal disease development and progression remain incompletely elucidated, and the treatment of these diseases still faces many challenges. Continuing basic research and clinical trials are crucial to developing more effective treatment options. Cell therapy has emerged as a promising approach in treating retinal degenerative diseases with significant advances in recent years. MSCs have garnered interest due to their remarkable accessibility and differentiation potential. Previous studies have demonstrated the capacity of MSCs to differentiate into photoreceptors.[5] The transplanted MSCs on rhodopsin knockout mice exhibited successful integration into the retinal tissue, displaying neuronal and glial morphologies, and promoting photoreceptor cell preservation.[6]

Mesenchymal stem cells are multipotent progenitor cells that exhibit a high self-renewal capacity, multilineage differentiation potential, and immunomodulatory properties.[7] MSCs were initially isolated from bone marrow and have subsequently been discovered in various tissues, including dental tissue, adipose tissue, umbilical cord, etc.[8–10] These cells can be identified and distinguished through their unique MSC-specific immunophenotype, characterized by the expression of cell surface cluster of differentiation (CD) markers such as CD73, CD90, and CD105 while lacking endothelial and hematopoietic cell markers CD31, CD34, and CD45.[11] Due to their proliferation capacity and multilineage differentiation potential, MSCs play an indispensable role in tissue engineering and regenerative medicine.

The extracted teeth, typically considered medical waste in clinics, represent an easily accessible and ethically acceptable source of stem cells. Dental-derived MSCs exhibit typical MSC characteristics and have been identified in various dental tissues, including dental pulp stem cells (DPSCs),[8] stem cells from human exfoliated deciduous teeth (SHED),[12] periodontal ligament stem cells (PDLSCs),[13] dental follicle stem cells (DFSCs),[14] stem cells from the apical papilla (SCAP),[15] etc. These cells originate from the neural crest during development and express neural markers such as neurotrophin receptor p75 and human natural killer-1 (HNK-1),[16] suggesting their potential application in regenerative therapy for neurodegenerative diseases.

DFSCs are recognized for their superior proliferation capacity, enhanced multipotency, and higher immunosuppressive properties than other dental sources.[17,18] Dental follicles also provide a larger tissue volume than sources like dental pulp or periodontal ligament (PDL), making them advantageous for tissue engineering. Beyond dental applications, DFSCs have also shown potential in various non-dental research areas. It has been demonstrated that DFSCs have great clinical prospects and value in neurological diseases with the natural advantages of their origin.[19] Based on these favorable characteristics, in this study, we investigated the potential of DFSCs to differentiate into neurons and retinal progenitor cells under specific culture conditions.

Materials and methods

Cell isolation and culture

The study source of dental tissues is the supernumerary tooth of the patient at the Dentistry Department of Tri-Service General Hospital (Taipei, Taiwan) and was approved by the Tri-Service General Hospital Institutional Review Board (No. A202105191). After extracting a supernumerary tooth, a scalpel blade was used to scrape off the gingiva and PDL around the tooth. Then, use a curette to remove the follicle tissue. After making an incision at the neck of the tooth, use a barbed broach to extract the pulp from both the crown and root. Place the extracted tissues separately into phosphate-buffered saline (PBS) containing 1 % Penicillin-Streptomycin and Amphotericin B, and subsequently compare their cultivation using two different methods.

Explant culture method

After thoroughly mincing and washing the tissues, centrifuge at 1000 rpm for 5 min to remove the supernatant. Wash with PBS and centrifuge again to remove the supernatant. Suspend tissues in the medium (MEM Alpha [1X] + GlutaMAXTM-I supplemented with 10 % Fetal Bovine Serum [FBS] [Gibco, Waltham, MA, USA]), then transfer to a 6 cm culture dish and incubate in a humidified environment at 37 °C, 5 % (vol/vol) CO_2. Replace the medium when cell attachment is observed, and passaging using 0.25 % trypsin/EDTA when cells reach 80 % confluence.

Enzymatic digestion method

Wash the tissues thoroughly and remove PBS. Add 3 mg/mL dispase II (Sigma Aldrich, Saint Louis, MO, USA) and 4 mg/mL collagenase I (Gibco). Vortex for 10 s every 15 min, repeating 4 times. Terminate enzymatic digestion by adding an equal volume of culture medium (MEM Alpha [1X] + GlutaMAXTM-I supplemented with 10 % FBS [Gibco]). Centrifuge at 1000 rpm for 5 min, remove the supernatant, and resuspend the cells in culture medium. Transfer to a 6 cm culture dish and incubate in a humidified environment at 37 °C, 5 % (vol/vol) CO_2. After 72 h, remove the medium and non-adherent cells and replace the medium. Detach cells using 0.25 % trypsin/EDTA for passaging when cells reach 80 % confluence.

MSCs stemness confirmation

Cells were expanded and collected for flow cytometry, fixed with 4 % formaldehyde for 15 min at room temperature, and then centrifuged with subsequent PBS wash. After removing PBS, suspended cells in 0.5 % bovine serum albumin (BSA) with antibodies of MSCs positive markers CD44 CD73, and CD90, and negative markers CD31, CD34, and CD45 (Invitrogen, Waltham, MA, USA), for 1 h at room temperature. Flow cytometer BD FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA, USA) were used for data acquisition and analysis.

Osteogenic differentiation of DFSCs and alizarin red staining

DFSCs were seeded into 6-well at a density of 3×10^5 cells/well and stimulated with osteogenic induction medium (Human Mesenchymal Stem cell [hMSC] Osteogenic Differentiation Medium BulletKit) (Lonza, Basel, Switzerland). Replace the differentiation medium every 3 days for 14 days. After removing the medium, wash cells twice with PBS and fix with 4 % formaldehyde for 15 min at room temperature. Wash with deionized (DI) water, incubate with 40 mM Alizarin Red staining dye (Sigma–Aldrich) at room temperature for 30 min, rinse with DI water to remove the red dye, and observe under a microscope.

Adipogenic differentiation of DFSCs and oil red-O staining

DFSCs were seeded into 6-well at a density of 3×10^5 cells/well and stimulated with adipogenic induction medium (Human Mesenchymal Stem Cell [hMSC] Adipogenic Differentiation Medium BulletKit) (Lonza). Replace the differentiation medium every 3 days for 14 days. Prepare Oil Red O (Sigma–Aldrich) by mixing with DI water in a ratio of 3:2. Incubate at room temperature for 30 min, then filter through a 0.22 μm filter. Remove the culture medium, wash the cells twice with PBS, and fix with 4 % formaldehyde at room temperature for 30 min. Wash with DI water, add premixed Oil Red O staining, and incubate at room temperature for 30 min. Wash with DI water until clean, then observe under a microscope.

Neurogenic differentiation of DFSCs

DFSCs were seeded into 6-well at a density of 3×10^4 cells/well. When the cells reached 60–70 % confluence, the medium was changed to the Mesenchymal Stem Cell Neurogenic Differentiation Medium (PromoCell, Heidelberg, Germany). According to the manufacturer's instruction, the cells should be incubated for at least 4 days, the present study replaced the differentiation medium every 3 days for 7 days.

Retinal progenitor cell differentiation of DFSCs

DFSCs were cultured according to Tucker et al., with modification on antibiotics.[20] Cells were seeded at a density of 50 cells/cm^2 in a 10 cm dish and stimulated with embryoid body (EB) medium consisting of DMEM/F12 containing 10 % knockout serum replacement, 2 % B27 supplement, 1 % N2 supplement, 1 % L-glutamine (Gibco), 1 % Penicillin-Streptomycin-Amphotericin B Solution, 1 % 100x non-essential amino acids (NEAA) (Biological Industries, Kibbutz Beit Haemek, Israel), 1 ng/ml Noggin, 1 ng/ml Dickkopf-related protein 1 (DKK-1), 1 ng/ml insulin-like growth factor 1 (IGF1), 0.5 ng/ml basic fibroblast growth factor (bFGF) (Sino Biological, Beijing, China). The medium was changed every 2 days. After 5 days of EB medium stimulation, the cells were passaged and seeded at a density of 30 cells/cm^2 in a 10 cm dish. Cells were then stimulated with differentiation medium I (DMEM/F12, 2 % B27, 1 % N2 supplement, 1 % L-glutamine [Gibco], 1 % Penicillin-Streptomycin-Amphotericin B Solution, 1 % 100x NEAA [Biological Industries], 10 ng/ml Noggin, 10 ng/ml DKK-1, 10 ng/ml IGF1, 1 ng/ml bFGF [Sino Biological]). The medium was changed every day for 10 days. After 10 days, the cells were stimulated with differentiation medium II (differentiation medium I + 10 μM Notch signaling inhibitor DAPT [Adooq Bioscience, Irvine, CA, USA]). The medium was changed every day and lasted for 6 days.

Immunofluorescence staining

Cells were fixed in 4 % formaldehyde at 4 °C for 15 min. After washing twice with 0.2 % phosphate buffered saline with Tween 20 (PBST), cells were permeabilized in 0.1 % Triton X-100 for 10 min at room temperature with subsequent 0.2 % PBST washing. Cells were blocked in 3 % BSA for 30 min with subsequent 0.2 % PBST washing and incubated with primary antibody solutions of Nestin (Abcam, Cambridge, UK), tubulin beta 3 (TUBB3), glial fibrillary acidic protein (GFAP), neuronal nuclei (NeuN) (Cell Signaling Technology, Danvers, MA, USA), microphthalmia-associated transcription factor (MITF), interferon-inducible double-stranded RNA-dependent protein kinase activator RAX, and paired box 6 (PAX6) (Abcepta, San Diego, CA, USA) at 4 °C overnight. Cells were then incubated with corresponding fluorescein isothiocyanate (FITC) and allophycocyanin (APC) secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature before counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. The slides were mounted with coverslips and observed under the THUNDER Imager (Leica Camera, Wetzlar, Germany).

Western blot

Briefly, cells were lysis using RIPA Lysis buffer (EMD Millipore Corp., Burlington, MA, USA). Protein was quantified using Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), separated by 10 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were incubated with primary antibody solutions of Nestin, Ceh-10 homeodomain-containing homolog (CHX10) (Abcam), TUBB3, NeuN, GFAP (Cell Signaling Technology), RAX, MITF, PAX6 (Abcepta), and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (Proteintech, Rosemont, IL, USA) at 4 °C overnight. Then incubate with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch) for 30 min at room temperature. The immunoreactive proteins were visualized using an enhanced chemiluminescence kit (ECL) (Amersham Biosciences, Buckinghamshire, UK) and detected by the luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software Inc., San Diego, CA, USA). A two-tailed Student's t-test was used to compare the means of the two groups. *P*-values less than 0.05 ($P < 0.05$) were considered statistically significant. Data are presented as mean ± standard error of the mean (SEM), and the sample size was 3 for each group ($n = 3$) with 2 replicates.

Results

Cultivation of various dental cells

The extracted follicle, gingiva, PDL, and pulp tissues were isolated and cultured using enzymatic digestion and explant culture methods (Fig. 1). In the enzymatic digestion method, cell attachment of dental follicle and gingival cells was observed under microscopy at 24 h, while dental pulp and PDL cells showed no significant cell adherence. Conversely, the explant culture method revealed few cell attachments of dental follicular cells from the tissue's periphery at 72 h, with no observable attachment for other cell types. By day 10, both methods demonstrated that dental follicular cells exhibited the fastest proliferation rate compared to gingival, PDL, and dental pulp cells. The enzymatic digestion method achieved approximately 90 % confluence, while the explant culture method reached only 60—70 % confluence.

Multi-lineage differentiation potency of DFSCs

Compared to the control group (Fig. 2A), DFSCs after osteogenic induction for 14 days revealed distinct calcium depositions with Alizarin Red staining (Fig. 2B), demonstrating the cells' osteogenic differentiation potential. Similarly, compared to the control group (Fig. 2C), DFSCs clearly showed lipid droplet accumulation with Oil Red-O staining following adipogenic induction for 14 days (Fig. 2D), indicating successful adipogenic differentiation of the cells.

DFSCs differentiate into neurons

After neurogenic differentiation induction of DFSCs, cells were observed for morphological changes. Fig. 3A—D represent the control group at days 1, 3, 5, and 7, respectively. In the differentiation group, day 1 (Fig. 3E) showed initial cell retraction. By day 3 (Fig. 3F), cells began to extend short, dendrite-like processes. Day 5 (Fig. 3G) revealed an increased number and length of these dendrite-like extensions, and day 7 (Fig. 3H) demonstrated prominent dendritic morphology. Additionally, other neural-like cell types observed during the culture period included: differentiated post-mitotic neuronal-like cell (Fig. 3I), restricted neural progenitor-like cell (Fig. 3J), and oligodendrocyte-like cell (Fig. 3K).

Immunofluorescence staining was performed with neural progenitor cell marker Nestin, immature and mature neuron marker TUBB3, mature neuron marker NeuN, and neural progenitor and astrocyte marker GFAP to evaluate the neural differentiation potential of DFSCs (Fig. 4A—T). In the differentiation group, expression of Nestin (Fig. 4C), TUBB3 (Fig. 4H), GFAP (Fig. 4M), and NeuN (Fig. 4R) is strongest on day 3 with subsequently declining on days 5 and 7, but remain more prominent than the control group (Fig. 4A—F, K, and P).

Western blot analysis was subsequently conducted to confirm neural differentiation with Nestin, TUBB3, NeuN, GFAP, and oligodendrocytes marker CNPase. Results demonstrated increased expression in some neural proteins during days 1 and 3, followed by a decline on days 5 and 7 (Fig. 4U). Quantitative analysis revealed statistically significant differences in TUBB3 with $P < 0.001$, while Nestin, NeuN, CNPase, and GFAP exhibited $P < 0.0001$ on day 3 of the differentiation group compared to the control group (Fig. 4V).

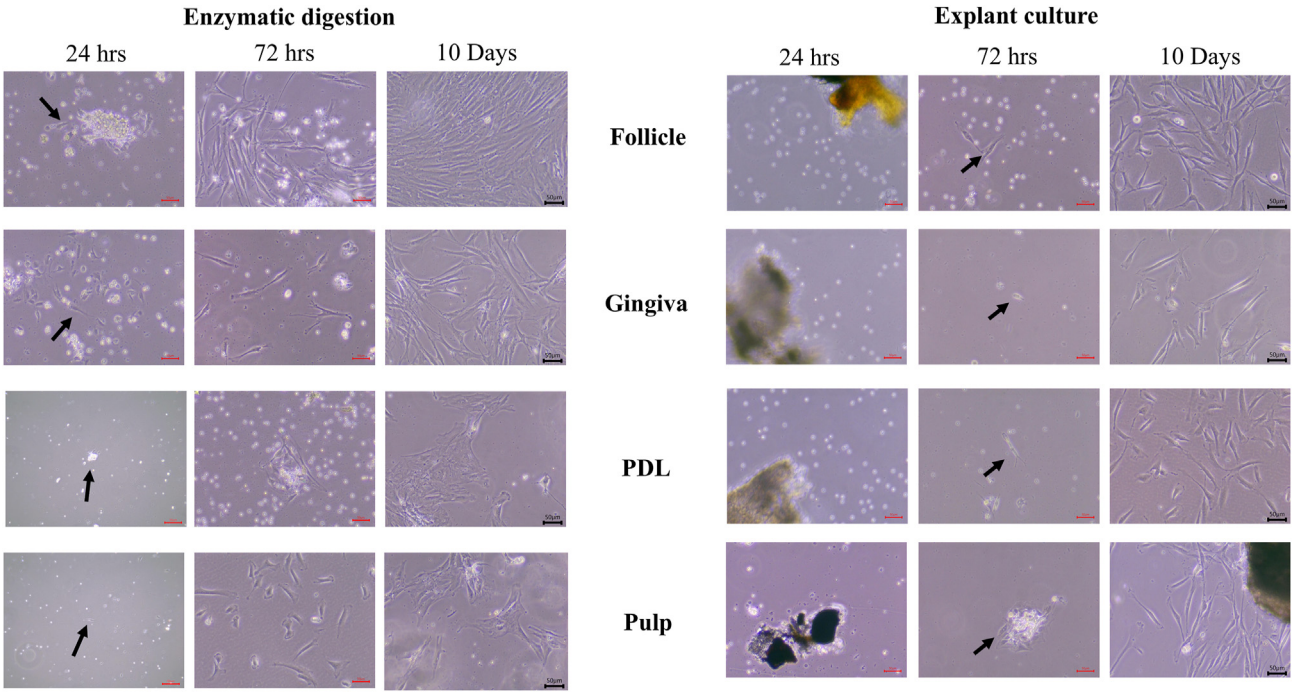

Figure 1 Comparison of enzymatic digestion and explant culture method of dental tissues. Microscopic observation of dental follicle, gingiva, PDL, and pulp tissue cultured by enzymatic digestion and explant culture at 24 h, 72 h, and day 10. Black arrows indicate initially attached cells. Scale bars, 50 μm. *Abbreviations:* PDL-periodontal ligament.

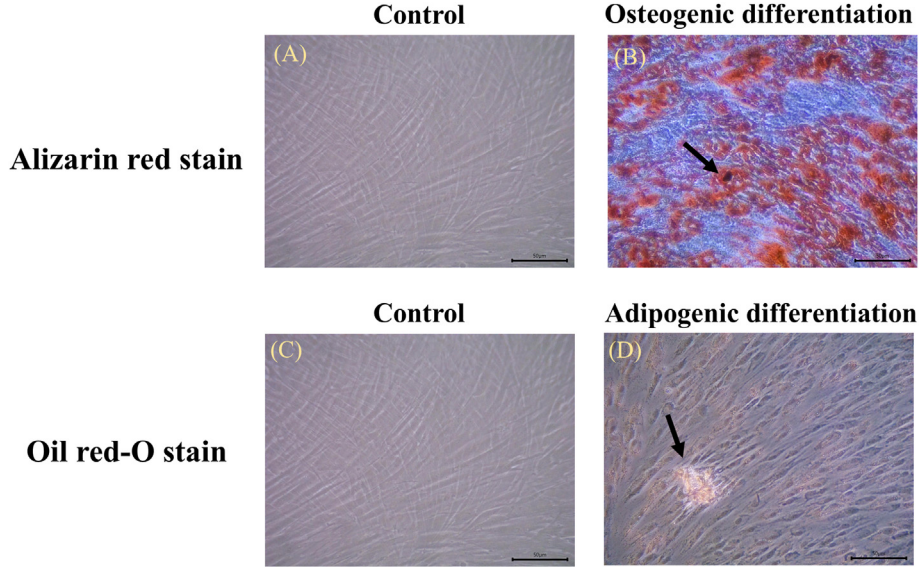

Figure 2 Multi-lineage differentiation potential of dental follicle stem cells (DFSCs). Control cells (A and C) compared to osteogenic induction of DFSCs under alizarin red stain with calcium deposition (black arrow) (B) and adipogenic induction of DFSCs under oil red-O stain with lipid droplet accumulation (black arrow) (D). Scale bars, 50 μm.

DFSCs differentiate into retinal progenitor cells

DFSCs were induced to differentiate into retinal progenitor cells using three culture media: EB medium, differentiation medium I (DMI), and differentiation medium II (DMII), with morphological changes observed and recorded microscopically over 21 days. Results revealed that EB medium stimulation showed only minimal morphological changes between control and differentiation groups (Fig. 5A–C and G-I). With DMI, the control group is shown in Fig. 5D–F and 5M. By day 9 of differentiation (Fig. 5J), cell bodies began to contract and extend multiple short processes. On day 11 (Fig. 5K), these processes and cell bodies started to elongate presenting an axon-like structure. On days 13 and 15 (Fig. 5L and Q), cells increased numerous dendrite-like processes. Following DMII stimulation, the control group is

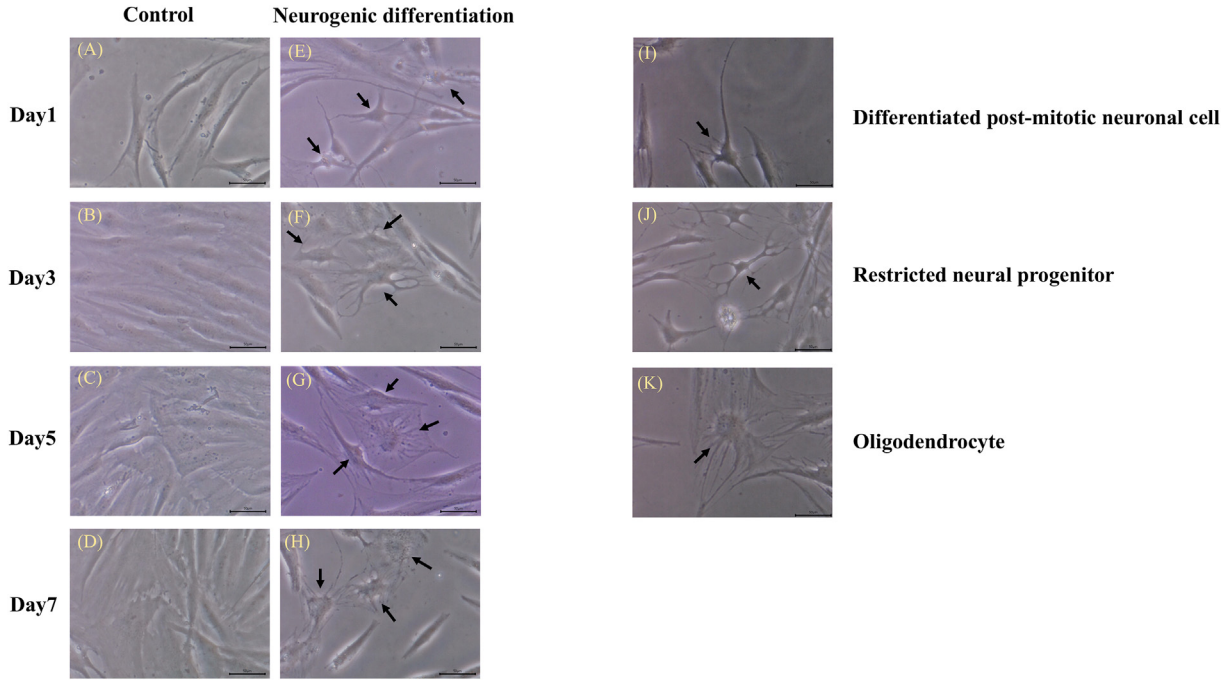

Figure 3 Microscopic observation of neurogenic induction of dental follicle stem cells (DFSCs) for 7 days (A—D) represent control cells on days 1, 3, 5, and 7, respectively (E—H) demonstrate neurogenic differentiation group on days 1, 3, 5, and 7, with black arrows pointing to neural-like cells. Additional observations of other cell morphology include differentiated post-mitotic neuronal-like cells (I), restricted neural-like progenitors (J), and oligodendrocyte-like cells (K). Scale bars, 50 μm.

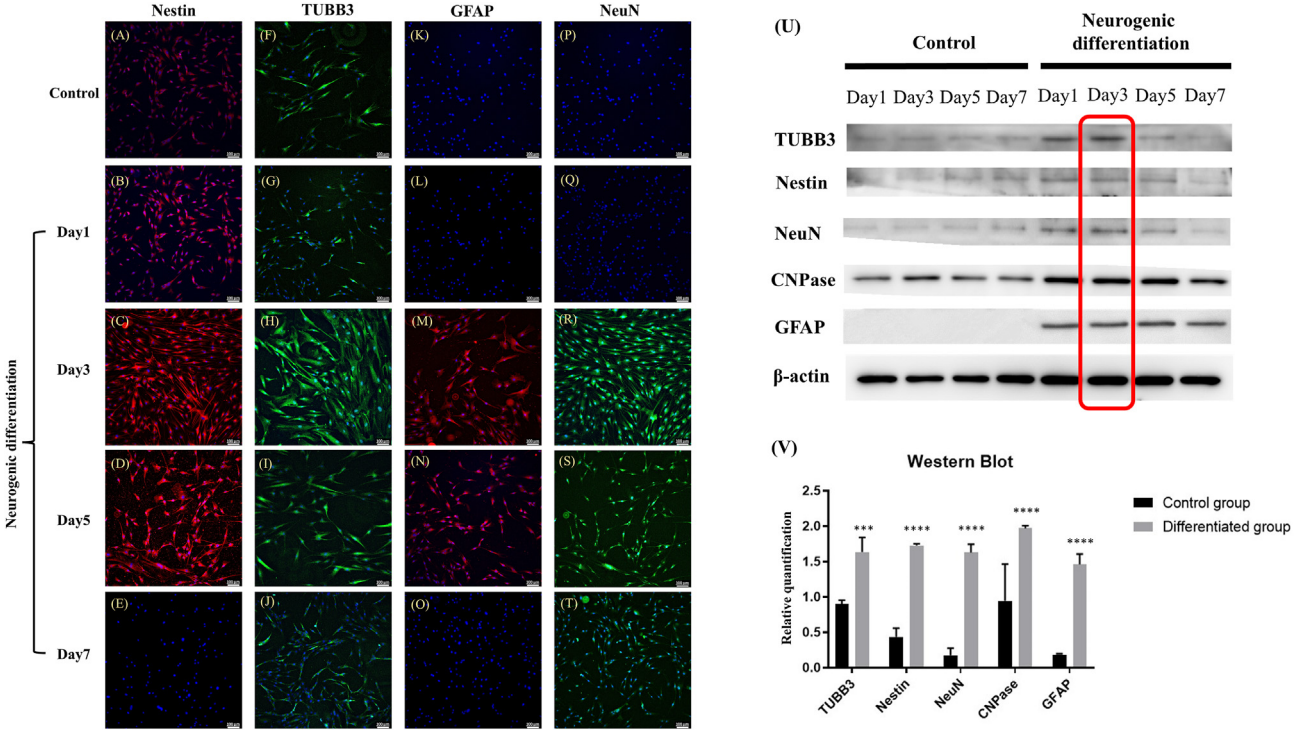

Figure 4 Immunofluorescence staining and Western blot analysis of neurogenic differentiation of dental follicle stem cells (DFSCs). Fluorescence microscopy observations for control cells (A, F, K, and P) and neurogenic induction of DFSCs on days 1, 3, 5, and 7 were presented. Staining with Nestin (red) (A—E), TUBB3 (green) (F—J), GFAP (red) (K—O), NeuN (green) (P—T), and DAPI (blue) nuclear staining. Scale bars, 100 μm. Western blot analysis was performed with TUBB3, Nestin, NeuN, CNPase, and GFAP (U), with quantitative analysis on day 3 showing significant differences in the differentiated group compared to the control group (V). Results represent mean ± SEM (n = 3). ***$P < 0.001$,****$P < 0.0001$. *Abbreviations:* TUBB3- tubulin beta 3, GFAP- glial fibrillary acidic protein, NeuN- neuronal nuclei, DAPI- 4′,6-diamidino-2-phenylindole, CNPase- 2′,3′-cyclic nucleotide 3′-phosphodiesterase.

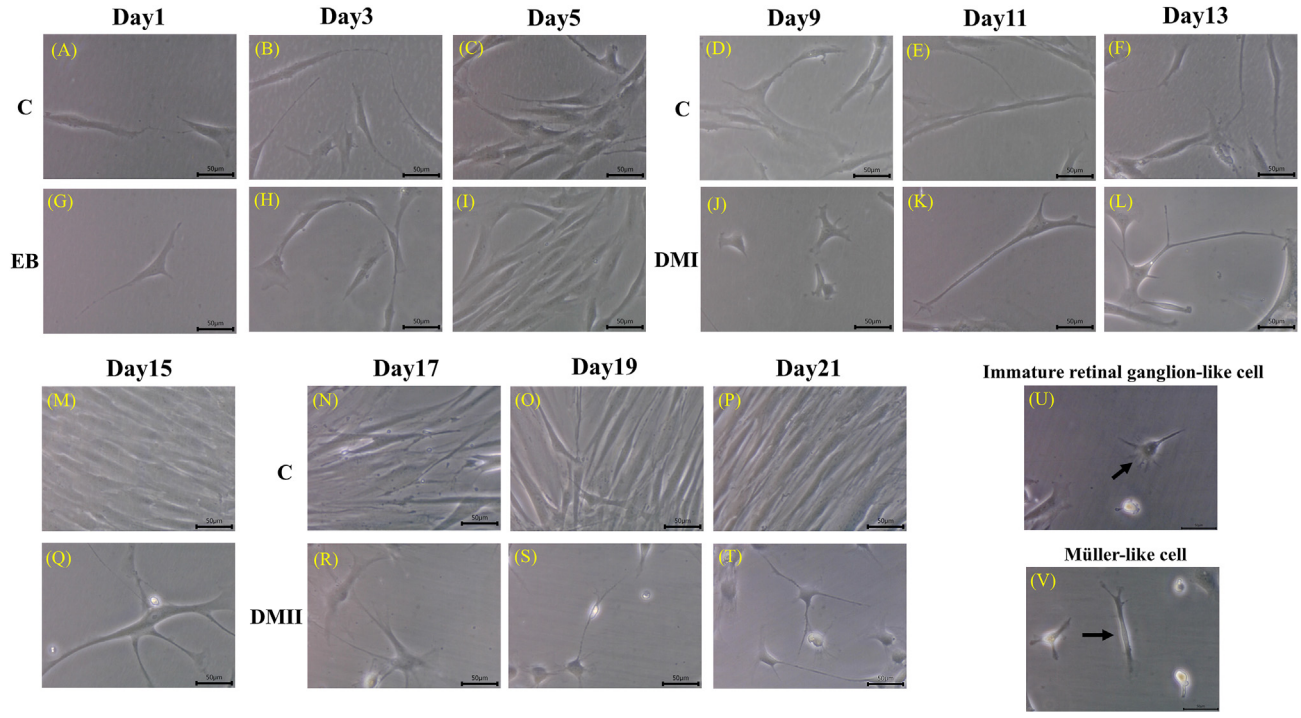

Figure 5 Microscopic observation of retinal progenitor cell induction of dental follicle stem cells (DFSCs) for 21 days (A-F and M–P) C represent control cells. The differentiation group was stimulated with retinal progenitor different mediums for 3 stages, during Day 1–5 with embryoid body (EB) medium (G–I), Day 6–15 with differentiation medium I (DMI) (J-L and Q), and Day 16–21 with differentiation medium II (DMII) (R–T). During the culture period, other retinal-like cells, including immature retinal ganglion-like cells (U) and Müller-like cells (V) were also observed (black arrows). Scale bars, 50 μm.

presented in Fig. 5N-P. On day 17 of differentiation (Fig. 5R), cell bodies decreased in size accompanied by an increase in dendrite-like processes. By days 19 and 21 (Fig. 5S and T), these dendrite-like extensions elongated further. Fig. 5U and V presents additional retinal-like cell morphologies observed on days 19 and 21, resembling immature retinal ganglion and Müller cells.

Immunofluorescence staining was performed with retinal progenitor cell marker MITF, RAX, and PAX6, retinal ganglion cells marker TUBB3, and Müller cell marker GFAP to evaluate retinal cell differentiation. Compared to the control group (Fig. 6A–E), the differentiation group demonstrated an increase in the expression of MITF (Fig. 6F), RAX (Fig. 6G), PAX6 (Fig. 6H), and GFAP (Fig. 6J).

Subsequently, Western blot analysis was performed to further confirm the retinal cell differentiation of DFSCs using the markers previously mentioned, as well as a retinal progenitor, ganglion, bipolar, amacrine, and Müller cells marker CHX10 (Fig. 6K). Quantitative and statistical analysis was conducted, and the differentiated group showed marked increases in the expression of these retinal cell-related proteins with TUBB3 exhibited $P < 0.001$, while RAX, PAX6, MITF, CHX10, and GFAP demonstrated $P < 0.0001$ (Supplement 1). This indicates highly significant expression of these markers, supporting the successful differentiation of retinal progenitor cells and the development of diverse retinal cell types (Fig. 6L).

Discussion

The enzymatic digestion method offers advantages in immediately determining cell presence and observing cell attachment within 24 h. In contrast, the explant culture method requires ensuring tissue contact with the culture dish, where insufficient contact surface area may require longer adherence time. The present study compared cell culture methods for dental tissues and demonstrated that the enzymatic digestion method was more effective and faster, consistent with Hilkens et al.'s research.[21] We further compared the growth rates of four cell types: dental follicle, gingival, pulp, and PDL cells. By day 10, dental follicular cells demonstrated a prominent growth rate compared to the other three cell types, similar to findings from Zhou et al.[18,22,23] Moreover, cells cultured by both methods showed comparable proliferation capacity and morphology after several times of thawing.

Following flow cytometry confirmation of MSCs immunophenotype of DFSCs (Supplement 2), we investigated their multipotent differentiation potential by inducing osteogenic and adipogenic differentiation. Morphological and staining results revealed calcium deposition after osteogenic stimulation and lipid droplet accumulation following adipogenic induction, confirming the stem cells' multilineage differentiation potential, consistent with Masaki J et al.'s research.[24]

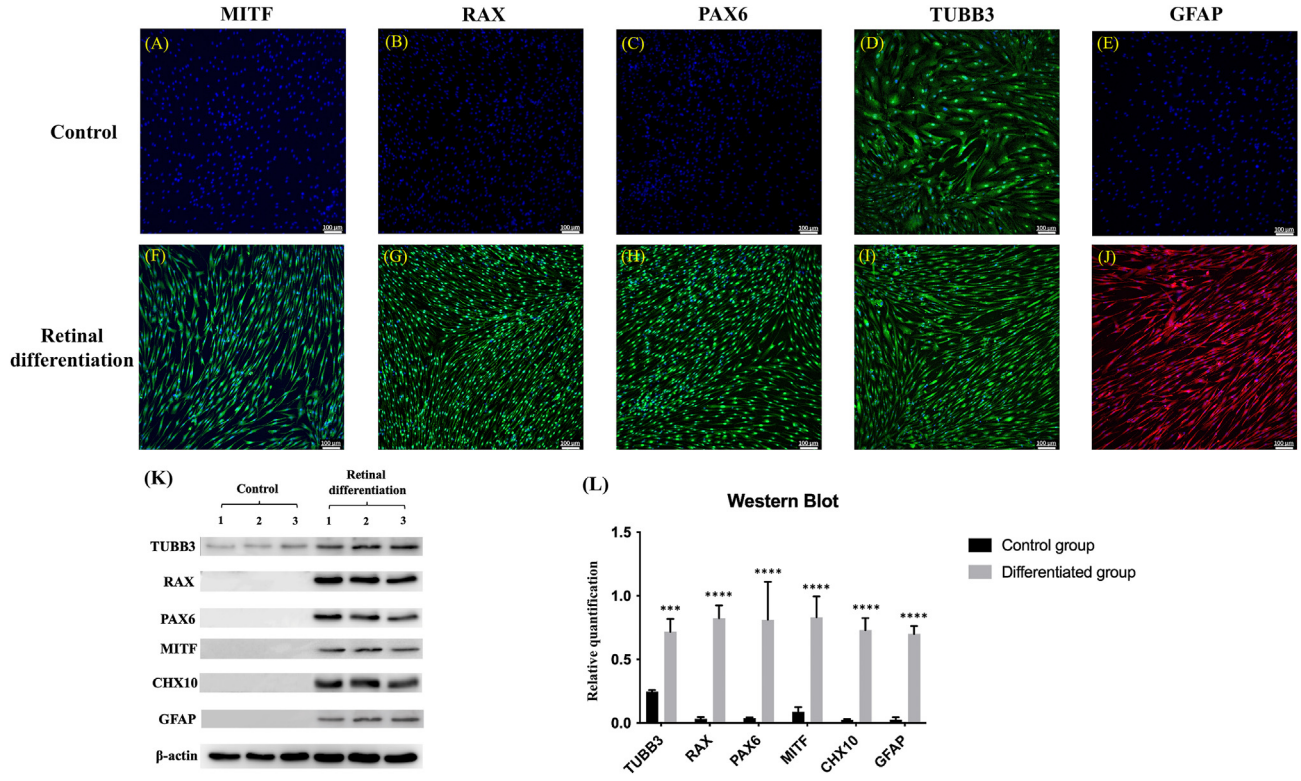

Figure 6 Immunofluorescence staining and Western blot analysis of retinal progenitor differentiation of dental follicle stem cells (DFSCs) on day 21. Fluorescence microscopy observations were presented for control cells (A—E) and retinal progenitor induction of DFSCs (F—J). Staining with MITF (green) (A and F), RAX (green) (B and G), PAX6 (green) (C and H), TUBB3 (green) (D and I), GFAP (red) (E and J), and DAPI (blue) nuclear staining. Scale bars, 100 μm. Western blot analysis was performed with TUBB3, RAX, PAX 6, MITF, CHX10, and GFAP (K), with quantitative analysis showing significant differences compared to the control group (L). Results represent mean ± SEM (n = 3). ***$P < 0.001$,****$P < 0.0001$. *Abbreviations:* MITF- microphthalmia-associated transcription factor, PAX6-paired box 6, TUBB3- tubulin beta 3, GFAP- glial fibrillary acidic protein, DAPI- 4′,6-diamidino-2-phenylindole, CHX10- Ceh-10 homeodomain-containing homolog.

The present study, through cellular morphology observation, immunofluorescence staining, and Western blot analysis, demonstrated that DFSCs possess the tendency to differentiate toward neural and neuronal cell lineages. Morphological observations showed the emergence and continuous elongation of dendrite-like processes, with an increasing number of differentiated post-mitotic neuronal-like cells. The growth of dendrites and axons is primarily associated with neurotrophic factors, which induce axonal and dendritic growth.[25] The length and branching of dendrites and axons vary significantly, with diameters ranging from <0.1 μm to >10 μm in the central nervous system and 0.1—20 μm in peripheral nerves.[26—28] However, our study did not measure the lengths, the future research will focus on quantifying axon length and exploring neuronal cell interconnection mechanisms. The immunofluorescence staining and Western blot expression of neural, neuronal, oligodendrocytes and astrocyte proteins are consistent with studies by Heng et al.[29] and Zhang et al.,[30] confirmed that DFSCs exhibit a potential to differentiate into neurons, oligodendrocytes, astrocyte, etc. when stimulated with neurogenic differentiation medium. Longer culture durations are generally associated with enhanced neuronal maturation.[31,32] However, it is essential to consider specific conditions and mediums that may influence the outcomes. In this study, the expression of neurogenic-related proteins began to decline after day 3 of differentiation stimulation in the Western blot. Nevertheless, the potential functional impact of further prolonging the culture time under these conditions remains to be verified through further experiments.

Retinal progenitor cells and most of the dental derived-MSCs share the same embryonic origin. Previous study has demonstrated the differentiation potential of dental pulp stem cells (DPSCs) into retinal neuron-like cells.[33] Since DFSCs exhibit a rapid growth rate than DPSCs, we investigate the potential of DFSCs to differentiate into retinal cells under 21 days of retinal progenitor cell differentiation medium stimulation. During the 21 days of morphological observation, we noted an increased presence of post-mitotic retinal precursors-like, immature retinal ganglion-like, and Müller cell-like features. Immunofluorescence and Western blot analysis using retinal cell markers MITF, RAX, PAX6, CHX10, TUBB3, and GFAP showed significantly increased expression compared to the control group. These results confirmed the DFSCs' differentiation tendency and potential towards retinal progenitor, ganglion, amacrine, bipolar, and Müller-like cells. Indicates significant developmental plasticity and remarkable cellular potential for multipotent differentiation of diverse retinal cell lineages

in DFSCs. Such multipotency suggests notable implications for regenerative medicine, potentially offering pathways for retinal stem cell therapy and tissue engineering.

Since our findings are based on in vitro molecular experiments, future investigations will focus on evaluating the functional properties of the differentiated cells to confirm their neuronal and retinal characteristics, which will involve patch-clamp recordings to assess their ability to generate action potentials, calcium imaging measurement to assess synaptic activity, and light-response assays to assess photosensitive capabilities of retinal progenitor cells. Furthermore, we aim to develop and optimize culture conditions for differentiating DFSCs into neurons and retinal cells under three-dimensional culture systems and conduct animal models to explore the therapeutic potential for clinical applications in neurological and retinal-related disorders.

In conclusion, the present study confirmed that enzymatic digestion is superior to the explant culture method and verified that DFSCs exhibit rapid proliferation capabilities and multi-lineage differentiation potential. After stimulation with neurogenic and retinal progenitor cell differentiation medium, DFSCs demonstrated significant potential for differentiation towards neural cells, retinal progenitor, ganglion, amacrine, bipolar, and Müller-like cells. Provide a more effective and promising cellular source for potential therapeutic application in retinal-related diseases.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2025.03.008.

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